Distribution of *Crenarchaeota* Representatives in Terrestrial Hot Springs of Russia and Iceland[†]

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Culture-independent (PCR with *Crenarchaeota*-specific primers and subsequent denaturing gradient gel electrophoresis) and culture-dependent approaches were used to study the diversity of *Crenarchaeota* in terrestrial hot springs of the Kamchatka Peninsula and the Lake Baikal region (Russia) and of Iceland. Among the phylotypes detected there were relatives of both cultured (mainly hyperthermophilic) and uncultured *Crenarchaeota*. It was found that there is a large and diverse group of uncultured *Crenarchaeota* that inhabit terrestrial hot springs with moderate temperatures (55 to 70°C). Two of the lineages of this group were given phenotypic characterization, one as a result of cultivation in an enrichment culture and another one after isolation of a pure culture, “*Fervidococcus fontis*,” which proved to be a moderately thermophilic, neutrophilic (optimum pH of 6.0 to 7.5), anaerobic organotroph.

Archaea of the phylum *Crenarchaeota* (19, 53, 54) represent one of the deep-branching phylogenetic lineages among prokaryotes. Since the pioneering work of T. Brock in Yellowstone National Park in the 1970s and subsequent findings of W. Zillig and K. Stetter in the 1980s, this group of organisms has been considered to be rather homogeneous phenotypically and to comprise hyperthermophiles that have a sulfur-dependent metabolism and inhabit terrestrial and marine hot springs, usually in volcanically active regions (47). Within the past two decades, the use of molecular techniques, including PCR-based amplification of 16S rRNA genes, allowed culture-independent assessment of microbial diversity (37). Remarkably, such techniques indicated a wide distribution of mostly uncultured archaea in nonthermal habitats, such as ocean waters (14, 17), lake waters (43), soils (25, 36), and other environments. However, the novel archaeal lineages remained elusive for a long time: no cultivated strains were available. Environmental genomic analyses provided clues about the potential metabolic strategies of several of the uncultivated and abundant groups of nonthermophilic terrestrial and marine *Crenarchaeota* (44). The first and so far the only low-temperature *Crenarchaeota* isolate turned out to be an ammonia oxidizer (29).

Although a great number of uncultured *Crenarchaeota* have been detected with molecular techniques in nonthermal environments, a significant diversity of the new *Crenarchaeota* phylotypes was also found in hot habitats. Studies by Barns et al. of Obsidian Pool, Yellowstone National Park (United States), more than doubled the known molecular diversity within the thermophilic crenarchaeotes, and novel, deeply branching 16S

rRNA sequences were found (2, 3). Subsequent studies of Obsidian Pool and other hot springs in the United States, Iceland, Japan, Italy, and Thailand extended this picture (24, 26, 30, 31, 33, 45, 46, 48).

The goal of this work was to study the diversity and distribution of crenarchaeotes in hot springs in the Kamchatka Peninsula and Lake Baikal region (Russia) and in Iceland. Kamchatka hot springs for a long time served as a source of novel thermophilic microorganisms (6), including several isolates of organotrophic anaerobic *Crenarchaeota* (4, 5, 39, 40, 41). The diversity of nonthermophilic crenarchaeotes in soils from permafrost and grassland areas of Kamchatka studied by culture-independent methods was reported (36). The application of molecular techniques to the studies of hot springs in Kamchatka showed that *Sulfolobus* strains were widespread (52). A diversity of thermophilic bacteria has been isolated from hot springs in the Lake Baikal region (35), but no data on the presence of *Crenarchaeota* in these habitats are available except for a recent paper describing “*Candidatus Nitrososphaera gargensis*” (20). Compared to hot springs in the Kamchatka and Lake Baikal regions, the biodiversity of Icelandic hot springs is rather well studied by both culture-dependent (47) and -independent methods (31, 32, 45).

Here, we present the results of an investigation of the distribution and diversity of thermophilic crenarchaeotes in terrestrial hot springs in the Kamchatka and Lake Baikal regions (Russia) and in Iceland by a new rapid and reliable approach based on PCR with *Crenarchaeota*-specific primers and subsequent denaturing gradient gel electrophoresis (DGGE), and we complement these data with the results of cultivation experiments.

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MATERIALS AND METHODS

Sample collection. Samples of water and mud from Kamchatka Peninsula, the Lake Baikal region, and Iceland hot springs were collected in sterile Falcon tubes and were then transferred to the laboratory for analysis as soon as possible. More

than 50 samples from Kamchatka Peninsula springs, which feature temperatures from 49 to 91°C and pH from 2.5 to 8.4, were taken in August and September, 2003 to 2006, on the East, Central, and Orange hydrothermal fields of the Uzon Caldera, in the Geyser Valley, and near Karymskii (54°03'N, 159°27'E) and Mutnovskii volcanoes (52°27'N, 158°12'E). Seven samples from hot springs of the Lake Baikal region, with temperatures from 59 to 71°C and pH from 8.4 to 8.9, were collected during the summer of 2003. Samples were taken from Urinskii spring (53°39' N, 110°07' E) and Gusihinskii spring situated in the Barguzin region of Buryat Republic. In Iceland, 18 samples were taken in spring 2006 from hot springs in the Hveragerði area in the valleys of Reykjadalur, Graendalur, and Hverakjalki, with temperatures of 55°C to 94°C and pH values of 2.0 to 7.5.

Primer design. The primers Cren7F and Cren518R (Fig. 1) were designed from an alignment of sequences retrieved from the Ribosomal Database Project II (RDP-II), release 8.0 (9). The set of aligned archaeal 16S rRNA sequences in RDP-II release 8 has not been updated since June 2000; therefore, toward the end of this work, the primers were reevaluated using aligned sequences retrieved from the GreenGenes database (15; <http://greengenes.lbl.gov/cgi-bin/nph-index.cgi>) updated 8 October 2007. The consensus sequences of *Crenarchaeota* and *Euryarchaeota* were deduced from the retrieved aligned sequences using a specially written program whose algorithm was based on a straightforward count of occurrence of particular nucleotides in particular positions. The reevaluation of the specificity of our primers was also done with the NCBI BLASTN program (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) (1) using Entrez queries to limit the search to nontarget organisms and with the Probe Match facility of RDP-II release 9.56 (10) (however, only bacterial sequences occurred in the latter database).

In vitro tests of the primer specificity used DNAs of the following crenarchaeotal, euryarchaeotal, and bacterial strains: as positive controls, *Staphylothermus marinus* DSM 3639^T, *Sulfophobococcus zilligii* DSM 11193^T, *Desulfurococcus mobilis* DSM 2161^T, *Thermoproteus tenax* DSM 2078^T, *Sulfolobus solfataricus* DSM 1616^T, *Ignicoccus pacificus* DSM 13166^T, and *Acidianus infernus* DSM 3191^T; as negative controls, *Thermococcus fumicolans* DSM 12820^T, *Thermococcus peptonophilus* DSM 10343^T, *Methanosarcina barkeri* DSM 800^T, *Methanococcus jannaschii* DSM 2661^T, and *Thermoanaerobacter siderophilus* DSM 12299^T. The strains were grown on the media and under the conditions recommended by the Deutsche Sammlung von Mikroorganismen und Zellkulturen.

DNA extraction. One to two milliliters of sample (mixture of mud and water) or 1 ml of enrichment culture was centrifuged at 12,000 × g, and the pellets were resuspended in 200 µl of TNE buffer (10 mM Tris-HCl, 10 mM NaCl, 5 mM EDTA, pH 8.0). Proteinase K (0.5 mg ml⁻¹), sodium dodecyl sulfate (0.5%, wt/vol), and 0.015 M EDTA were added, and the mixture was incubated at 50°C for 1 to 2 h. Then, a phenol-chloroform (1:1) mixture was added, and the samples were shaken prior to centrifugation (12,000 × g for 10 min). The supernatant was extracted twice with an equal volume of chloroform. To the aqueous phase, 0.1 part of 3 M sodium acetate (pH 5.2) and 2 parts of 100% ethanol were added to precipitate DNA. The DNA was collected by centrifugation (12,000 × g for 15 min), washed in 70% ethanol, dried, and resuspended in sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA isolated was used as a template for PCR.

PCR amplification. For amplification of fragments of 16S rRNA genes, the *Crenarchaeota*-specific Cren7F-Cren518R primer pair was used. The PCR mixture (20 µl) contained PCR buffer [1 × a mixture of 75 mM Tris-HCl, pH 8.8, 20 mM (NH₄)₂SO₄, 0.01% (vol/vol) Tween 20; Fermentas, Lithuania], 1.5 mM MgCl₂, a 20 µM concentration of each deoxynucleoside triphosphate, a 0.5 µM concentration of each primer, 1 U of *Taq* polymerase (Fermentas, Lithuania), and 1 µl of template DNA solution (1 to 10 ng DNA). The temperature program of the reaction, adjusted experimentally, was as follows: 3 min at 95°C and 32 cycles of denaturation at 94°C for 7 s, annealing and extension at 72°C for 1 min, and final extension at 72°C for 10 min. In all experiments, a reaction mixture without DNA was included as a negative control. PCR was run in a Tertsik multichannel DNA amplifier (DNA-Tekhnologiya, Russia). The PCR products (5 µl) were analyzed by agarose electrophoresis (42).

DGGE. DGGE was used for phylogenetic characterization of the crenarchaeotal component of each site. The Cren7F primer was attached to a 40-bp GC clamp (34) to facilitate analysis with DGGE and was used in PCR together with Cren518R. In some cases, amplification with the primer pair Cren7F-Cren518R was followed by reamplification with the pair of primers with the GC clamp. The reaction mixture for the PCR was the same as for the PCR with the primers without the GC clamp. The PCR was run in a Perkin Elmer amplifier (Perkin Elmer Cetus), using the following program: 94°C for 5 min and one cycle of denaturation at 94°C for 30 s, annealing at 75°C for 30s, and extension at 72°C for 1 min. Then, the annealing temperature was decreased by 1°C every second cycle until a touchdown at 65°C, at which point 10 additional annealing and

primer Cren7F			TTCCGGTTGATCCYGCCGGACC
<i>Crenarchaeota</i> ,	RDP-II-8,	90%, -	RAGGCCAACTAGGRCGGCCTGG
<i>Crenarchaeota</i> ,	GreenGenes,	90%, -	DAGGCCAACTAGGRCGGCCTGG
<i>Euryarchaeota</i> ,	RDP-II-8,	90%, -	DAGRCMAACTAGGRCGGCYCTcc
<i>Euryarchaeota</i> ,	GreenGenes,	90%, -	AAGRCCAACTAGGACSGCYCTcc
" <i>Nanoarchaeota</i> ",	GreenGenes,	100%, -	GAGGgCAACTAGGACgCCYcc
" <i>Korarchaeota</i> ",	GreenGenes,	100%, -	. .GGCCAAACAWGRACGGCCTcc
<i>Bacteria</i> ,	RDP-II-8,	95%, -	HtctCaAACTARKAccGagTcy
<i>Eukaryota</i> ,	GreenGenes,	99%, -	HbctCaARCTAGKAMcGagTcc
primer Cren518R			GCTGGTWTTACCGCGCGGCTGA
<i>Crenarchaeota</i> ,	RDP-II-8,	95%, +	CGACCAWAATGGCGCGCGGACT
<i>Crenarchaeota</i> ,	GreenGenes,	95%, +	CGACCAWAATGGCGCGCGGACT
<i>Euryarchaeota</i> ,	RDP-II-8,	95%, +	CGVCCAYAAATGGCGCGCGGACT
<i>Euryarchaeota</i> ,	GreenGenes,	95%, +	CGSCCAYAAATGGCGCGCGGACT
" <i>Nanoarchaeota</i> ",	GreenGenes,	100%, +	CccCCAYAAgGGRCCGCGGAGc
" <i>Korarchaeota</i> ",	GreenGenes,	100%, +	CGACCAAAATGGCGCGCGGACT
<i>Bacteria</i> ,	RDP-II-8,	99%, +	NRdRCABARTGGCGCGGAGCAGCc
<i>Eukaryota</i> ,	GreenGenes,	99%, +	RRdRCDAATGGCGCGGAGCAGCc

FIG. 1. Primers Cren7F and Cren518R (5'→3') and consensus sequences of the potential annealing sites of 16S rRNA genes (3'→5') in organisms belonging to the phyla *Crenarchaeota*, *Euryarchaeota*, "*Korarchaeota*," and "*Nanoarchaeota*" and the domains *Bacteria* and *Eukaryota*. In the primer names, the numbers 7 and 518 refer to the locations of the sites in the 16S rRNA molecules (*E. coli* numbering). The percentages show the consensus levels (percentage of sequences having a particular nucleotide in a particular position). +, plus-strand DNA; -, minus-strand DNA. Mismatches are shown in lowercase letters; 3' mismatches, crucial for primer specificity, are shown in bold lowercase. Periods in the nucleotide sequences indicate a lack of information. Consensuses were deduced for sequences retrieved from the RDP-II release 8 and GreenGenes databases.

extension cycles were carried out. Final primer extension was carried out at 72°C for 10 min (our modification of the program from reference 16). Negative control amplifications without template DNA were performed routinely. Amplification products were confirmed by agarose gel electrophoresis and analyzed by DGGE. DGGE was performed with the Scie-Plas system (Warwickshire, England). The PCR samples were applied directly onto 8% (wt/vol) polyacrylamide gels in 0.5 × TAE (20 mM Tris acetate, pH 7.4, 10 mM sodium acetate, 0.5 mM EDTA) buffer. The 8% (wt/vol) polyacrylamide gels (acrylamide-*N,N'*-methylenebisacrylamide, 37.5:1; Bio-Rad Laboratories, Inc.) were made with denaturing gradients ranging from 35 to 65%, where 100% denaturant contains 7 M urea (Bio-Rad) and 40% (vol/vol) formamide (Fluka) deionized with AG501-X8 mixed-bed resin (Bio-Rad). The electrophoresis was performed at 70 V and a temperature of 60°C for 16 h. After electrophoresis, the gels were rinsed in water and stained with Sybr Gold (1:10,000 dilution; Molecular Probes, Leiden, The Netherlands) for 40 min, after which the banding patterns were examined under UV light. The bands of interest were punched from the gel with sterile pipette tips and placed in sterilized vials, and 20 µl of sterilized water was added. The DNA was allowed to passively diffuse into water at 4°C overnight. Three microliters of eluate was used as the template DNA in PCR with the Cren7F-Cren518R primer pair without a GC clamp, as described above.

Sequencing and phylogenetic inference. PCR products obtained after reamplification of the DNA from DGGE bands were visualized on agarose gels to confirm that the correctly sized band was present and to evaluate the amount of DNA. The PCR products were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, USA) and were then sequenced using a Big Dye Terminator kit, version 3.1, on an automatic ABI 3730 sequencer (Applied Biosystems, Inc.). The sequences were compared to 16S rRNA gene sequences from GenBank by BLASTN to retrieve closest relatives. The sequences were aligned with the use of Multalin software (11; <http://bioinfo.genopole-toulouse.prd.fr/multalin/>). Phylogenetic trees were inferred by performing neighbor joining with a Jukes-Cantor distance correction method implemented in the TREECON software package (51).

Enrichment cultures. Enrichments were set up by 10% inoculation of samples of water and mud into anaerobically prepared basal medium (39) with 200 mg liter⁻¹ yeast extract (Difco) and with or without elemental sulfur (10 g liter⁻¹). The medium was supplemented with the following substrates (2 g liter⁻¹): peptone, starch, a variety of individual sugars, yeast extract, individual amino acids, albumin, cellulosic substrates, or chitin. The enrichments from sample BL1017 (Lake Baikal region) were cultivated on 2 g liter⁻¹ of peptone at various temperatures (40, 50, 60, 70, and 80°C), various pH values (6.0, 6.5, 7.0, 7.5, 8.0, 9.0, and 10.0), and under various redox conditions (anaerobically with a reducing agent, anaerobically without reducing agent, and aerobically). The enrichment cultures from samples Kam940 and Kam920 (Uzon Caldera, Kamchatka) were obtained at 82°C, pH 6.2 to 6.5, and with 2 g liter⁻¹ of chitin; subsequently, culture Kam940 was cultivated with starch or peptone (2 g liter⁻¹) at 70°C and pH 6.0.

For isolation of colonies, the same medium was used with the addition of 1.5% agar. Colonies were obtained at 60°C after incubation for about 1 week. Individual colonies were picked and transferred to liquid medium. The purity of the isolates was tested by phase-contrast microscopy of cultures grown under various conditions.

Monitoring of enrichment cultures. DNA from the enrichment cultures was subjected to PCR amplification with the *Crenarchaeota*-specific primers Cren7F and Cren518R. Then, the PCR products were subjected to DGGE analysis, and the bands were sequenced.

Nucleotide sequence accession numbers. The sequences of the 16S rRNA gene fragments retrieved in this study from natural samples have been deposited in GenBank under accession numbers EU586809 to EU586828, and the 1,269-nucleotide 16S rRNA gene fragment of "*F. fontis*" strain Kam940 has been deposited under accession number EF552404.

RESULTS

Site description. The Kamchatka Peninsula is located in the northeast of Russia in the volcanically active area between the Central and Eastern Volcanic Belts. Kamchatka has numerous hydrothermal systems that constantly release geothermal gases and fluids out to the earth's surface. Geothermal gases such as N₂ and CO₂ dominate in the outflows, but H₂, CH₄, and H₂S also frequently occur. Hot spring waters in Kamchatka may have multiple origins, including meteoric and magmatic water. The temperature of these springs ranges from ~20°C to more than 90°C. Water chemistry also varies dramatically, with pH ranging from 3.1 to 9.8. Hydrothermal fluids are sodium chloride waters and contain various dissolved constituents including K⁺, H₃BO₃, H₄SiO₄, Ca²⁺, and SO₄²⁻ (56). The Uzon Caldera is situated 180 km northeast of Petropavlovsk. The Caldera contains numerous active hydrothermal fields that demonstrate a large variety in chemical composition, temperature, and pH of the springs. Significant hydrothermal activity also occurs in Geyser Valley, located to the east of the Uzon Caldera, and near Karymskii (54°03'N, 159°27'E) and Mutnovskii volcanoes (52°27'N, 158°12'E) (27, 50, 56).

The Lake Baikal region is located in the middle of Russia. Geologically, this area is a rift zone. In contrast to the hydrothermal vents in the areas of modern volcanism, the formation of hydrothermal vents of the Lake Baikal region is not affected by magmatic processes. The temperatures of venting waters reach 81 to 83°C, total salt content does not exceed 1 g liter⁻¹, pH is up to 10, and sulfide does not exceed 15.6 mg liter⁻¹. Hot springs of the Lake Baikal region contain typical alkaline waters with rather high concentrations of silicates (up to 100 mg liter⁻¹), HCO₃-Na, and SO₄-Na (35). In the waters of Urinskii spring, sulfide concentration was below the method of detection. The dominating gas in the Urinskii and Gusihinskii hot springs is N₂ (98% to 99%) (8).

Iceland is located on the Mid-Atlantic Ridge which traverses

the island from southwest to northeast, where the active spreading axis appears as a zone of active rifting and volcanism. The volcanic rift zone is characterized by active volcanoes, fissure swarms, numerous normal faults, and high-temperature geothermal fields. The Hveragerði high-temperature geothermal field, located about 50 km southwest of Reykjavik, is on the southern margin of the Hengill neovolcanic area. In the Hveragerði field, geothermal manifestations consist of fumaroles dominating in the north and hot springs, which are most abundant in the south. In the springs of this area, different values of temperatures and pH are observed, and volcanic gases occur, including H₂S, CO₂, and N₂ (55).

Primer design. The 16S rRNA gene sites chosen for *Crenarchaeota*-specific primers are shown in Fig. 1. The discriminating ability of these primers with respect to organisms other than *Crenarchaeota* is rooted in the presence of very strong unique signatures in positions 27, 28, and 518 (*Escherichia coli* numbering) in crenarchaeotal 16S rRNA genes. As shown by our analysis, these signatures, described by Winker and Woese (53) based on the analysis of a limited number of archaeal sequences, are still specific to *Crenarchaeota* a decade and a half after their original description. These signatures are adjoined by conserved sites of the 16S rRNA genes. Thus, primers Cren7F and Cren518R form perfect duplexes with 16S rRNA genes of most *Crenarchaeota* and exhibit 3'-terminal mismatches with 16S rRNA genes of all other organisms, and they should selectively prime PCR of *Crenarchaeota* 16S rRNA genes when used with a polymerase lacking 3'-to-5' exonuclease activity. A similar forward primer has previously been used successfully in combination with a reverse primer of narrower specificity, Cren457R, to amplify nonthermophilic *Crenarchaeota* from freshwater lake sediments (43).

In specificity tests, the annealing temperature was varied from 60°C to 85°C. A single PCR product of the expected size (about 500 bp) was produced on the DNA of all *Crenarchaeota* strains at temperatures between 65°C and 75°C though at higher temperatures no amplification occurred for some of the strains. No products were formed on the DNAs of negative controls at temperatures of 65°C and higher. For further work, the annealing temperature of 72°C was chosen as providing a safety margin for primer specificity.

Detection and phylogeny of *Crenarchaeota* in the samples. Eighty-four samples from different regions of the Kamchatka Peninsula: Uzon Caldera (47 samples), Geyser Valley (5 samples), Karymskii (5 samples), and Mutnovskii (2 samples) volcano areas, as well as from the Lake Baikal region (7 samples) and Iceland (18 samples), were analyzed by PCR with *Crenarchaeota*-specific primers (Table 1).

The presence of *Crenarchaeota* in 41 samples from the Kamchatka Peninsula could be inferred from positive PCR results (Table 1). For nine of these samples (all from Uzon Caldera), PCR products were analyzed by DGGE, which, however, produced no more than two distinct bands for each sample. The bands were analyzed by sequencing (Table 2; phylogenetic evaluation of crenarchaeotal sequences is shown in Fig. 2). Four samples (Kam1506, Kam1509, Kam1523, and Kam1529) from sites with temperatures rather low (57 to 70°C) for hyperthermophiles contained *Crenarchaeota* that were similar (with the level of similarity from 98 to 99%) to those representing the deep-branching phylogenetic lineage of clone

TABLE 1. Occurrence of *Crenarchaeota* in terrestrial hot springs of Kamchatka, the Lake Baikal region, and Iceland as studied by PCR

Region	No. of samples analyzed	pH	Temp (°C)	No. of <i>Crenarchaeota</i> -positive samples
Uzon Caldera, Kamchatka	47	2.5–7.0	50–86	33
Geyser Valley, Kamchatka	5	4.0–8.4	57–78	4
Karymskii Volcano, Kamchatka	5	5.1–7.5	48–91	3
Mutnovskii Volcano, Kamchatka	2	4.5–5.0	65–82	1
Lake Baikal region	7	8.4–8.9	59–71	1
Iceland	18	2.0–7.5	55–94	16

pSL12 found in Yellowstone National Park (2). In sample Kam1509 (58°C and pH 6.2), we also found a crenarchaeotal component related to *Caldisphaera draconis* (95% similarity). Two crenarchaeotal sequences detected in the Uzon samples Kam1514 and Kam1615 were related to the *Thermoproteales*. The phylotype from sample Kam1514 (80 to 82°C; pH 5.5) fell within the clade containing *Thermoproteus neutrophilus* (96% similarity). The phylotype from sample Kam1615 (75°C; pH 5.5) fell within the clade containing *Thermofilum pendens* (97% similarity). One sequence from sample Kam1521 was related to the group called “miscellaneous uncultured *Crenarchaeota*” by Takai et al. (49) and Kvist et al. (31). *Sulfolobales* and *Desulfurococcales* were not detected in the samples from Uzon Caldera, Kamchatka.

Of the seven Baikal region samples, only one, taken from a spring with a temperature of 59°C and pH 8.8 (sample BL1017), appeared to contain *Crenarchaeota*. The sequencing of the PCR product from sample BL1017 was carried out directly and showed the presence of only one phylotype, belonging to the deep-branching uncultivated crenarchaeotal group earlier detected in the Obsidian Pool, Yellowstone National Park, and represented by environmental clone pJP41 (3).

Of 16 *Crenarchaeota*-positive samples from Iceland, one sample (Is2; 72°C and pH 7.0) yielded a phylotype that fell into the deep-branching phylogenetic group of clone pSL12. Two sequences from samples V4 and Is6 fell into the group of the so-called “unknown *Desulfurococcales*” (31, 33). One sequence from sample G14 was related to the *Thermoproteales* group and fell within the clade containing *Thermocladium modestius* (92% similarity). Five crenarchaeotal sequences (V3, V5, Is5, Is9, and V6) found in samples from Iceland were related to the group of “miscellaneous uncultured *Crenarchaeota*.” The crenarchaeotal sequence from sample V6 (71°C; pH 6.5) was related to clone SUBT-13 from the clone library obtained from a subterranean hot spring in Iceland (32).

Cultivation of new representatives of *Crenarchaeota*. In order to get some information on the metabolic properties of the detected uncultured *Crenarchaeota*, we tried to obtain them in laboratory cultures on diverse media and under diverse conditions. Growth of several groups of thermophilic crenarchaeotes was obtained in enrichments inoculated with samples from Kamchatka and the Lake Baikal region.

(i) Uzon Caldera cultures. Two positive enrichments were obtained by inoculating samples Kam920 and Kam940 into

anaerobic basal medium with chitin as the growth substrate at pH 6.0 and an incubation temperature of 85°C. Cells in both enrichments were regular cocci. The enrichment cultures were monitored by PCR with *Crenarchaeota*-specific primers over the whole process of pure culture isolation. A pure culture was obtained by the isolation of a single colony from the enrichment Kam940. Cells of the new isolate were regular cocci of various sizes (1 to 3 µm) without flagella (Fig. 3). Isolate Kam940 was an anaerobic thermophile, growing in the temperature range of 55 to 85°C, with an optimum at 65 to 70°C. The pH optimum of growth was 5.5 to 6.0. Kam940 grew on peptone, starch, and yeast extract. Sulfur did not stimulate growth; H₂ (100% in gas phase) inhibited growth. Phylogenetic analyses of the 16S rRNA gene of strain Kam940 (1,291 bp) showed that strain Kam940 was not closely related to any cultured organism (no more than 87 to 89% sequence similarity). In the phylogenetic tree (Fig. 2), strain Kam940 clustered with uncultured clones from Yellowstone National Park and Iceland (31, 33; also this work) that were previously designated “uncultured *Desulfurococcales*.” On the basis of its phylogenetic position and new combination of phenotypic features, this isolate will be described elsewhere as a new genus, “*Fervidococcus*,” with the type species “*F. fontis*” (Perevalova et al., unpublished).

(ii) Lake Baikal region culture. Sample BL1017, which was shown to contain *Crenarchaeota* of the deep-branching phylogenetic lineage of clone pJP41, was used as the inoculum for organotrophic enrichment cultures. Primary enrichments were obtained under aerobic and anaerobic conditions with peptone as the growth substrate at pH 7.0 and at different temperatures (40, 50, 60, 65, 70, and 82°C). The enrichments were monitored by PCR with the specific primers Cren7F-Cren518R for the presence of *Crenarchaeota*. Only two of the enrichment variants were *Crenarchaeota* positive, namely, those incubated under anaerobic conditions at 60 or 65°C. The enrichment was subcultured on various substrates (starch, sugars, yeast extract or amino acids), at different pH values (6.0, 6.5, 7.0, 7.5, 8.0, 9.0, 10.0), under various redox conditions (anaerobic with and without reducing agent), and in the presence or absence of elemental sulfur (10 g liter⁻¹), and it was found to contain *Crenarchaeota* only when grown with peptone, starch, or yeast extract as the substrates, at pH 7.0 and 7.5, without elemental sulfur, and in the presence of a reducing agent. All of the *Crenarchaeota*-positive cultures contained only rod-shaped cells of different sizes. Thus, the pJP41-related organism from Urinskii hot spring could be a rod-shaped strict anaerobe, an organotroph not dependent on the presence of sulfur that is capable of growth within a rather narrow temperature interval (60°C to 65°C) and a pH interval of 7.0 to 7.5.

DISCUSSION

In this work we used *Crenarchaeota*-specific primers to detect *Crenarchaeota* in thermal habitats of Kamchatka, the Lake Baikal region, and Iceland, which had different pH and temperature values. Furthermore, we adopted these primers for carrying out DGGE analysis in order to reveal the phylogenetic position of the detected *Crenarchaeota*. All sequences obtained in the course of this work belonged to *Crenarchaeota*. These results, as well as our previous data (38), demonstrate

TABLE 2. Distribution of *Crenarchaeota* in hot springs of Kamchatka, the Lake Baikal region, and Iceland as studied by PCR and DGGE

Sample	Spring name and location	Temp (°C)	pH	Sample description	GPS	E _h (mV)	Closest relative (% 16S rRNA gene identity)
Kam1506	White Spring, Eastern thermal field, Uzon Caldera, Kamchatka	57	5.7	White sediment	54°29'58.3"N, 160°00'59.0"E	−160 (top); −300 (2 cm)	pSL12, hot spring clone (99)
Kam1509	Zavarzin Pool, Eastern thermal field, Uzon Caldera, Kamchatka	58	6.2	Mat	54°49'81.0"N, 160°01'46.0"E	−296	1509_1: pSL12, hot spring clone (98); 1509_2: <i>Caldisphaera draconis</i> (95)
Kam1521	Vertoletnyi Spring, Eastern thermal field, Uzon Caldera, Kamchatka	68	7.0	Gray filaments			YNP_SSp_A70, hot spring clone (96)
Kam1523	Zashchepin Pool, Eastern thermal field, Uzon Caldera, Kamchatka	70	7.0	Mat with filaments on the top			pSL12, hot spring clone (98)
Kam940 ^a	Treshchinnyi Spring, Eastern thermal field, Uzon Caldera, Kamchatka	84	6.5	Black mud	54°29'56.3"N, 160°00'55.9"E	−270	Hverd031N, hot spring clone (95)
Kam920 ^a	Eastern thermal field, Uzon Caldera, Kamchatka	78	5.5				Hverd031N, hot spring clone (92)
Kam1514	Central field, Uzon Caldera, Kamchatka	80–82	5.5	Black filaments		−180	<i>Thermoproteus neutrophilus</i> (96)
Kam1529	Orange Neutral Spring, Orange field, Uzon Caldera, Kamchatka	65–71	5.8–6.0	Mat	54°30'26.9"N, 160°00'02.7"E	−316	pSL12, hot spring clone (98)
Kam1615	Izvilistyi Spring, Central field, Uzon Caldera, Kamchatka	75	5.5	Gray sediment with white filaments		−250	<i>Thermofilum pendens</i> (97)
BL1017	Urinskii Spring, Lake Baikal region, Barguzin Valley	59.1	8.8	Bottom of stream, sediment	53°39'N, 110°07'E	−130	pJP41, hot spring clone (93)
Is2	Hverakjalki, Hveragerði region, Iceland	72	7.0	Black sediment			pSL12, hot spring clone (95)
Is3	Hverakjalki, Hveragerði region, Iceland	94	2.5	Mud			<i>Sulfolobus solfataricus</i> (100)
Is5	Reykjadalur, Hveragerði region, Iceland	73	ND ^b	Mud			pJP89, hot spring clone (94)
Is6	Reykjadalur, Hveragerði region, Iceland	55	6.0	Gray filaments			Hverd031N, hot spring clone (99)
Is9	Reykjadalur, Hveragerði region, Iceland	68.6	6.5	Gray filaments			YNP_ObP_A5, hot spring clone (92)
V5	Reykjadalur, Hveragerði region, Iceland	66	6.5	Gray filaments			Hverd044N, hot spring clone (89)
V6	Reykjadalur, Hveragerði region, Iceland	71	6.5	Yellow sediment			SUBT-13, subterranean hot environment clone (94)
G14	Reykjadalur, Hveragerði region, Iceland	70	5.0	Gray filaments			<i>Thermocladium modestius</i> (92)
V3	Graendalur, Hveragerði region, Iceland	69	7.5	White filaments			Hverd066N, hot spring clone (94)
V4	Graendalur, Hveragerði region, Iceland	71	7.0	Gray filaments			Hverd031N, hot spring clone (96)

^a Enrichment culture.^b ND, not determined.

the specificity of the Cren7F-Cren518R primer pair to *Crenarchaeota* representatives.

So far, the majority of the cultured *Crenarchaeota* are hyperthermophilic neutrophiles which have an optimal growth

temperature of 80 to 90°C. Scarce extreme thermophiles with growth temperature optima of 65 to 70°C are acidophiles and predominantly aerobes or facultative anaerobes belonging to *Sulfolobales*, with *Thermocladium modestius* and the two spe-

FIG. 2. Phylogenetic positions of crenarchaeotal phylotypes revealed in the present work in hot springs and enrichment cultures. Sequences of the ca. 500-bp 16S rRNA gene fragments retrieved from DGGE bands and reference sequences downloaded from the GenBank database were used for the construction of the neighbor-joining tree. Bootstrap values (per 1,000 trials) of 90% and higher are shown. Bar, 1 substitution per 10 sequence positions.

cies of *Caldisphaera* as the only obligate anaerobes in this group (7, 21, 22, 23). Among the crenarchaeotal sequences detected by us, there were sequences related to cultivated organisms of the orders *Thermoproteales* and *Sulfolobales* and to the *Acidilobus* group. The temperature and pH characteristics of the sampling sites where these sequences were detected were in good agreement with those of known cultivated representatives of the genera *Thermoproteus*, *Thermofilum*, *Thermocladium* (22), *Sulfolobus* (21), and *Caldisphaera* (7, 23).

In our search for new thermophilic *Crenarchaeota*, we stud-

ied many neutral hot springs that had water temperatures ranging from 55 to 70°C, i.e., exhibiting conditions not optimal for known cultured *Crenarchaeota*. Five crenarchaeotal sequences, four from different sites of Uzon Caldera, Kamchatka, and one from Hverakjalki, Iceland, belonged to the deep-branching *Crenarchaeota* lineage of clone pSL12, which has been proposed to form a clade with the marine nonthermophilic group of the *Crenarchaeota* (2). Sequences belonging to this lineage were also found in hot springs of Yellowstone National Park, as well as of Iceland, Italy, and Thailand (26, 31,

FIG. 3. Electron micrograph of strain Kam940 negatively stained whole cells. Bar, 1 μ m.

33, 46). Another deep-branching lineage of *Crenarchaeota* (clone pJP89 lineage), previously found only in Obsidian Pool, Yellowstone National Park (3, 33, 46), was detected by us in Icelandic hot springs (phylotypes Is9, Is5, and V5).

In one of the samples from Lake Baikal region (BL1017), we found a phylotype distantly related to a deep-branching *Crenarchaeota* lineage (clone pJP41 lineage), observed previously in Obsidian Pool, Yellowstone National Park (3), and the Hveragerði area, Iceland (31). Our attempts to cultivate this organism under various cultivation conditions led us to the conclusion that the pJP41-related *Crenarchaeota* could be moderately thermophilic, neutrophilic, or moderately alkali-philic anaerobic organotrophs not dependent on the presence of elemental sulfur.

Given the trees constructed by Pace et al. (2, 12) and our tree (Fig. 2), at least two thermophilic lineages occur within the radiation of nonthermophilic *Crenarchaeota*—the clone pSL12 lineage and the clone pJP89 lineage. In contrast, the clone pJP41 phylogenetic lineage appears to be an early offspring of the (hyper)thermophilic *Crenarchaeota*. These phylogenetic positions are supported by the occurrence of signatures 289C: 311G, 501G, and 504A in clones pSL12 and pJP89 and our phylotypes related to them and by the presence of only 289C: 311G in pJP41 and our phylotypes related to it (Table 3).

The distribution of the four groups of uncultured thermophilic crenarchaeotes (the pSL12, pJP89, pJP41, and “*Fervidococcus*” groups) in natural environments is shown in Fig. 4 in temperature-pH coordinates. It appears from the diagram that pSL12-related sequences were previously found in a rather narrow range of temperatures (74 to 81°C) and pH values (5.7 to 7.6). Geochemical data for the hot springs of Iceland were not reported, whereas Obsidian Pool is known to contain H_2S and high concentrations of H_2 , Fe^{2+} , and NH_4^+ (3, 33, 46). Our data on Uzon Caldera, Kamchatka, showed that this group of organisms is also present in hot springs having temperatures from 57°C to 75°C. The habitats of these organisms in Uzon Caldera are characterized by the presence of sulfide and low redox conditions. These observations confirm that the deep-branching phylogenetic lineage of nonthermophilic crenarchaeotes is not limited to low-temperature environments but actually includes thermophilic subbranches. These organisms are most probably moderate thermophiles and neutro-

TABLE 3. Sequence signatures of 16S rRNAs of uncultured moderately thermophilic *Crenarchaeota*

Sequence position(s) ^a	Base(s) specific to ^b :					
	<i>Euryarchaeota</i>	<i>Thermoprotei</i>	NTCR	pSL12 lineage	pJP89 lineage	pJP41 lineage
289:311	Y:R	G:C	C:G	C:G	C:G	C:G
501	H	C	G	G	G	C
504	Y	G	W	A	A	G

^a Sequence positions are specified in *E. coli* numbering.

^b Aligned sequences of *Euryarchaeota* and *Thermoprotei* (orders *Desulfurococcales*, *Thermoproteales*, and *Sulfolobales*) were retrieved from the GreenGenes database. Aligned sequences of nonthermophilic *Crenarchaeota* (NTCR) were retrieved from the RDP-II release 8 database. pSL12 lineage here means clone pSL12 and our clones Kam1529, Kam1509, Kam1523, Kam1506, Is2; pJP89 lineage means clone pJP89 and our clone Is9 (V5 and Is5 sequences were too short for analysis); pJP41 lineage means clone pJP41 and our clones BL107 and V3. Data for clones pJP89 and pJP41 are from Barns et al. (3). For all bases, frequency was at least 97%. Underlining indicates 100% occurrence of particular nucleotides. Colons indicate base pairing.

philes. Furthermore, as we mentioned above, the clone pSL12 lineage is adjacent to the group of nonthermophilic marine and soil *Crenarchaeota*, in which the presence of *amoA* genes (44) or nitrification capacity (13, 20, 29) has repeatedly been demonstrated by metagenomics or cultural methods, respectively. It cannot be excluded that the microorganisms related to the clone pSL12 lineage function as nitrifiers in the hot springs of Uzon Caldera, where ammonia ions are present.

The information on the habitats of pJP41-related and pJP89-related organisms is rather scarce and consists only of Obsidian Pool parameters (74 to 81°C and pH 5.7 to 7.0). This information is extended by our data for the Icelandic hot spring (69°C; pH 6.5), where a pJP89-related phylotype was detected, and for the Baikal region hot spring (59°C; pH 8.8), where the pJP41-related organism was found. The growth characteristics of the pJP41-related crenarchaeote present in enrichment culture BL1017 (60 to 65°C and pH 7.0 to 7.5) were

FIG. 4. Temperature and pH of hot springs where phylotypes related to the pSL12 (squares), pJP89 (asterisks), pJP41 (triangles), and “*Fervidococcus*” (circles) lineages were found. Filled symbols represent data obtained in this work; empty symbols represent data of other researchers (3, 31, 33, 46). The relatedness groups were defined as sharing at least 93% identity. Actually, the identity values were usually higher than 97%, with few or no sequences with identities in the range of 93 to 97%.

somewhere in between the parameters of these two natural habitats. Baikal habitat differs from Obsidian Pool by the absence of sulfide although the redox potential of water there was also low, corresponding to the anaerobic nature of the pJP41-related crenarchaeote BL1017.

Monitoring with *Crenarchaeota*-specific primers also allowed us to isolate the first representative of “uncultured *Desulfurococcals*,” which we have named “*F. fontis*.” Environmental clones of the “*Fervidococcus*” group were found in Yellowstone National Park in the Sylvan and Bison springs (33), which have temperatures of 81 to 82.6°C and pH values of 5.5 to 8.1, and in the Hveragerði area (Iceland) (31), but geochemical data on the hot springs of Iceland were not reported. According to our data, the temperature and pH range of the hot springs where the “*Fervidococcus*”-related phylotypes were detected is very broad. These data are in good agreement with the “*F. fontis*” growth parameters.

It follows from the diagram in Fig. 4 that there could be a large and diverse group of *Crenarchaeota* that inhabit terrestrial hot springs with temperatures that are moderate (55 to 70°C) compared with the parameters of the usual habitats of hyperthermophilic cultured *Crenarchaeota*. The adaptation of the former *Crenarchaeota* group to moderate-temperature habitats is evidenced by the data on the G+C contents of their 16S rRNAs. The G+C contents of the 16S rRNAs are known to correlate with the temperature growth optima of the organisms (18, 28). Our analysis showed that the G+C content of the 16S rRNA fragment flanked by Cren7F and Cren518R primers also shows good correlation with the temperature optimum. For example, the G+C content of this fragment in the *Desulfurococcals* in the GreenGenes database is 62 to 72 mol%, whereas that of marine nonthermophilic *Crenarchaeota* is 45 to 51 mol%. The G+C content of this fragment in our pSL12-related phylotypes was 56 to 57 mol%, which correlates with the temperature characteristics of their habitats. In our “*Fervidococcus*”-related phylotypes, this value was 62 to 63 mol%, which correlates with the temperature characteristics of habitats and with those of “*F. fontis*” growth.

Thus, the combination of culture-independent and cultivation-based methods applied to microbial communities of hot springs of Russia and Iceland allowed us to extend the knowledge of the biodiversity of *Crenarchaeota* representatives. This work also sheds light on the ecological function of some *Crenarchaeota* groups commonly referred to as uncultivated. “*F. fontis*” is a strict anaerobe, organotroph, and neutrophile, growing at temperatures lower than other anaerobic neutrophilic *Crenarchaeota*. Thus, the organisms of this group share an ecological niche with many thermophilic bacteria. This seems to explain how these organisms have so far escaped cultivation under laboratory conditions as they are outcompeted by bacteria at lower temperatures and by hyperthermophilic archaea at higher temperatures.

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