Isolation of the anaerobic thermoacidophilic crenarchaeote *Acidilobus saccharovorans* sp. nov. and proposal of *Acidilobales* ord. nov., including *Acidilobaceae* fam. nov. and *Caldisphaeraceae* fam. nov.

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An anaerobic acidophilic, hyperthermophilic archaeon, designated strain 345-15^T, was isolated from an acidic hot spring of Kamchatka (Russia). Cells of strain 345-15^T were regular or irregular cocci, 1–2 µm in diameter, with flagella. Strain 345-15^T grew optimally at 80–85 °C and pH 3.5–4.0 and fermented a wide range of carbohydrates, including polysaccharides. Acetate, ethanol and lactate were the fermentation products. Growth was stimulated by elemental sulfur and thiosulfate, which were reduced to hydrogen sulfide. The G + C content of the DNA was 54.5 mol%. The 16S rRNA gene sequence analysis indicated that strain 345-15^T belonged to the genus *Acidilobus*. The level of DNA–DNA hybridization between strain 345-15^T and *Acidilobus aceticus* 1904^T was 61 %. Thus, strain 345-15^T was considered as representing a novel species of the genus *Acidilobus*, with the name *Acidilobus saccharovorans* sp. nov. (type strain, 345-15^T=DSM 16705^T=VKM B-2471^T), which shared the main morphological and physiological properties of the genus but differed by the presence of flagella and the spectrum of substrates utilized. Phylogenetic analysis showed that the genus *Acidilobus*, with its species *Acidilobus aceticus*, *Acidilobus saccharovorans* sp. nov. and '*Acidilobus sulfurireducens*', and the genus *Caldisphaera*, represented by *Caldisphaera lagunensis* and '*Caldisphaera draconis*', formed a separate cluster that adjoins the cluster formed by the species of the order *Desulfurococcales*. Members of the *Acidilobus*–*Caldisphaera* cluster are thermophilic, organotrophic anaerobic cocci that can be distinguished from all species of the order *Desulfurococcales* on the basis of acidophily. Based on these considerations, we propose a new family, *Acidilobaceae* fam. nov., to accommodate the subcluster of hyperthermophiles represented by the genus *Acidilobus*, a new family, *Caldisphaeraceae* fam. nov., for the subcluster of extreme thermophiles represented by the genus *Caldisphaera*, and a new order, *Acidilobales* ord. nov., to accommodate the two new families.

Obligately anaerobic thermoacidophilic archaea are represented by only a few genera. The only representative of the phylum *Euryarchaeota* is '*Aciduliprofundum boonei*' (Reysenbach *et al.*, 2006), the first thermoacidophilic micro-organism isolated from deep-sea hydrothermal vents. All of the other anaerobic thermoacidophiles belong

to the phylum *Crenarchaeota* and were isolated from terrestrial hot springs and sea-shore hot vents. Obligately respiring, anaerobic, chemolithotrophic thermoacidophiles that grow by means of reduction of elemental sulfur with molecular hydrogen belong to the genus *Stygiolobus* (Seeger *et al.*, 1991). Organotrophic thermoacidophilic crenarchaeota representing the genera *Thermocodium* (Itoh *et al.*, 1998), *Caldivirga* (Itoh *et al.*, 1999) and *Vulcanisaeta* (Itoh *et al.*, 2002) were described as anaerobes able to tolerate low levels of oxygen. Another group of organotrophic thermoacidophilic archaea includes the genera *Acidilobus* (Prokofeva *et al.*, 2000) and

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 345-15^T is AY350586.

A table showing the characteristics of strain 345-15^T and closely related species is available as supplementary material with the online version of this paper.

Caldisphaera (Itoh *et al.*, 2003). Whereas the type species of the first genus is an obligate anaerobe, *Caldisphaera lagunensis* has been reported to be able to grow in the presence of low oxygen concentrations. Initially the genera *Acidilobus* and *Caldisphaera* were represented by a single species – *Acidilobus aceticus* isolated from an acidic hot spring near Moutnovskii Volcano (Kamchatka, Russia) and *Caldisphaera lagunensis* obtained from an acidic hot spring at Mt Maquiling (Laguna, Philippines). Later, another two species of these genera – ‘*Acidilobus sulfurireducens*’ and ‘*Caldisphaera draconis*’ – were isolated from acidic hot springs of Yellowstone National Park (Boyd *et al.*, 2007). It was shown that micro-organisms of this group comprise a significant part of microbial communities in sulfur-rich, acidic hot springs (12.2–32.3 % of the total microbial population for ‘*Caldisphaera draconis*’ and 5.3–7.9 % for ‘*Acidilobus sulfurireducens*’). Strains and 16S rRNA clones exhibiting a 93–97 % nucleotide sequence similarity with the type species of the genera *Acidilobus* or *Caldisphaera* were obtained from acidic hot springs of different areas of Kamchatka (Prokofeva *et al.*, 2005), Yellowstone National Park (Meyer-Dombard *et al.*, 2005; Korf *et al.*, 2006; Boyd *et al.*, 2007), and Lassen Volcanic National Park in California, USA (Siering *et al.*, 2006), showing the wide distribution of these genera.

Here we present the characterization of a novel thermoacidophilic archaeon belonging to the genus *Acidilobus* and propose a new order, *Acidilobales* ord. nov., which includes the families *Acidilobaceae* fam. nov. and *Caldisphaeraceae* fam. nov.

Sample collection, enrichment and isolation

For cultivation, the following basal medium was used (g l⁻¹): NH₄Cl, 0.33; KCl, 0.33; KH₂PO₄, 0.33; CaCl₂·2H₂O, 0.33; MgCl₂·6H₂O, 0.33; trace elements (Kevbrin & Zavarzin, 1992), 10 ml l⁻¹; vitamins (Wolin *et al.*, 1963), 10 ml l⁻¹. Starch or sucrose (2 g l⁻¹) were added as substrates in the presence of yeast extract (0.1 g l⁻¹). The medium was prepared anaerobically (10 min of boiling and reduction with 600 mg Na₂S·9H₂O l⁻¹) and dispensed as 10 ml portions into 15 ml Hungate tubes; CO₂ was used as the gas phase. The pH of the medium, measured at 20 °C using a pH meter calibrated at 20 °C, was adjusted with 3 M HCl or H₂SO₄. The pH and cultivation temperature were close to those at the respective sampling sites. Enrichment cultures were obtained at 85 °C and pH 3.5–4.0 from samples of Uzon Caldera and Moutnovskii Volcano, Kamchatka and Golovnin Caldera, Kunashir, Kuril Islands. These enrichments have been described previously (Prokofeva *et al.*, 2005). The dominant micro-organisms from these cultures were isolated from the highest positive dilution after two rounds of serial dilutions on medium containing 2 g maltose l⁻¹. Strain 345-15^T was isolated from a sample (mixture of water and mud) from a hot acidic pool (T=85 °C, pH 3.5–4.0) with grey loamy mud (54° 30' 40" N 160° 00' 06" E) located on the Orange Thermal Field

(Uzon Caldera, Kamchatka, Russia; see also Perevalova *et al.*, 2008) and was chosen for detailed characterization.

Morphology and growth characteristics

Cells of strain 345-15^T were regular to irregular cocci, 1–2 µm in diameter. Motility was not observed using a light microscope (MBI-3 with phase-contrast; LOMO). However, in electron micrographs of whole exponential-phase cells of strain 345-15^T stained negatively [with 2 % phosphotungstic acid on Formvar (Serva)-coated copper grids and examined using a JEM-100B (JEOL) electron microscope operated at 60 kV], they were found to possess flagella (Fig. 1a). To obtain thin sections, cells were fixed with 5 % glutaraldehyde for 2 h at 4 °C. Additional fixation was also performed with 1 % OsO₄ for 4 h at 4 °C. Cells were embedded in epon-812, and were thin-sectioned on microtome LKB-3R and stained with uranyl acetate and lead citrate. The cell envelope was about 30 nm thick (Fig. 1b).

Growth of isolate 345-15^T occurred at a temperature range of 60–90 °C, with optimum growth at 80–85 °C (no growth occurred at 58 or 94 °C) and at a pH range of

Fig. 1. Electron micrographs of exponential-phase cells of strain 345-15^T: (a) whole cells negatively stained with phosphotungstic acid; (b) ultrathin sections stained with uranyl-acetate and lead citrate. Bars, 0.5 µm.

2.5–5.8, with optimum growth at 3.5–4.0 (no growth occurred at pH 2.0 or 6.0).

Potential growth substrates were added to the basal medium at concentrations of 2 g l⁻¹ (organic acids as their sodium salts). Potential electron acceptors added to the basal medium were elemental sulfur as sulfur powder (10 g l⁻¹), thiosulfate and nitrate as sodium salts (each as 0.5, 1.0 and 2.5 g l⁻¹) or Fe(III) citrate (20 mM). The headspace was filled with 100 % CO₂; when molecular hydrogen was tested as the growth substrate, the gas phase used was a mixture of H₂ and CO₂ (8:2, v/v). All growth experiments were carried out in triplicate and (minimum) in three transfers. Growth was measured by direct cell count under a light microscope. Gases, alcohols and volatile fatty acids were determined on GC 'Cristal 5000.1' with FID and TCD (Chromatek, Russia) and lactate – by HPLC on GC 'Stayer' with UV-detector (220 nm, Akvilon, Russia) and column Rezex RDA (Phenomenex, USA), eluent – 0.2 % H₃PO₄. H₂S was determined as described previously (Prokofeva *et al.*, 2005).

Strain 345-15^T was able to grow on yeast extract, beef extract, soya extract, peptone, starch, glucose, lactose, maltose, sucrose, fructose, galactose, lichenan, laminarin, arbutin, amygdalin and xylan. Yeast extract (0.1 g l⁻¹) and CO₂ in the gas phase were required for growth. No growth occurred with acetate, arabinose, cellulose, formate, glycine, gelatin, gum guar, malate, methanol, pectin, pyruvate, propionate, trehalose, ribose, xylose, fumarate, microcrystalline cellulose, glycine or succinate. The products of glucose and yeast extract fermentation were acetate, ethanol and lactate. Molecular hydrogen was not detected. Growth on sugars and yeast extract was stimulated by elemental sulfur and thiosulfate, which were reduced to hydrogen sulfide, whereas nitrate and Fe(III) citrate did not stimulate growth of strain 345-15^T.

Chloramphenicol (100 mg l⁻¹), penicillin (500 mg l⁻¹) and streptomycin (500 mg l⁻¹) were used to test antibiotic resistance. Strain 345-15^T appeared to be resistant to all of the antibiotics tested.

DNA composition and phylogenetic analysis

DNA was isolated by using the procedure of Marmur (1961). The G + C content of the DNA was determined by using the thermal denaturation method (Owen *et al.*, 1969). The DNA G + C content was 54.5 ± 0.5 mol% (SEM). 16S rRNA genes were amplified using the *Archaea*-specific primer 4F (5'-TCCGGTTGATCCT-GCCRG-3') and the universal primer 1492R (5'-CGGTTACCTTGTTAGGACTT-3'). The PCR products were purified from low-melting agarose using a 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 on an ABI 3730 DNA automatic sequencer (Applied Biosystems). Preliminary phylogenetic analysis of the newly determined sequences was done with the NCBI BLAST server ([http://](http://www.ncbi.nlm.nih.gov/BLAST/)

www.ncbi.nlm.nih.gov/BLAST/). The nucleotide sequences were aligned using MAFFT v. 6 (Katoh *et al.*, 2002) with sequences retrieved from GenBank. Positions exhibiting more than 50 % variability were discarded using a specially written program. Phylogenetic trees were reconstructed using TREECONW (Van de Peer & De Wachter, 1994) with the neighbour-joining method after calculation of the matrix of evolutionary distances with the Jukes and Cantor correction (Jukes & Cantor, 1969). 16S rRNA gene analysis showed that the new isolates belonged to the *Crenarchaeota* phylum of the domain *Archaea* and were closely related to *Acidilobus aceticus*. The level of 16S rRNA gene similarity between strain 345-15^T and *Acidilobus aceticus* 1904^T was 98.1 % [when distinctions due to Ns (nucleotides determined ambiguously) were neglected]. The phylogenetic position of strain 345-15^T is shown in Fig. 2. DNA–DNA hybridization between strain 345-15^T and *Acidilobus aceticus* 1904^T was performed by using the method of De Ley *et al.* (1970) and showed a level of 61 % DNA–DNA hybridization.

Comparison with related species

Strain 345-15^T was found to be close to *Acidilobus aceticus* both phylogenetically and phenotypically: it is a hyperthermophilic acidophilic coccus that grows anaerobically on organic substrates; its growth is stimulated by sulfur. However, it differs from *Acidilobus aceticus* by having a much wider spectrum of substrates utilized: in addition to yeast extract and starch, which are the only growth substrates used by *Acidilobus aceticus*, strain 345-15^T can also utilize mono- and disaccharides. Based on these phenotypic differences, as well as the level of 16S rRNA gene sequence similarity and the results of DNA–DNA hybridization with *Acidilobus aceticus* 1904^T, we propose that strain 345-15^T should be assigned as representing a novel species of the genus *Acidilobus* – *Acidilobus saccharovorans* sp. nov. Phylogenetically, strain 345-15^T is most close to '*Caldococcus noboribetus*' (Aoshima *et al.*, 1996): the level of 16S rRNA gene similarity between strain 345-15^T and '*Caldococcus noboribetus*' was 99 %. The two organisms have certain phenotypic features in common (see Supplementary Table S1, available in IJSEM Online). However, '*Caldococcus noboribetus*' still lacks a detailed physiological description. Another *Acidilobus* species, '*Acidilobus sulfurireducens*', was described recently by Boyd *et al.* (2007), but the name has not been validly published. Strain 345-15^T differed from '*Acidilobus sulfurireducens*' in the list of substrates used and in the optimum pH (Supplementary Table S1). The level of 16S rRNA gene similarity between strain 345-15^T and '*Acidilobus sulfurireducens*' was 96.6 %.

GDGT composition

The isoprenoid glycerol dialkyl glycerol tetraether (GDGT) composition of strain 345-15^T was determined by Pearson *et al.* (2008) and included GDGT-3 (3 cyclopentane rings,

Fig. 2. Phylogenetic dendrogram showing the position of strain 345-15^T within the phylum *Crenarchaeota*. The dendrogram was constructed proceeding from 16S rRNA gene sequences of the type strains of type species of all recognized crenarchaeotal genera, as well as some additional sequences. Numbers at branch points specify the reliability of the branching order determined for 1000 resamplings; only bootstrap values of 90 % and higher are shown. The triangles represent the *Sulfolobales* representatives *Acidianus infernus* DSM 3191^T (D85505), *Metallosphaera sedula* DSM 5348^T (X90481), *Stygiolobus azoricus* DSM 6296^T (D85520), *Sulfolobus acidocaldarius* DSM 639^T (D14876) and *Sulfurisphaera ohwakuensis* DSM 12421^T (D85507), and the *Thermoproteales* representatives *Thermofilum pendens* DSM 2475^T (X14835), *Caldivirga maquilensis* DSM 13496^T (AB013926), *Pyrobaculum islandicum* DSM 4184^T (L07511), *Thermocodium modestius* JCM 10088^T (AB005296), *Thermoproteus tenax* DSM 2078^T (M35966), and *Vulcanisaeta distributa* DSM 14429^T (AB063630).

m/z 1296), GDGT-4 and GDGT-4' (4 cyclopentane rings, *m/z* 1294), isocrenarchaeol GDGT-5' (4 cyclopentane + 1 cyclohexane rings, *m/z* 1292), as well as GDGT-6 and GDGT-6' (six rings, *m/z* 1290), representing 1, 14, 2, 27, 54 and 2 % of the total GDGTs in the lipid fraction of strain 345-15^T. In the same work, the GDGT composition of the type strain of *Acidilobus aceticus* was shown to include acyclic caldarchaeol GDGT-0 (*m/z* 1302), GDGT-4 and GDGT-5', representing 20, 34 and 46 % of the total GDGTs. The GDGT composition of '*Acidilobus sulfuri-reducens*' includes GDGT-4, GDGT-5', GDGT-6 and GDGT-6', representing 4, 36, 55 and 5 % of the total GDGTs (Boyd *et al.*, 2007).

Conclusion and proposal of new taxa

In the latest edition of *Bergey's Manual* (Garrity *et al.*, 2005), the genus *Acidilobus* was included in the order *Desulfurococcales*. At the same time, it was proposed to establish the order *Caldisphaerales* to include a single genus, *Caldisphaera* (Itoh *et al.*, 2003), which at present contains two species, *Caldisphaera lagunensis* and '*Caldisphaera draconis*' (Boyd *et al.*, 2007). The genera *Caldisphaera* and *Acidilobus* are distantly but specifically related, as shown by their clustering in the 16S rRNA gene-based phylogenetic trees with a high level of bootstrap support (Fig. 2), and the presence of common nucleotide signatures in their 16S rRNA genes, distinguishing them from other representatives of the *Crenarchaeota* (Table 1). Organisms of this group exhibit common phenotypic

properties, such as a coccoid cell shape and acidophily (optimum pH 3.0–4.0); they are obligate anaerobes, extremely thermophilic (*Caldisphaera* spp.) or hyperthermophilic (*Acidilobus* spp., '*Caldococcus noboribetus*') and organotrophic micro-organisms, capable of using elemental sulfur as electron acceptor. This group also includes poorly characterized strains and environmental rRNA gene clones. Strains and clones exhibiting 93–97 % nucleotide sequence similarity with *Acidilobus aceticus* or with *Caldisphaera lagunensis* were obtained from various acidic hot springs of different areas (Prokofeva *et al.*, 2005; Meyer-Dombard *et al.*, 2005; Korf *et al.*, 2006; Siering *et al.*, 2006). These strains and clones differ markedly in the DNA G + C content (66.9–69.1 mol% for *Acidilobus* relatives and 61.2–63.9 mol% for related *Caldisphaera* species), which is known to be positively correlated with the temperature optimum of growth (Galtier & Lobry, 1997; Lebedinsky *et al.*, 2007).

Taking into account the separate position of the *Acidilobus*–*Caldisphaera* group in the 16S rRNA gene-based phylogenetic tree, the presence in the 16S rRNA gene of common specific signatures, and common phenotypic properties distinguishing the representatives of this group from *Desulfurococcales* and other *Crenarchaeota*, as well as their wide distribution in natural thermal ecosystems, we propose a new order, *Acidilobales* ord. nov., to accommodate this group. The evolutionary divergence of this group may be related to adaptation to anaerobic environments in terrestrial hot springs with low pH values. It should be noted that, in *Archaea*, acidophily usually correlates with

Table 1. 16S rRNA sequence signatures that distinguish *Acidilobus* and *Caldisphaera* from other *Crenarchaeota* and from each other

Signatures were deduced from alignments retrieved from the GreenGenes database updated on June 16 2008 (623 crenarchaeotal sequences). A particular letter signifies no less than 97 % occurrence of the corresponding nucleotide.

Sequence position*	Corresponding nucleotides			
	<i>Acidilobus</i>	<i>Caldisphaera</i>	<i>Desulfurococcales</i> , <i>Thermoproteales</i> , <i>Sulfolobales</i>	All <i>Crenarchaeota</i>
62	U	C	U	U
321 : 332	C : G	C : G	A : G	A : G
678	C	U	U	U
1056	U	C	U	U
1310 : 1327	G : C	A : U	A : U	A : U
1335	C	C	G	A, C, G
1393	U	U	C	C

**Escherichia coli* numbering.

phylogenetic position: among the *Crenarchaeota*, apart from the members of the group under consideration, all members of the order *Sulfolobales* (aerobes) and certain representatives of the order *Thermoproteales* (anaerobes) are acidophilic. In *Euryarchaeota*, all members of the order *Thermoplasmatales* and the only cultured representative of the deep DHVE2 phylogenetic lineage – ‘*Aciduliprofundum boonei*’ are acidophilic.

Based on the distinct positions of *Acidilobus* and *Caldisphaera* in the phylogenetic tree, the existence of specific 16S rRNA signatures that distinguish them (Table 1), as well as different ranges of growth temperatures, we propose two families within the order *Acidilobales*: family *Acidilobaceae* fam. nov., represented by the hyperthermophilic genus *Acidilobus* and by the phylogenetically close species ‘*Caldococcus noboribetus*’ and environmental clones; and the family *Caldisphaeraceae* fam. nov., represented by the extremely thermophilic genus *Caldisphaera*, and by related environmental clones.

Description of *Acidilobales* ord. nov.

Acidilobales (A.ci.di.lo.ba'les. N.L. masc. n. *Acidilobus* the type genus of the order, suff. -ales ending denoting an order; N.L. fem. pl. n. *Acidilobales* the order of *Acidilobus*).

Cells are regular to irregular cocci. Anaerobic. Extremely thermophilic or hyperthermophilic. Acidophilic. Organotrophic, able to use carbohydrates and peptides in the absence of electron acceptors or in the presence of sulfur. Elemental sulfur stimulates growth. Habitats are terrestrial acidic hot springs. The type genus is *Acidilobus* Prokofeva *et al.* 2000.

Includes *Acidilobaceae* fam. nov. and *Caldisphaeraceae* fam. nov.

Description of *Acidilobaceae* fam. nov.

Acidilobaceae (A.ci.di.lo.ba.ce'a.e. N.L. masc. n. *Acidilobus* the type genus of the family, suff. -aceae ending denoting a

family; N.L. fem. pl. n. *Acidilobaceae* the family of *Acidilobus*).

Cells are regular to irregular cocci, 1–2 µm in diameter. Strictly anaerobic. Hyperthermophilic. Acidophilic. Organotrophic, able to use carbohydrates and peptides. Hydrogen is not formed even in the absence of electron acceptors. Growth is stimulated by the presence of elemental sulfur. Resistant to chloramphenicol, penicillin and streptomycin. Habitats are acidic terrestrial hot springs. The type genus is *Acidilobus* Prokofeva *et al.* 2000.

Description of *Caldisphaeraceae* fam. nov.

Caldisphaeraceae (Cal.di.spha.e.ra.ce'a.e. N.L. fem. n. *Caldisphaera* the type genus of the family, suff. -aceae ending denoting a family; N.L. fem. pl. n. *Caldisphaeraceae* the family of *Caldisphaera*).

Cells are regular cocci, 0.8–1.1 µm in diameter. Anaerobic. Extremely thermophilic. Acidophilic. Organotrophic, able to use carbohydrates and peptides in the absence of electron acceptors. Growth is stimulated by the presence of sulfur. Resistant to chloramphenicol, kanamycin, oleandomycin, streptomycin and vancomycin. Habitats are terrestrial acidic hot springs. The type genus is *Caldisphaera* Itoh *et al.* 2003.

Emended description of the genus *Acidilobus*

Cell envelope consists of an S-layer covering the cytoplasmic membrane. Hyperthermophiles with a temperature range of 60–92 °C and an optimum of 80–85 °C. Acidophiles with a pH range of 2.0 to 6.0 (optimum at pH 3.5–4.0). Obligate anaerobes. Organotrophs. Peptides, polysaccharides, and monosaccharides can serve as energy and carbon sources in the absence of electron acceptors. Acetate, ethanol and lactate are the fermentation products. Hydrogen is not produced. Elemental sulfur stimulates growth and is reduced to hydrogen sulfide. Resistant to

chloramphenicol, penicillin and streptomycin. The G+C content of the total DNA is around 53–60 mol%. The type species is *Acidilobus aceticus*. Habitats: terrestrial acidic hot springs.

Description of *Acidilobus saccharovorans* sp. nov.

Acidilobus saccharovorans (sac.cha.ro.vo'rans. Gr. neut. n. saccharon sugar; L. part. adj. vorans devouring; N.L. masc. adj. saccharovorans sugar-devouring).

Exhibits the following properties in addition to those given in the genus description. Cells are regular to irregular cocci, 1–2 µm in diameter. Growth occurs at 60–90 °C (optimum, 80–85 °C) and at pH 2.5–5.8 (optimum, 3.5–4.0). Strictly anaerobic. Heterotrophic. Growth occurs on yeast extract, peptone, starch, glucose, lactose, maltose, sucrose, fructose, galactose, lichenan, laminarin, arbutin, amygdalin and xylan. Elemental sulfur and thiosulfate stimulate growth on yeast extract but are not obligately required. Growth product from sugars are acetate, ethanol, lactate and, in the presence of S⁰, H₂S. The DNA G+C content of the type strain is 54.5 mol%.

The type strain, 345-15^T (=DSM 16705^T=VKM B-2471^T), was isolated from a hot acidic pool in the Orange Thermal Field of Uzon Caldera, Kamchatka, Russia.

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