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 ºC 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 (Segerer et al., 1991). Organotrophic thermoacidophilic crenarchaeota representing the genera Thermocladium (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...
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 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 (Siring et al., 2006), showing the wide distribution of these genera.

Here we present the characterization of a novel thermoacidophilic archaean 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 345-15ᵀ 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 345-15ᵀ 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 345-15ᵀ 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 microtone LKB-3R and stained with uranil acetate and lead citrate. The cell envelope was about 30 nm thick (Fig. 1b).

Growth of isolate 345-15ᵀ 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 3.5–4.0.

**Fig. 1.** Electron micrographs of exponential-phase cells of strain 345-15ᵀ: (a) whole cells negatively stained with phosphotungstic acid; (b) ultrathin sections stained with uranil-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 % CO\(_2\); when molecular hydrogen was tested as the growth substrate, the gas phase used was a mixture of H\(_2\) and CO\(_2\) (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 % H\(_2\)PO\(_4\). H\(_2\)S 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 CO\(_2\) 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 *Archaean*-specific primer 4F (5’-TCCGTTGATCCT-GCCRG-3’) and the universal primer 1492R (5’-CGGTACCTTGGAGACTT-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://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 showing more than 50 % variability were discarded using a specially written program. Phylogenetic trees were reconstructed using TREEXONW (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 restrictions 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,
contains two species, Caldisphaera genus, Acidilobus 2005), the genus to establish the order Caldisphaerales Desulfurococcales. 'Organisms of this group exhibit common phenotypic from other representatives of the signatures in their 16S rRNA genes, distinguishing them based phylogenetic trees with a high level of bootstrap related, as shown by their clustering in the 16S rRNA gene- Fig. 2), and the presence of common nucleotide support (Fig. 2), and the presence of common nucleotide related, as shown by their clustering in the 16S rRNA gene- and Caldisphaera draconis GDGT-6 (AB087496).

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'. In the same way, 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 sulfurifraudens' 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

Fig. 2. Phylogenetic dendrogram showing the position of strain 345-15T 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 3191T (D85505), Metallosphaera sedula DSM 3548T (X90481), Stygiolobus azoricas DSM 6296T (D85520), Sulfolobus acidocaldarius DSM 639T (D14876) and Sulfurisphaera ohwakuensis DSM 12421T (D85507), and the Thermoproteales representatives Thermomethyllum pendens DSM 2475T (X14835), Caldivigia maquinlingensis DSM 13496T (AB013926), Pyrobaculum islandicum DSM 4184T (L07511), Thermocladium modestus JCM 10088T (AB005296), Thermoproteus tenax DSM 2078T (M35966), and Vulcanaeta distributa DSM 14429T (AB068360).
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.

<table>
<thead>
<tr>
<th>Sequence position*</th>
<th>Acidilobus</th>
<th>Caldisphaera</th>
<th>Desulfurococcales, Thermoproteales, Sulfolobales</th>
<th>All Crenarchaeota</th>
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<td>C</td>
<td>U</td>
<td>U</td>
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<td>A;G</td>
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<td>C</td>
<td>U</td>
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<td>A;U</td>
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<td>1393</td>
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<td>U</td>
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</table>

*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 Thermoplasmales 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. 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.

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

**Description of Acidilobaceae fam. nov.**


**Description of Caldisphaeraceae fam. nov.**

*Caldisphaeraceae* (Cal.di.spha.e.rah.ee.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...
chlordamphenicol, 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 S°, H₂S. The DNA G+C content of the type strain is 54.5 mol%.

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

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