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Reinhard Dirmeier · Martin Keller · Doris Hafenbradl
Franz-Josef Braun · Reinhard Rachel · Siegfried Burggraf
Karl O. Stetter

Thermococcus acidaminovorans sp. nov., a new hyperthermophilic alkalophilic archaeon growing on amino acids

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Abstract From a shallow marine hydrothermal system at Vulcano (Italy), a new hyperthermophilic member of the *Archaea* was isolated. The cells are coccoid – shaped and possess up to five flagella. They grow between 56° and 93°C (optimum 85°C) and pH 5.0–9.5 (optimum 9.0). The organism is strictly anaerobic and grows heterotrophically on defined amino acids and complex organic substrates such as casamino acids, yeast extract, peptone, meat extract, tryptone, and casein. Polysulfide and elemental sulfur are reduced to H_2S. In the absence of polysulfide or elemental sulfur, the isolate grows at a significantly reduced rate. Growth is not influenced by the presence of H_2. DNA–DNA hybridization and 16S rRNA partial sequences indicated that the new isolate belongs to the genus *Thermococcus*, and represents a new species, *Thermococcus acidaminovorans*. The type strain is isolate AEDII10 (DSM 11906).

Key words Archaea · Hyperthermophiles · *Thermococcus acidaminovorans* · Defined amino acids · Polysulfide reduction · Alkaline pH

Introduction

Within their hot biotopes, the *Thermococcales* play an important role as decomposers of organic matter (Stetter 1995). These obligately heterotrophic hyperthermophiles grow on complex substrates, such as yeast extract, meat extract, peptone, bacterial and archaeal cell homogenates, and polymers such as casein, gelatine, starch, and chitin (Fiala and Stetter 1986; Zillig et al. 1983; Neuner et al. 1990; Huber et al. 1995; Kobayashi et al. 1994; Kengen and Stams 1994). Peptides are fermented, forming fatty acids, CO_2, and H_2 (Schönheit and Schäfer 1995). With the exception of *Thermococcus fumicolans* (Godfroy et al. 1996) and *Thermococcus litoralis* (Rinker and Kelly 1996), members of *Thermococcales* are unable to grow on amino acids. From a shallow marine hydrothermal system in Vulcano, Italy, a new *Thermococcus* isolate was obtained which uses casamino acids or defined amino acids as the sole carbon- and energy-source, with optimum growth at pH 9.0.

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R. Dirmeier(✉) · M. Keller · D. Hafenbradl · F-J. Braun · R. Rachel
S. Burggraf · K. O. Stetter
Lehrstuhl für Mikrobiologie, Universitätsstraße 31, D-93053 Regensburg, Germany
Tel. +49-941-943-3180; Fax +49-941-943-2403
e-mail: Reinhard.Dirmeier@biologie.uni-regensburg.de

Materials and methods

Collection of samples and isolation

Samples (AEDII10–AEDII12) were taken anaerobically (Stetter 1982) at a shallow beach (depth 1.0 m) situated at the base of the reef close to Porto di Levante at Vulcano Island, Italy. The original temperature of the sampling site was 95°C. For enrichment, 1 ml of sample was inoculated into 10 ml medium at pH 8.5 containing per liter: NaCl, 19.45 g; $MgCl_2 \cdot 6\ H_2O$, 12.6 g; Na_2SO_4, 3.24 g; $CaCl_2 \cdot 2\ H_2O$, 2.38 g; KCl, 0.55 g; $Na_2CO_3 \cdot 10\ H_2O$, 0.26 g; KBr, 0.08 g; $SrCl_2 \cdot 6\ H_2O$, 57 mg; H_3BO_3, 22 mg; sodium metasilicate, 4 mg; NaF, 2.4 mg; KNO_3, 1.6 mg; $Na_2HPO_4 \cdot 2\ H_2O$, 10 mg; casamino acids, 0.2%; polysulfide, 6 mM; glycine, 0.1 M. The enrichments were incubated at 85°C (gas phase: 200 kPa N_2). Pure cultures were obtained by serial dilution of positive enrichments followed by plating. For plating, the medium was solidified by 2% agar (Oxoid, Basingstoke, UK). The plates were incubated at 75°C in a pressure cylinder (Balch and Wolfe 1976) under an atmosphere of 150 kPa N_2.

Strains, culture conditions, and substrate utilization

Type strains of *Thermococcus stetteri* (Miroshnichenko et al. 1989), *Thermococcus litoralis* (Neuner et al. 1990), *Thermococcus celer* (Zillig et al. 1983), *Pyrococcus furiosus* (Fiala and Stetter 1986), *Thermococcus alcaliphilus* (Keller

et al. 1995), and *Thermococcus chitonophagus* (Huber et al. 1995) were from the culture collection of our institute and were grown as described. *Thermococcus* strains were cultivated anaerobically (Balch and Wolfe 1976) under N_2 in "SME" medium as described previously (Stetter et al. 1983) with 0.2% sulfur (Aldrich, Germany), 0.2% peptone, and 0.2% yeast extract, adjusted to pH 6.0. Polysulfide was prepared as described previously (Ikeda et al. 1972). Unless mentioned otherwise, cultures were grown in 100-ml serum bottles at 85°C. Large-scale cultures were grown in a 50-l enamel-protected fermentor (HTE Bioengineering, Wald, Switzerland) with stirring (100 rpm) at 85°C.

To determine which possible substrates supported growth, yeast extract, peptone, meat extract, tryptone, casein, casamino acids, a mixture of amino acids (see Results section), glucose, fructose, xylose, lactose, maltose, saccharose, xylane, and starch were added to the basic medium to a final concentration of 0.2% (w/v).

Light microscopy and electron microscopy

Light microscopy was carried out as described (Huber et al. 1989). Bacterial growth was determined by direct cell counting using a Thoma chamber (depth 0.02 mm; Brandt, Wertheim, Germany). Electron microscopy was performed as described (Huber et al. 1995).

Isolation of DNA, DNA base composition, and DNA homology

DNA was isolated by extraction with phenol/chloroform as described (Lauerer et al. 1986). The G + C content was determined by direct analysis after digestion of the DNA with nuclease P1 and separation by HPLC (Völkl et al. 1993). As a reference, calf thymus DNA (42 mol% G + C) was used. DNA preparations obtained from *Thermococcus celer, Thermococcus litoralis, Thermococcus stetteri, Thermococcus alcaliphilus, Thermococcus chitonophagus, Pyrococcus furiosus,* and isolate AEDII10 were labeled in vitro using the digoxigenin labeling and detection system (Boehringer, Mannheim, Germany). Isolated DNA was immobilized on nylon membranes (Boehringer) and was subsequently hybridized to various probes. The hybridization reaction was incubated for 12 h at 42°C under either "stringent" (Tm-15°C), "optimal" (Tm-25°C), or "relaxed" (Tm-32°C) conditions (Brenner 1973; Meyer and Schleifer 1978). Hybridization signals were identified by chemiluminescence (30 min, Kodak X-ray film).

16S rRNA analysis

A 16S rRNA gene fragment corresponding to bases 23–1390 in *Escherichia coli* 16S rRNA (Brosius et al. 1978) was amplified using the polymerase chain reaction (PCR; Saiki et al. 1988). The PCR product was cloned using the CLONEAMP system (Gibco-BRL, Bethesda, MD, USA) and partially sequenced using a Sequenase version 2.0 DNA sequencing kit (US Biochemical, Cleveland, OH, USA). The sequence was aligned to a set of representative archaeal sequences (RDP; Larsen et al. 1993).

DNA fingerprinting

DNA fingerprinting was performed by arbitrarily primed PCR reaction as described by Welsh and McClelland (1990). A 50-µl reaction volume contained: 10x Taq buffer (5 µl); forward primer (5.39 µl, approximately 50 pmol); nucleotides at a 1:1:1:1 ratio (4 µl); H_2O (30.36 µl); *Taq* polymerase (0.25 µl, 5 U/µl); and DNA (5 µl, at a concentration of 10 ng/ml). The primer used was 5′-GTAAAACGACGGCCAGT-3′. Two cycles of reaction were conducted for 5 min at 94°C, 5 min at 40°C, and 5 min at 72°C. Forty cycles of reaction were conducted for 1 min at 94°C, 1 min at 40°C, and 2 min at 72°C. The reaction was stopped by reducing the temperature of the mixture to 4°C, and the products were separated on a conventional 1.5% agarose gel.

Lipid composition

Lipids were extracted according to Hafenbradl et al. (1993) and Nishihara and Koga (1987) and were analyzed by two-dimensional thin-layer chromatography (TLC) (solvent A: $CHCl_3$/MeOH/7 M NH_3 7:3.5:0.8 by vol.; solvent B: $CHCl_3$/MeOH/acetic acid/H_2O 20:6:3:1 by vol.). The total lipid extract was hydrolyzed in 1 M methanolic HCl to cleave the polar headgroups. After methanolysis, the core lipids were separated by TLC using the solvent *n*-hexane/ethyl acetate 4:1 (v/v). All compounds were detected by spraying with anisaldehyde, followed by heating at 150°C for 5 min. The Dittmer and Lester reagent was used for phospholipids, α-naphthol/H_2SO_4 for glycolipids, and ninhydrin for amino lipids (Kates 1995).

Results

Enrichment and isolation

For enrichment of hyperthermophiles, 10 ml of anaerobic culture medium was inoculated with about 1 ml of the sample and incubated at 85°C. After 2 days, round coccoid cells became visible in the enrichment culture from sample AEDII10. The enrichment culture was successfully transferred into fresh medium and purified by serial dilutions, followed by plating. On agar plates, after 10 days of anaerobic incubation at 75°C, brownish colonies of 1–2 mm in diameter became visible. One single colony served as the inoculum for a liquid culture of the isolate, which was designated the same as the original sample (AEDII10).

Morphology

Cells of isolate AEDII10 were round to slightly irregular cocci, occurring singly or in pairs. The size of the organisms

was 0.8–1.3 μm in diameter. Isolate AEDII10 was motile and exhibited monopolar polytrichous flagellation, with up to five flagella (Fig. 1a). Ultrathin sections (Fig. 1b) showed that the cell envelope was composed of a cytoplasmic membrane (7 nm), a uniformly stained periplasmic space (approx. 10 nm), and a surface layer (approx. 5 nm). Freeze-fracturing and -etching revealed that the surface layer had p6 symmetry, with a lattice constant of approx. 16 nm (Fig. 1c,d).

Growth requirements

The isolate AEDII10 grew at between 56° and 93°C with an optimum around 85°C (120-min doubling time; Fig. 2a). The pH range of growth was 5.0–9.5 (optimum around pH 9.0; Fig. 2b). No growth was observed at pH 4.5 or pH 10. The isolate AEDII10 grew in media containing 1%–6% NaCl with an optimum between 2% and 3%.

Nutrition and metabolic products

Isolate AEDII10 grew under strictly anaerobic culture conditions on either a mixture of amino acids or casamino acids as the sole carbon- and energy-source. The final cell titers were about 8×10^8/ml. The growth rate was enhanced by the addition of polysulfide or elemental sulfur to the medium (doubling time: with polysulfide 2 h; without polysulfide 5.5 h). However, there was no difference in the final growth yield. Growth was obtained on a mixture of defined amino acids [alanine, arginine, aspartate, histidine, isoleucine, leucine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, valine, and lysine, each 0.02% (w/v)], casamino acids, yeast extract, peptone, meat extract, tryptone, or casein. No growth was observed on glucose, fructose, xylose, lactose, maltose, sucrose, xylane, or starch. The growth of isolate AEDII10 was not inhibited by molecular hydrogen, either in the absence or in the presence of

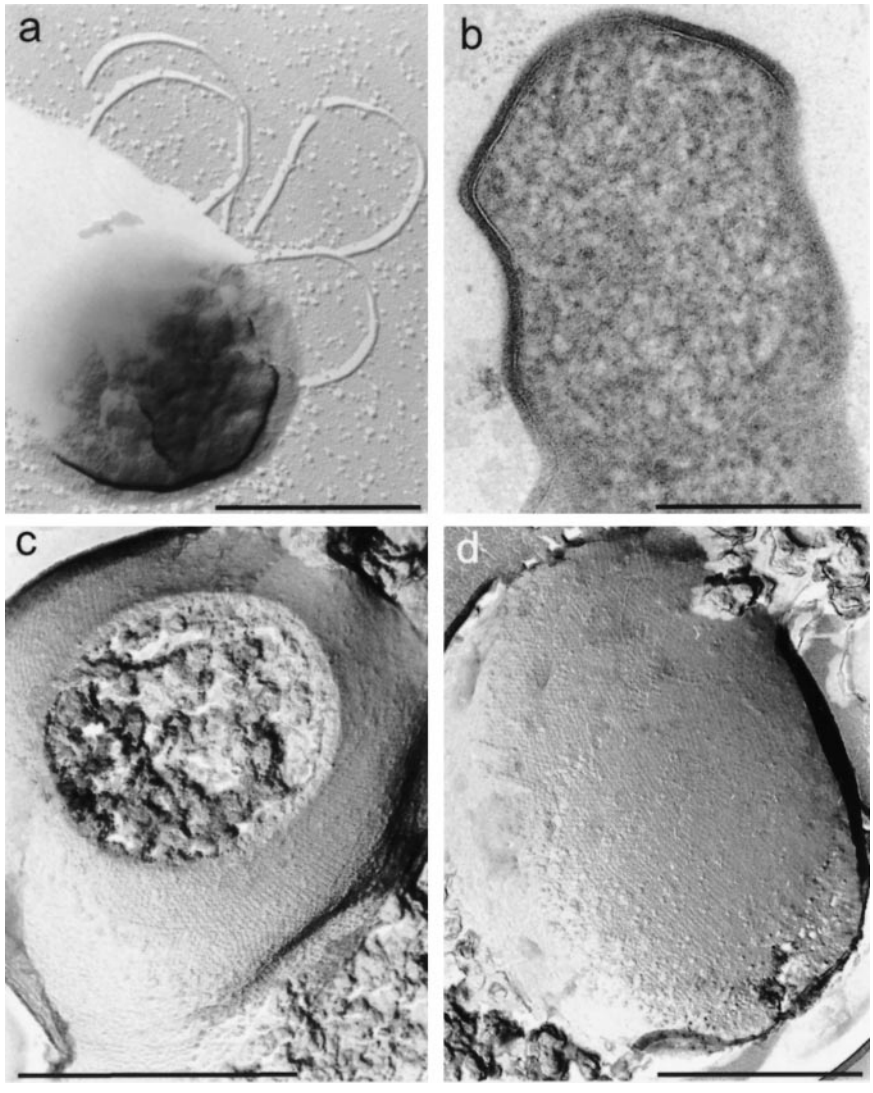

Fig. 1. Electron micrographs of isolate AEDII10: **a** chemically fixed with glutaraldehyde, air-dried, and Pt-shadowed (*bar* 0.5 μm); **b** ultrathin section of a freeze-substituted cell (*bar* 0.25 μm); **c** freeze-fractured; **d** freeze-etched (*bar* for **c** and **d**, 0.5 μm)

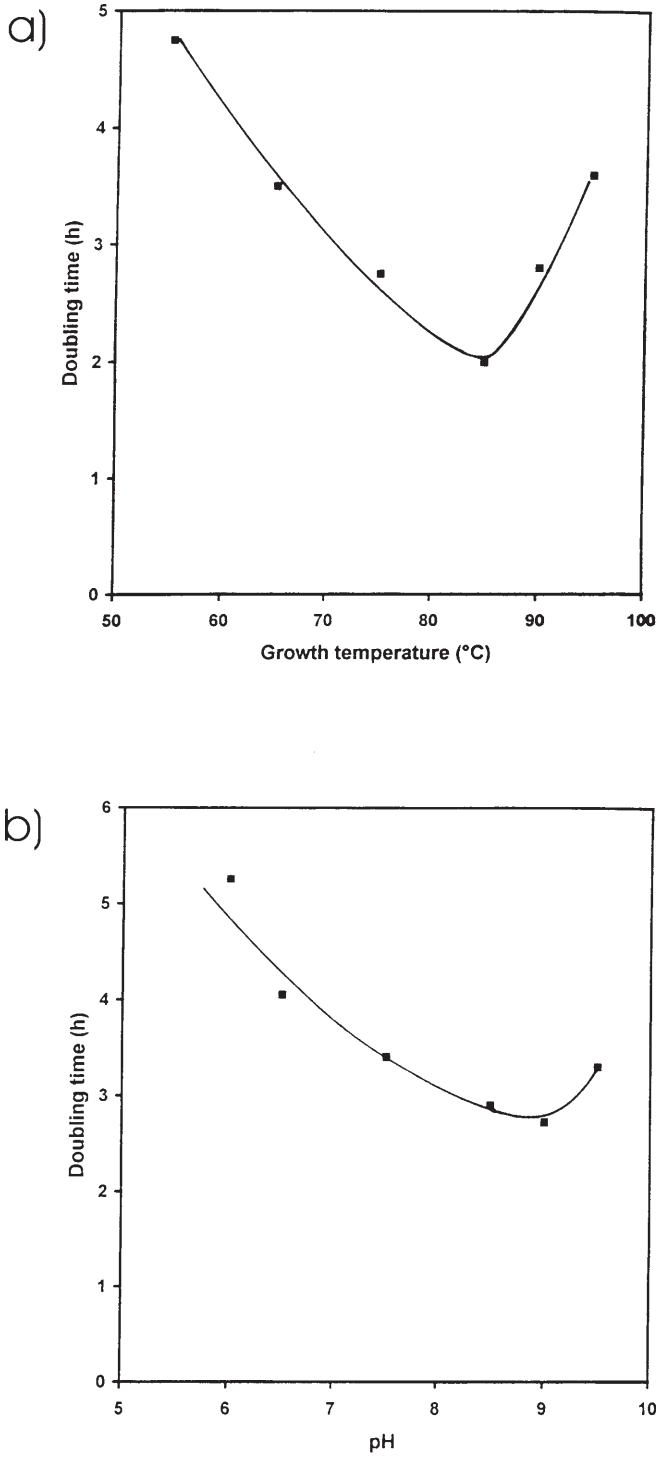

Fig. 2. a Effect of temperature on growth of *Thermococcus acidaminovorans*. Doubling times were calculated from the slopes of the growth curves (not shown) at pH 9.0. **b** Influence of pH on growth of *Thermococcus acidaminovorans*. Doubling times were calculated from the slopes of the growth curves (not shown) at 85°C

polysulfide or elemental sulfur. In the presence of H_2 and polysulfide or sulfur, H_2S was formed. However, no growth was observed under autotrophic culture conditions.

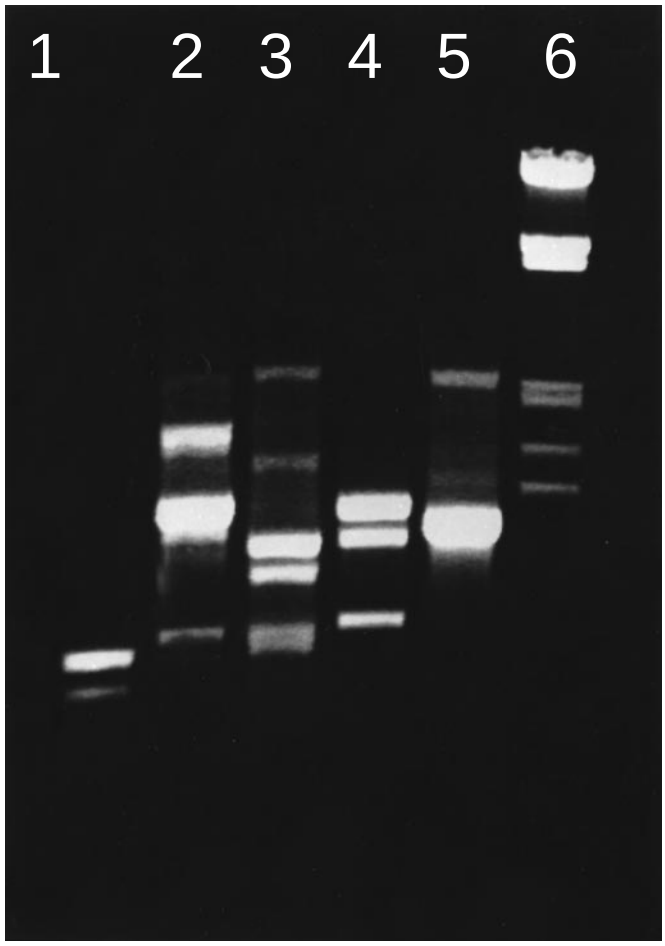

Fig. 3. Patterns of fragments from DNA fingerprinting. *Lane 1*, *Thermococcus stetteri*; *Lane 2*, *Thermococcus litoralis*; *Lane 3*, *Thermococcus celer*; *Lane 4*, *Thermococcus alcaliphilus*; *Lane 5*, *Thermococcus acidaminovorans*; *Lane 6*, lambda DNA digested by *Eco*RI and *Hind*III (21227 bp, 5148 bp, 4268 bp, 2027 bp, 1904 bp, 1584 bp, 1375 bp)

DNA and 16S rRNA analyses

The isolate AEDII10 exhibited a G + C content of 49 mol% as determined by analysis of the mononucleotides after digestion of the DNA with nuclease P1 (Völkl et al. 1993). Comparisons of the partially sequenced 16S rDNA of isolate AEDII10 showed that it is a member of the *Thermococcales.* Partial 16S rRNA sequences of isolate AEDII10 have been deposited in the EMBL nucleotide sequence database under accession number Y15935.

In DNA/DNA hybridization studies, there was no hybridization signal of the DNA of isolate AEDII10 with the DNA of *P. furiosus, T. celer, T. stetteri, T. litoralis,* or *T. alcaliphilus* under stringent hybridization conditions. The PCR profile of an arbitrarily primed PCR reaction obtained on a 1.5% agarose gel showed a pattern clearly different from those of *T. stetteri, T. litoralis, T. celer,* and *T. alcaliphilus* (Fig. 3).

Lipid composition

The complex lipid pattern of isolate AEDII10 analyzed by TLC showed only phosphoglycolipids. Aminolipids, phospholipids, and glycolipids were absent. Hydrolysis of complex lipids yielded 2,3-di-*O*-phytanyl-*sn*-glycerol and glycerol-dialkyl-glycerol tetraethers as core lipids.

Discussion

Based on the content of phytanyl ether lipids (De Rosa and Gambacorta 1988) and the phylogenetic characterization by partial 16S rRNA sequencing, the new marine isolate AEDII10 is a (hyperthermophilic) member of the archaeal domain belonging to the genus *Thermococcus* (Woese et al. 1990). DNA from AEDII10 did not hybridize under "restrictive" conditions to DNA obtained from *T. litoralis* (Neuner et al. 1990), *T. stetteri* (Miroshnichenko et al. 1989), *T. celer* (Zillig et al. 1983), or *T. alcaliphilus* (Keller et al. 1995). This is in line with the DNA fingerprinting experiment, which revealed different polymerase chain reaction profiles for all *Thermococcus* strains compared. The G + C content of the new isolate is 49 mol%, which is closest to *T. stetteri* (G + C content 50.2 mol%). However, the new isolate AEDII10 has a much higher optimal growth temperature (85°C) and pH optimum (9.0) than *T. stetteri* (75°C and pH 6.0). In contrast to *T. profundus* (Kobayashi et al. 1994) and *T. peptonophilus* (González et al. 1995), the new isolate AEDII10 grew on defined amino acids or casamino acids as the sole carbon- and energy-source. By its growth on defined amino acids, the new isolate AEDII10 is similar to *T. fumicolans* (Godfroy et al. 1996). However, it is clearly distinguished by its higher pH optimum (pH 9.0 compared with pH 8.0), its inability to grow on maltose, and its different G + C content (49 mol% compared with 55–56 mol%). In addition, isolate AEDII10 showed the same growth yield with or without sulfur or polysulfide. Based on its phylogenetic relationship and physiological differences, the new isolate represents a new species of the genus *Thermococcus*. Considering its ability to grow on amino acids, we named it *Thermococcus acidaminovorans.* The type strain is isolate AEDII10 (DSM 11906).

Description of *Thermococcus acidaminovorans* sp. nov.

Thermococcus acidaminovorans sp. nov. (a.cid.a.mi.no. vo'rans M.L.n. *acidum*. acid; M.L.pref. *amino*. with an amino group; M.L. *vorare*. swallow; M.L.part.adj. *acidaminovorans*. growing on amino acids). Coccoid-shaped cells, of 0.8–1.3 μm in diameter. Obligately anaerobic. Temperature range for growth from 56° to 93°C; optimum around 85°C. pH range for growth from pH 5.0 to 9.5 with the optimum around pH 9.0, and growth in 1%–6% NaCl with an optimum around 2%–3%. Strictly heterotrophic. Defined amino acids, casamino acids, yeast extract, peptone, meat extract, tryptone, and casein are fermented. Growth not inhibited by H_2. Growth with and without polysulfide or sulfur, with a significant stimulation of the growth rate in the presence of polysulfide or sulfur. G + C content of DNA is approximately 49 mol%. Core lipids consist of 2,3-di-*O*-phytanyl-*sn*-glycerol and glycerol-dialkyl-glycerol tetraethers. Isolated from a shallow marine hydrothermal system at Vulcano, Italy.

Type strain: *Thermococcus acidaminovorans* AEDII10 (DSM 11906)

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