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ORIGINAL PAPER

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Cultivated anaerobic acidophilic/acidotolerant thermophiles from terrestrial and deep-sea hydrothermal habitats

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Abstract Metabolic and phylogenetic diversity of cultivated anaerobic microorganisms from acidic continental hot springs and deep-sea hydrothermal vents was studied by molecular and microbiological methods. Anaerobic organotrophic enrichment cultures growing at pH 3.5–4.0 and 60 or 85°C with organic energy sources were obtained from samples of acidic hot springs of Kamchatka Peninsula (Pauzhetka, Moutnovski Volcano, Uzon Caldera) and Kunashir Island (South Kurils) as well as from the samples of chimneys of East Pacific Rise (13°N). The analyses of clone libraries obtained from terrestrial enrichment cultures growing at 60°C revealed the presence of archaea of genus *Thermoplasma* and bacteria of genus *Thermoanaerobacter*. Bacterial isolates from these enrichments were shown to belong to genera *Thermoanaerobacter* and *Thermoanaerobacterium*, being acidotolerant with the pH optimum for growth at 5.5–6.0 and the pH minimum at 3.0. At 85°C, domination of thermoacidophilic archaea of genus *Acidilobus* in terrestrial enrichments was found by both molecular and microbiological methods. Five isolates belonging to this genus possessed some phenotypic features that were new for this genus, such as flagellation or the ability to grow on monosaccharides or disaccharides. Analyses of clone libraries from the deep-sea thermoacidophilic enrichment cultures showed that the representatives of the genus *Thermococcus* were present at both 60 and 85°C. From the 60°C deep-sea enrichment, a strain belonging to *Thermoanaerobacter siderophilus* was isolated. It grew optimally at pH 6.0 with the minimum pH for growth at 3.0 and with salinity optimum at 0–2.5% NaCl and the maximum at 7%, thus differing significantly from the type strain. These data show that fermentative degradation of organic matter may occur at low pH and wide temperature range in both terrestrial and deep-sea habitats and can be performed by acidophilic or acidotolerant thermophilic prokaryotes.

Keywords Acidic hot springs · Deep-sea hydrothermal vents · Thermophiles · Anaerobes · Acidophiles · Acidotolerant microorganisms

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Introduction

The ability to grow at low pH values with an optimum growth below pH 4.0 is rather common among thermophilic prokaryotes. Among them, organisms with respiratory type of metabolism predominate (Johnson 1998). These are aerobic sulfur-oxidizing and/or organotrophic archaea of genera *Sulfolobus* (Brock et al. 1972), *Sulfurococcus* (Golovacheva et al. 1987), *Metallosphaera* (Huber et al. 1989), *Acidianus* (Segerer et al. 1986), *Sulfurisphaera* (Kurosawa et al. 1998), *Picrophilus* (Schleper et al. 1995), *Thermoplasma* (Segerer et al. 1988) and the microaerophiles *Thermocladium* (Itoh et al. 1998) and *Caldivirga* (Itoh et al. 1999). Some of these organisms are facultative anaerobes, able to oxidize organic substrates in the course of anaerobic respiration with sulfur compounds as the electron

acceptors; representatives of the genus *Acidianus* are capable of anaerobic lithoautotrophic growth with molecular hydrogen and sulfur. This process is the only one supporting the growth of the obligately anaerobic thermoacidophilic archaea of the genus *Stigiolobus* (Segerer et al. 1991). Other obligate anaerobes are the organotrophs *Acidilobus* (Prokofeva et al. 2000), *Vulcanisaeta* (Itoh et al. 2002) and *Caldisphaera* (Itoh et al. 2003). *Acidilobus*, *Thermoplasma* and *Caldisphaera* are capable of anaerobic growth via fermentation in the absence of external electron acceptors. Thermoacidophilic bacteria are not numerous and are represented mostly by aerobes such as the organotrophic *Alicyclobacillus* (Darland and Brock 1971; Wisotzkey et al. 1992) and the lithotrophic *Sulfobacillus* (Golovacheva and Karavaiko 1978) and *Acidithiobacillus* (Hallberg and Lindström 1994). Among anaerobic thermophilic bacteria, only moderate acidophiles with fermentative metabolism are known such as *Thermoanaerobacterium aotearoense* with a pH optimum of 5.2 (Liu et al. 1996).

Most thermoacidophilic prokaryotes were isolated from terrestrial hot springs with low pH values of water: Yellowstone National Park (Brock et al. 1972), Kamchatka (Golovacheva et al. 1987; Prokofeva et al. 2000), Japan (Schleper et al. 1995; Itoh et al. 1998, 2002), Iceland (Huber and Stetter 1991), Phillipines (Itoh et al. 1999, 2003); some were isolated from the sea-shore hot vents of Italy (Segerer et al. 1986), Azores (Segerer et al. 1991) and tropical swamps (Segerer et al. 1988). Thermoacidophiles were also isolated from anthropogenic sources such as thermogenesis zones of workable sulfide ore deposits, uranium mines, self-heated coal refuse piles (Golovacheva and Karavaiko 1978; Segerer et al. 1988; Fuchs et al. 1995) and some food products (Matsubara et al. 2002; Goto et al. 2003). Here we report the enrichment, isolation and molecular identification of anaerobic microorganisms from acidic terrestrial hot springs and deep-sea vents.

Materials and methods

Sampling

Samples of water and sediment were taken in 1996, 1997, 1999 and 2000 from terrestrial hot springs in different volcanic areas of Kamchatka: Uzon Caldera, Geyser Valley, Pauzhetka area, Moutnovsky Volcano area and, in 1998, on Kunashir Island (Kuril Islands). Deep-sea hydrothermal samples were collected at the 13°N hydrothermal vent field on the East Pacific Rise (2,650 m depth) during the AMISTAD cruise in 1999 (Nercessian et al. 2003). At 13°N, active black smokers and diffuse flow vents were generally colonized by dense macrofaunal assemblages including vestimentiferas and polychaetes. Sulfide structures were collected by the arm of the submersible *Nautile* and placed in the submersible insulated basket for the trip to the surface. Terrestrial and marine hydrothermal samples were transferred to 50-ml flasks with screw caps, closed hermetically and transported to the laboratory without temperature control. Characteristics of samples are given in Table 1.

Enrichment and isolation

For the enrichment of thermoacidophilic anaerobic microorganisms from continental hot springs, the modified basal medium described by Pfennig (1965) was used as the mineral background (mg l^{-1}): $MgCl_2 \cdot 6H_2O$, 330; $CaCl_2 \cdot 2H_2O$, 330; KCl, 330; KH_2PO_4, 330; NH_4Cl, 330. For the cultivation of thermoacidophilic microorganisms from the deep-sea samples, modified MG basal medium (Balch et al. 1979) was used as the mineral background (mg l^{-1}): NaCl, 18,000; KCl, 325; $MgCl_2 \cdot 6H_2O$, 4,260; $MgSO_4 \cdot 7H_2O$, 3,450; $CaCl_2 \cdot 2H_2O$, 150; NH_4Cl, 250; K_2HPO_4, 150. Trace elements (Kevbrin and Zavarzin 1992) and vitamins (Wolin et al. 1963) stock solutions were added at 1 ml l^{-1} to both basal media. Yeast extract (50 mg l^{-1}) was added as a source of growth factors. The media were reduced by boiling and subsequent addition of 700 mg l^{-1} $Na_2S.9H_2O$. Headspace of the enrichment vials was filled with 100% oxygen-free CO_2. The pH of the media was adjusted by the addition of 6 N HCl and was controlled after autoclaving (120 or 110°C when elemental sulfur was added). Starch, glucose, sucrose, yeast extract, acetate (2 g l^{-1}) or a mixture of starch, maltose and peptone (1.5 g l^{-1} each) were added as growth substrates to the basal media. If molecular hydrogen was tested as a growth substrate, no organic substrates were added and CO_2 was replaced with a sterile mixture of H_2/CO_2 (8/2 vol/vol) after autoclaving. Elemental sulfur (10 g l^{-1}), sulfate, thiosulfate or nitrate as sodium salts (2 g l^{-1}) were tested individually as possible electron acceptors for growth. Flasks of 30- or 100-ml (when H_2 was used as the growth substrate) volume, containing 10 ml of the medium, were inoculated with 1 ml of sample and incubated at 60 and/or 85°C. Microbial growth was monitored using light microscopy.

For the isolation of pure cultures, serial dilutions were used to inoculate a solid medium of the same composition as used for the enrichments, with 1.5% agar (pH 5.0) or 1.2% Gelrite + 0.05% $MgSO_4$ (pH 3.5), in shake-tubes or 90-ml Bellco flat bottles. Individual colonies were picked up with a thick needle or a loop and transferred into the corresponding liquid medium. The purity of cultures was examined by light microscopy.

Morphology and cell structure studies

The morphology of microorganisms was studied using an MBI-3 phase contrast light microscope (LOMO, Leningrad, Russia). Cells were negatively stained with 2% phosphotungstic acid on Formvar (Serva, Heidelberg, Germany) coated copper grids and examined using

Table 1 Summary of the samples for enrichment of acidophilic organisms

No. of samples	Sampling site	Habitat	Location	Properties[a]
Kamchatka				
124	Pauzheta area	–	–	–
345	Moutnovsky area	Water and gray clay from a hot lake	–	pH 4.0, 82°C
711	Geyser valley	Mud (red to brown color) from the mud lake	54° 26.357′ N, 160° 08.573′ E	pH 3.8, 60°C
722	Uzon caldera, Central thermal field	Mud (gray to brown color) from a small mud pool	54° 29.990′ N, 160° 00.369′ E	pH 3.4, 91°C
739	Uzon caldera, North thermal field	Mud (white clay with sulfur deposits) and water from the hot spring	54° 30.638′ N, 160° 00.072′ E	pH 3.0, 63°C
743	Uzon caldera, Orange field	Mud with organic compound (plant's leafs) from a mud pool	54° 30.389′ N, 160° 00.072′ E	pH 3.3, 84°C
760	Uzon caldera, Orange field	Mud from a pool surrounded with grass	–	pH 3.8, 38°C
761	Uzon caldera, Orange field	Mud	54° 30.237′ N, 160° 00.038′ E	pH 3.8, 48°C
764	Moutnovsky area	Mud (white to gray clay) from a pool surrounded with grass	–	pH 3.1, 79°C
768	Moutnovsky area	Water and mud (brown clay) from the spring surrounded with ferric oxide deposits	52° 31.988′ N, 158° 12.163′ E	pH 3.0, 90°C
816	Moutnovsky area	Water and clay from the hot spring	–	pH 3.0, 60°C
Kunashir (Kurils Islands)				
405	Golovnin caldera, hot lake	Mud from the botton of hot lake	–	pH 2.5, 65–95°C
East Pacific Rise (13°N, 2,650 m depth)				
502	Site Genesis	Inner part of a chimney	12°48.666′N, 103°56.429′W	Max. 90–100°C in the area colonized by *Alvinella* spp.
518	Site Elsa	Outer and inner part of a chimney	12°48.193′N, 103°56.338′W	Max. 344°C vent water
520	Site Genesis	Outer and inner part of a chimney	12°48.666′N, 103°56.429′W	Max. 72°C vent water
527	Site La Chaînette	Outer and inner part of a chimney	12°50.342′N, 103°56.903′W	Max. 106°C vent water
530	Site Genesis	Outer and inner part of a chimney	12°48.666′N, 103°56.429′W	Max. 344°C vent water

[a] "–"Indicates that no physical parameters were recorded. The temperatures were recorded before collection of hydrothermal chimneys.
[b] Of 43 samples used, only those that gave growth to stable thermoacidophilic enrichments are included.

a JEM-100B (Jeol, Tokyo, Japan) electron microscope operated at 60 kV.

Determination of metabolic products

H_2S formed in the cultures was determined by colorimetric method (Trüper and Schlegel 1964). H_2 in the head space was determined by using a Crom-5 gas chromatograph (Laboratorni Pristroje, Praha, Czechoslovakia) equipped with a thermal conductivity detector and a column packed with AG-3 activated carbon and operated at room temperature with argon as a carrier gas at a flow rate of 40 ml min^{-1} . Volatile fatty acids and ethanol were determined on Crom-5 equipped with a flame ionization detector and a column filled with Chromosorb-10 (Sigma, St. Louis, MA, USA). The temperature was 180°C for fatty acids and 120°C for ethanol. Argon was used as a carrier gas at a flow rate of 40 ml min^{-1} .

DNA extraction, PCR and cloning

DNA was extracted from 4 ml of culture using the method described by Charbonnier et al. (1995). Bacterial 16S rRNA genes were amplified using the universal reverse primers 1492R (5′-CGG TTA CCT TGT TAC GAC TT-3′) or 1407R (5′ GAC GGG CGG TGW GTR CAA 3′) and the *Bacteria*-specific forward primer 8F (5′-AGA GTT TGA TYM TGG CTC AG-3′). The universal primer 1492R and the *Archaea*-specific primers 4F (5′-TCC GGT TGA TCC TGC CRG-3′) or 341F (5′-CCT AYG GGG YGC ASC AGG CG-3′) were used to amplify archaeal 16S rRNA genes. PCR reaction mixtures containing 1 µl of DNA extract consisted of (as final concentrations) 50 mM KCl, 10 mM Tris–HCl (pH 9 at 25°C), 0.1% Triton ×100, 1.5 mM $MgCl_2$, 0.25 mM of each dATP, dCTP, dGTP and dTTP, 0.2 µM of each amplification primer and 1.25 U of *Taq* polymerase (Promega Corp., Charbonnieres, France) in a final volume of 25 µl. Reaction mixtures were

incubated in a Robocycler Gradient 96 (model Robocycler 96; Stratagene, La Jolla, CA, USA). Negative and positive controls were performed without the DNA template and with DNA from *Archaeoglobus fulgidus* (DSM 4304) and *Desulfovibrio giganteus* (DSM 4123), respectively. The initial denaturation step consisted of heating the reaction mixture at 95°C for 5 min, and the thermal profile then consisted of 30 cycles of denaturation at 95°C for 1.5 min, annealing at 53°C for 1.5 min and extension at 72°C for 2.5 min. A final extension step was carried out at 72°C for 8 min. PCR products and negative controls were cloned in a TOPO XL vector with a cloning kit following the manufacturer's instructions (Invitrogen, Groningen, The Netherlands).

Restriction fragment length polymorphism (RFLP) analysis

Clones containing inserts of the expected size (about 1,400 and 1,100 bp) were categorized using enzymatic digestion with the four-base recognition site restriction endonucleases, *Hae*III (Promega) or *Bst*UI (New England Biolabs), according to the manufacturer's instructions. Digestion products were separated by gel electrophoresis in 2.5% agarose gel (agarose 3 : 1, Eurobio, Les Ulis, France) run in TAE buffer. The gels were stained with ethidium bromide and photographed under UV light. One or two clones of each RFLP type were selected for sequencing.

Sequencing of 16S rRNA genes

16S rRNA genes sequences of the selected clones were obtained by cycle sequencing using the Thermo Sequenase Primer Cycle Sequencing Kit (Amersham Pharmacia Biotech) and M13 forward (5′-GTA AAA CGA CGG CCA G-3′) and M13 reverse (5′-CAG GAA ACA GCT ATG AC-3′) primers. Automated DNA sequencing was performed on a LI-COR 4000 sequencer (Sciencetec).

The 16S rDNA genes of the bacterial isolates were amplified using 11-27F and 1492R or 519R primers (Edwards et al. 1989) and for archaea A8F and A537R primers were used (Kolganova et al. 2002), respectively. The PCR mixtures (20 µl) consisted of 1× PCR buffer (17 mM $(NH_4)_2SO_4$, 6 mM Tris–HCl, 2 mM $MgCl_2$, pH 8.8), 0.25 mM of each dNTP, 10–50 ng of template DNA, 100 pM of each primer and 2 U of BioTaq DNA Polymerase ("Dialat Ltd", Russia). The initial denaturation step consisted of heating the reaction mixture at 94°C for 9 min, amplification of samples through 30 three-step cycles of DNA denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min and elongation at 72°C for 2 min with a final extension step of 72°C for 7 min. PCR products were analyzed by electrophoresis in 1% agarose gel stained with EtBr. PCR fragments were purified on low melting point agarose using "Wizard PCR Preps" kit (Promega, USA).

Sequencing of 16S rRNA genes PCR fragments was performed by the Sanger method (Sanger et al. 1977) using the "Silver Sequencing" kit (Promega, USA) according to the manufacturer's instructions with minor modifications. Electrophoresis was run on SQ3 Sequencer (Hoefer, USA) in 0.19 mm thick polyacrylamide gel. Universal prokaryotic primers (Lane 1991) and Sequenase (Biochemicals, Cleveland, OH, USA) were used for sequencing in both directions.

16S rRNA gene sequence analysis

For phylogenetic placement, clone sequences were subjected to BLASTN searches within the GenBank database [http://www.ncbi.nlm.nih.gov/blast] to determine their phylogenetic affiliations approximately. The percentage identity values were recalculated proceeding from BLASTN alignments, but omitting from consideration the positions containing uncertainties.

Clone sequences and sequences downloaded from GenBank were aligned using the CLUSTAL X program (Thompson et al. 1994). Phylogenetic trees were constructed using methods available in the TREECONW (Van de Peer and De Wachter 1994) and PHYLIP (Felsenstein 1993) program packages.

16S rRNA genes sequences of clones and isolates are available from GenBank under accession numbers AY350582–AY350611.

Results

Enrichment cultures of thermoacidophilic anaerobes

Flasks with anaerobically prepared Pfennig medium (pH 3.5) or marine MG medium (pH 3.5–4.0) supplemented with different growth substrates (molecular hydrogen, sugars, starch, yeast extract, acetate) and electron acceptors (S°, SO_4^{2-}, $S_2O_3^{2-}$, NO_3^-) (19 combinations) were inoculated with 43 samples from continental and deep-sea habitats. Of them, 19 enrichments with stable growth (three or more transfers) were obtained (Table 2). Stable enrichments were obtained only on fermentable substrates. No enrichments were obtained when acetate was used as a growth substrate irrespective of the electron acceptor used. Several enrichments were obtained in the presence of H_2 with S° or without electron acceptors; however, no significant decrease of H_2 was observed (data not shown). Nitrate, added to the medium with sucrose, yeast extract and starch, did not stimulate the growth of microorganisms in the enrichment cultures at all tested conditions or, in some cases, inhibited it. Elemental sulfur stimulated the growth of microorganisms in the enrichments with organic substrates but did not influence their morphological diversity.

Table 2 Thermophilic microorganisms identified in terrestrial and hydrothermal samples

Enrichment number	pH, *T* (°C)	Substrate	Morphology	Fermentation products	Stimulatory compounds	Isolates or 16S rDNA clones (accession numbers)	Closest validated taxa (% identity, blastN[a])
124	3.5, 85	Yeast extract	Cocci 1–2 μm	Acetate	S°	Isolate 124-87 (AY350585)	*Acidilobus aceticus* (95.4)
345	3.5, 85	Starch	Cocci 1–2 μm with flagella	Acetate	S°	Isolate 345-15 (AY350586)	*Acidilobus aceticus* (96.0)
405	3.5, 85	Starch	Cocci 1–2 μm	Acetate	S°	Clones 405-a1–405-a3 (AY350582–AY350584)	*Acidilobus aceticus* (93.9–97.3)
						Isolate 405-16 (AY350587)	*Acidilobus aceticus* (95.2)
711	4.0, 60	Sugars	Rods	Acetate, ethanol, H_2	$S_2O_3^{2-}$, S°	Clones 711-a1–711-a3 (AY350604, AY350595, AY350605)	*Thermococcus* spp. (97.2–99.5)
						711-b12, 711-b25 (AY350590, AY350591)	*Thermoanaerobacter* spp. (96.2–99.0)
						Isolate 711-75 (AY350593)	*Thermoanaerobacter ethanolicus* (98.9)
722	3.5, 85	Yeast extract	Cocci 1–2 μm	Acetate	S°	Isolate 722-67 (AY350588)	*Acidilobus aceticus* (95.1)
739	3.5, 60	Yeast extract	Irregular cocci, rods	Acetate	S°	Clones 739-a1, 739-a3, 739-a6–739-a8 (AY350606–AY350610)	*Thermoplasma acidophilum* (98.3–99.0)
743	3.8, 85	Glucose, starch	Rods, cocci	Acetate	n.d.	–	–
760	3.8, 60	Sugars	Rods	Acetate, ethanol	n.d.	–	–
761	4.0, 60	Sugars	Rods	Acetate, ethanol, H_2	$S_2O_3^{2-}$, S°	Isolate 761-119 (AY350594)	*Thermoanaerobacterium lactoethylicum* (98.6)
764	3.5, 85	Glucose, starch	Rods, cocci	Acetate, H_2	n.d.	–	–
768	4.0, 85	Yeast extract	Rods	Acetate	S°	Isolate 768-28 (AY350589)	*Vulcanisaeta distributa* (96.8)
816	3.5, 60	Yeast extract	Irregular cocci, rods	Acetate	S°	Clone 816-a9 (AY350611)	*Thermoplasma acidophilum* (96.3)
502a	3.7, 85	Organic[b]	Cocci	Acetate, H_2S	S°	–	–
502b	3.7, 60	Organic	Cocci, rods	Acetate, H_2S	S°	–	–
518a	3.7, 85	Organic	Cocci	Acetate, H_2S	S°	–	–
518b	3.7, 60	Organic	Cocci and rods	Acetate, ethanol, H_2S	S° $S_2O_3^{2-}$	Clones 518b-a1, 518b-a2 (AY350596, AY350597)	*Thermococcus* spp. (97.5–99.5)
						Isolate 518-21 (AY350592)	*Thermoanaerobacter siderophilus* (99.3)
520a	3.7, 85	Organic	*Cocci*	Acetate, H_2S	S°	Clones 520a-a1, 520a-a2 (AY350598, AY350599)	*Thermococcus* spp. (98.7–99.8)
520b	3.7, 60	Organic	Cocci	Acetate, H_2S	S°	–	–
527	3.7, 60	Organic	Rods	Acetate, H_2S	S°	Clones 527-a1–527-a4 (AY350600–AY350603)	*Thermococcus* spp. (97.8–99.5)

[a] The percentage identity values were recalculated proceeding from blastN alignments but omitting from consideration positions containing uncertainties.
[b] Organic means mixture of starch, maltose and peptone.

Short rods dominated in the terrestrial enrichments at 60°C (Fig. 1a); formation of acetate (up to 24 mM), hydrogen (up to 20%) and, in some cases, ethanol (up to 15 mM) was detected in these cultures. In the enrichments 739 and 816, a mixture of cocci and rods was present. After several transfers both enrichments consisted only of irregular cocci (Fig. 1b).

At 85°C organisms with coccoid cells dominated in the enrichments with sugars, starch and yeast extract as the growth substrates. Their growth was stable, although the cell yield never exceeded 2×10^7 cells ml^{-1}. Acetate (up to 2 mM) was the only growth product in these cultures; no hydrogen formation was observed. Long rods were present in enrichments 722, 764 and 768 at 85°C (Fig. 1f).

All deep-sea enrichments growing at 85°C consisted of coccoid microorganisms (Fig. 1d). At 60°C both cocci and short rods were observed (Fig. 1c, e). Cell yields achieved 10^8 cells ml^{-1}, and significant amounts of acetate were produced (up to 24 mM at 80°C and up to 13 mM at 60°C). A prominent stimulating effect of sulfur on the growth of all marine cultures was observed; in this case, significant amounts of hydrogen sulfide were produced (up to 40 mM).

Analyses of clone libraries from terrestrial and deep-sea enrichments

DNAs from terrestrial enrichment cultures 405, 711, 739 and 816 and deep-sea cultures 520a and 518b were used to construct 16S rRNA gene clone libraries. Only the archaeal representatives were amplified from enrichments 739 and 816. Attempts to amplify the bacterial 16S rDNA from these samples were unsuccessful. Restriction analysis of archaeal clones derived from enrichment cultures 739 (28 clones analyzed) and 816 (17 clones analyzed) showed the presence of three distinct RFLP patterns, which were similar in both cultures. An additional pattern was observed only in enrichment 739. Comparison of 16S rRNA gene sequences of each category of clones with sequences available in the databases revealed that they were all affiliated to the genus *Thermoplasma* (Table 2, Fig. 2). Bacterial and archaeal 16S rRNA genes were amplified from the DNA isolated from enrichment culture 711 (ten archaeal clones and 22 bacterial clones analyzed). Restriction analysis of 16S rRNA of bacterial clones revealed the presence of two distinct RFLP patterns. A majority of clones (19) and the minor group (three clones) were represented by clones 711-b12 and 711-b25, respectively, affiliated to genus *Thermoanaerobacter* (Table 2, Fig. 3). Archaeal clones exhibited three distinct RFLP patterns, representatives of which belonged to the genus *Thermococcus* (Table 2, Fig. 4).

Positive archaeal and bacterial amplifications were obtained with DNA isolated from the terrestrial enrichment 405 and the deep-sea enrichments 520a, 527 and 518b, but only their archaeal PCR products were cloned. Restriction analysis of archaeal clones from enrichment culture 405 (ten clones analyzed) showed the

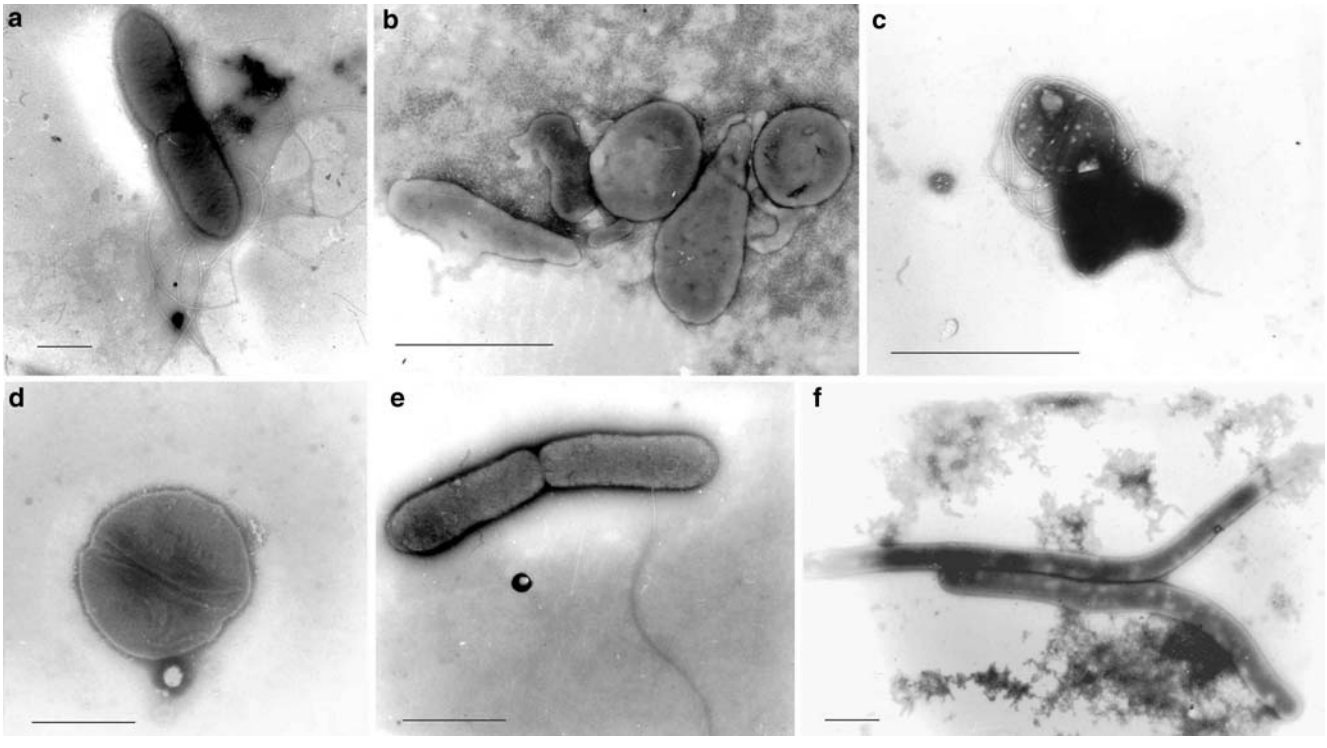

Fig. 1 Electron micrographs of negatively stained whole cells of enrichment cultures: (**a**) enrichment 711, pH 4.0, 60°C, sucrose; (**b**) enrichment 816, pH 3.5, 60°C, yeast extract; (**c**, **e**) deep-sea enrichment 518b, pH 3.7, 60°C, mixture of starch, maltose and peptone + S°; (**d**) enrichment 502a, pH 3.7, 85°C, H_2 + S°; (**f**) enrichment 764, pH 3.5, 85°C, starch. Bar = 1 μm

presence of three distinct RFLP patterns, represented by clone 405-a1 (group of five clones), clone 405-a2 (group of four clones) and the only clone 405-a3, all identified as members of the genus *Acidilobus* (Table 2, Fig. 5).

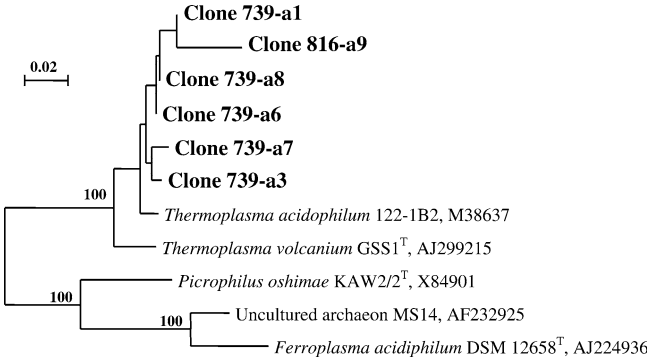

Fig. 2 Phylogenetic tree showing the position of *Thermoplasma* clones based on a comparison of 16S rDNA sequences. The phylogenetic tree was constructed by the neighbor-joining method. The *bar* represents 2% of sequence difference. Bootstrap values (expressed as percentage of 100 replications) are shown at branching points; only maximal values (100%) are indicated

Restriction analysis of archaeal 16S rDNA clones obtained from the deep-sea enrichments 518b, 520a and 527 (ten clones from each analyzed) showed 4, 2 and 2 RFLP patterns, respectively. The phylogenetic analysis of representative sequences of each pattern indicated that all of them belonged to the genus *Thermococcus* (Table 2, Fig. 4).

Isolation of anaerobic thermoacidophiles from terrestrial hot springs

Eight strains were isolated in pure culture (Table 2). Terrestrial strains 761-119 and 711-75 from enrichments 761 and 711, respectively, possessed rod-shaped cells with flagella. Both isolates used diverse sugars and peptides as growth substrates and formed acetate, ethanol, H_2 and CO_2. H_2S was formed when they were cultivated in the presence of $Na_2S_2O_3$ or S°. The optimal growth temperatures of isolates 761-119 and 711-75 were 66 and 60°C, respectively (data not shown). Both strains grew optimally in slightly acidic medium (pH 5.0–6.3), but were also able to grow at rather low pH values, having minimum pH of

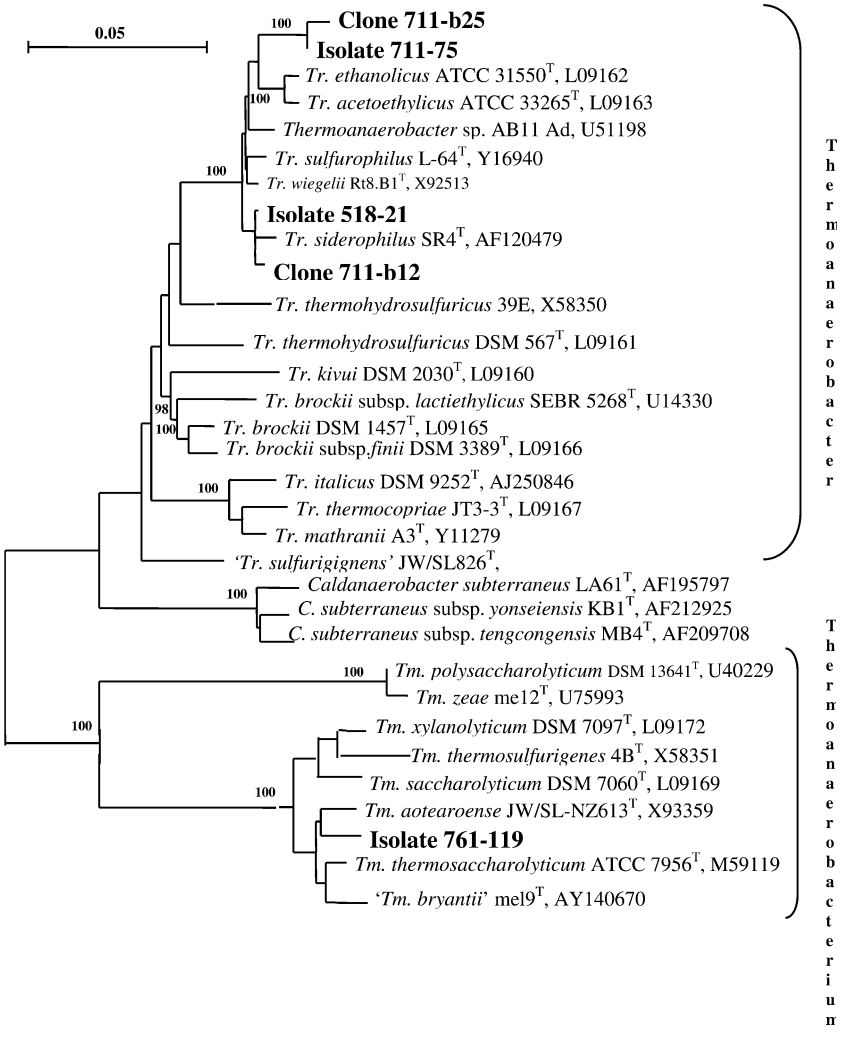

Fig. 3 Phylogenetic tree showing the relationship of bacterial clones and isolates with other bacteria based on a comparison of 16S rDNA sequences. The phylogenetic tree was constructed by the neighbor-joining method. The *bar* represents 5% of sequence difference. Bootstrap values (expressed as percentage of 100 replications) are shown at branch points; only maximal values (100%) were considered significant

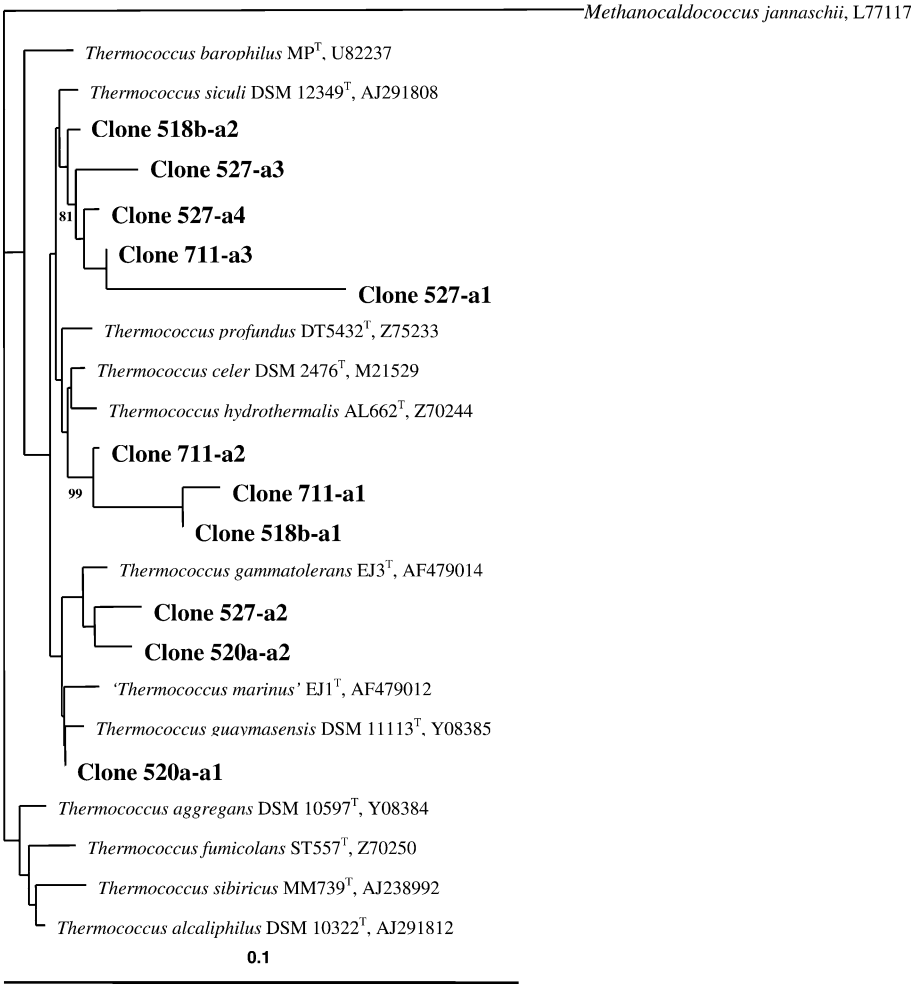

Fig. 4 Phylogenetic tree showing the positions of *Thermococcus* clones based on a comparison of 16S rDNA sequences. The phylogenetic tree was constructed by the Fitch–Margoliash method (Felsenstein 1993). The sequence of *Methanocaldococcus jannaschii* was included as an outgroup. The *bar* represents 10% of sequence difference

growth around 3.0. 16S rDNA sequence analysis revealed that strain 761-119 belonged to the genus *Thermoanaerobacterium* and strain 711-75 belonged to the genus *Thermoanaerobacter* (Table 2, Fig. 3).

Isolates 124-87, 345-15, 405-16 and 722-67 were obtained from the corresponding terrestrial enrichments and had cells of coccoid shape, 1–2 μm in diameter. Strain 345-15 was the only one possessing flagella. All these strains grew optimally at 80–85°C and pH 3.5–4.0. All strains used yeast extract and starch as growth substrates; strains 345-15 and 405-16 were also able to use monosaccharides, disaccharides and peptone. Elemental sulfur was not required but stimulated growth of all isolates, being reduced to hydrogen sulfide. Acetate was the main growth product, and no hydrogen formation was observed. 16S rDNA sequence analysis placed all these isolates in the *Acidolobus* group (Table 2, Fig. 5). Within the group, the closest validated species is *Acidilobus aceticus* (Prokofeva et al. 2000) with 95.1–96.0% similarity; however, all new isolates exhibited higher similarity (96.1–97.4%) with the sequence of " *Caldococcus noboribetus*" (Aoshima et al. 1996) available from GenBank.

Cells of strain 768-28, also originating from terrestrial enrichment, were long, straight rods, 5.0 × 0.5 μm in size. This strain grew optimally at 80–85°C and pH 5.0–5.5. Growth was supported by different fermentable substrates, peptone and yeast extract supporting the best growth. Thiosulfate and elemental sulfur stimulated the growth and were reduced to hydrogen sulfide. The 16S rDNA sequence of strain 768-28 was 96.8% similar to that of *Vulcanisaeta distributa* (Itoh et al. 2002), thus suggesting that this isolate may represent a new species in this genus (Table 2, Fig. 5).

Strain 518-21 obtained from the deep-sea enrichment 518c possessed rod-shaped cells (0.6 × 3–6 μm). The temperature range for growth of this isolate was 40–85°C with the optimum at 60°C. It was found to be acidotolerant, with a pH optimum for growth at 6.0 and a pH minimum at 3.0. Isolate 518-21 was able to grow at an NaCl concentration of up to 7%, with optimal growth at 0–2.5% NaCl. Sugars, starch, peptone and yeast extract were used as growth substrates. Thiosulfate significantly stimulated growth and was reduced to H_2S. 16S rDNA sequence analysis revealed that strain 518-21 belonged to the genus *Thermoanaerobacter* (Table 2, Fig. 3), where its closest relative was *Thermoanaerobacter siderophilus* (Slobodkin et al. 1999) with 99.3% similarity.

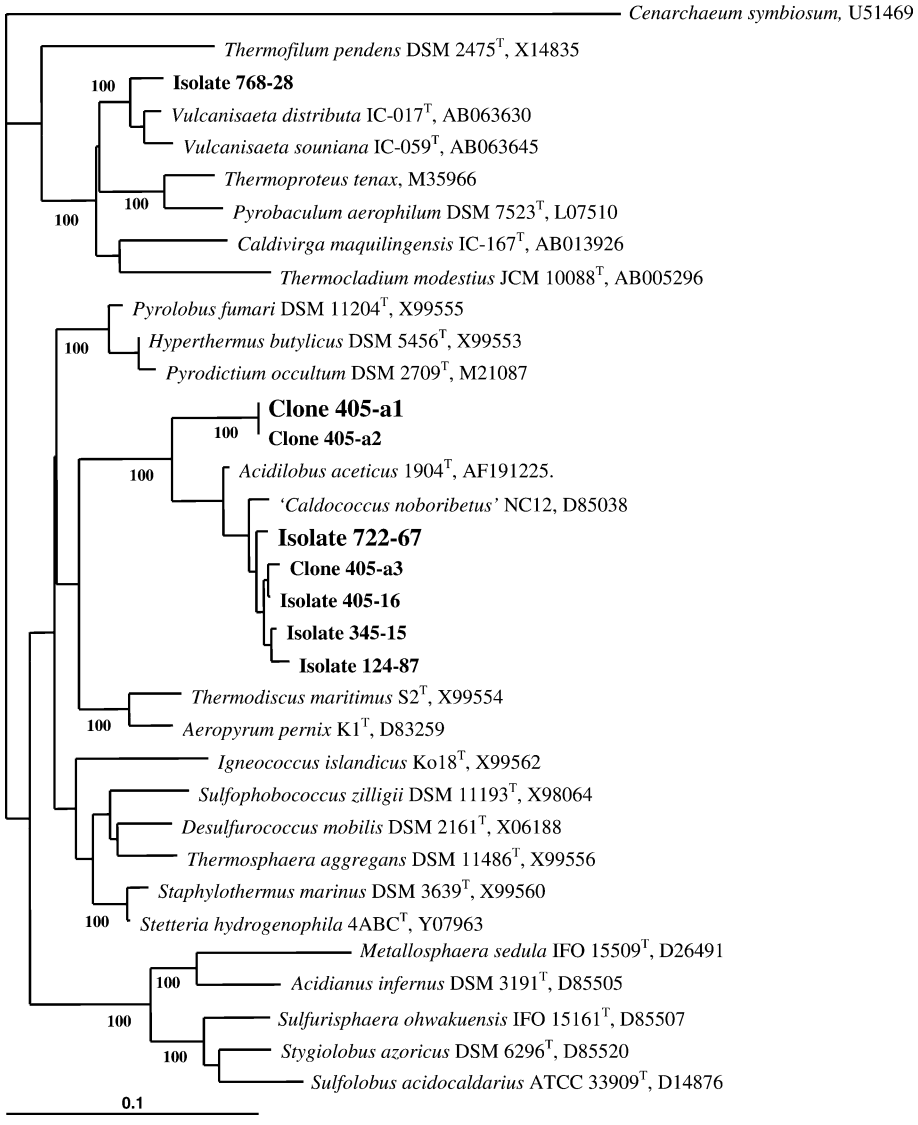

Fig. 5 Phylogenetic tree showing the phylogenetic positions of clones and isolates related to *Crenarchaeota* based on comparison of 16S rDNA sequences. The phylogenetic tree was constructed by the Fitch–Margoliash method. The sequence of *Cenarchaeum symbiosum* was included as an outgroup. The *bar* represents 10% of sequence difference. Bootstrap values (expressed as percentage of 100 replications) are shown at branching points; only maximal values (100%) are indicated

Discussion

Low pH values of water are common in terrestrial hot springs, where sulfuric acid (pK 1.8) is one of the main buffer compounds (Brock 1978). Sulfuric acid is formed in the reaction of volcanic H_2S with atmospheric oxygen; thus, sites with low pH values should be present mostly as aerobic environments. However, the solubility of oxygen at high temperature is very low and discharging gases in most of the acidic hot springs contain no oxygen (Baskov and Surikov 1989); thus, the existence of anaerobic zones is expected to occur beneath the zone of contact with the atmosphere.

Deep-sea hydrothermal vent chimneys are formed by metal sulfides precipitated from hot acidic anaerobic fluids during their contact with cold oxic seawater. Inside, outside and across these chimneys, microniches occur where microbial processes are based on gradients of nutrients and physicochemical conditions (temperatures from 350 to 2°C and pH from 3–4 to 8 within a few centimeters, oxygen levels from highly reduced to oxygenated). The mixing of anoxic, hot, acidic hydrothermal fluids with oxic, cool, moderately alkaline bottom seawater increases their pH and oxygen level. The steepness of pH and temperature gradients might explain the lack of successful attempts to cultivate aerobic thermoacidophiles from the deep-sea hydrothermal systems. The existence of discrete niches with temperatures and pH appropriate for thermoacidophiles could, however, be expected inside the chimney structures. On the basis of the retrieval of *Thermoplasma* related phylotypes in Mid-Atlantic Ridge hydrothermal samples, Reysenbach et al. (2000) suggested that thermoacidophiles probably occupy certain niches in the deep-sea vent ecosystem.

Anaerobic microorganisms inhabiting acidic thermal environments could obtain energy either from molecular hydrogen of volcanic origin, or from organic matter

synthesized by autotrophs or supplied by the turnover of water from cooler neutral zones. Thus, in this work both molecular hydrogen and organic substrates were used for the enrichment of thermoacidophilic anaerobes from terrestrial and deep-sea sources and caused the abundant anaerobic growth at pH 3.0–4.0. However, since no evident chemolithotrophic growth was obtained, organotrophic cultures became the main subjects of study. Fermentative microorganisms were found to dominate in anaerobic thermoacidophilic enrichments. Stimulating effect of electron acceptors should, most probably, be attributed to "facilitated fermentation" found in many thermophilic anaerobes.

Under hyperthermophilic and acidophilic conditions (at 85°C and pH 3.5) terrestrial communities were represented by archaea of the *Acidilobus* group. Most numerous clones were closely related to *A. aceticus*. Another group consisted of four new isolates also identified as *Acidilobus* spp. and only one clone. New isolates possessed main phenotypic characteristics of genus *Acidilobus*, of which the most important was the lack of molecular hydrogen formation during fermentation (Prokofeva et al. 2000). However, the new isolates exhibited several new phenotypic features, such as flagellation (strain 345-15) or the ability to ferment monosaccharides and disaccharides (strains 345-15, 405-16 and 722-67), and most probably represented a new species of genus *Acidilobus*.

In the same physicochemical conditions, only representatives of the genus *Thermococcus* were detected in deep-sea enrichments. The occurrence of *Thermococcus* at deep-sea hydrothermal vents is ubiquitous and members of this genus are among the most numerous newly described hyperthermophiles at deep-sea vents (Kobayashi 2001). Although these organisms are known to thrive generally at neutral pH, our data are not in contradiction with the characteristics of some *Thermococcus* species that have minimum pH of growth around 3.5–4.0 (Neuner et al. 1990; González et al. 1995; Huber et al. 1995; Godfroy et al. 1997). However, significant cell yields and products formation observed in our deep-sea enrichments might indicate the presence of new *Thermococcus* species for which low pH values are not suboptimal, but optimal.

At 60°C, both archaeal and bacterial representatives were detected in terrestrial and deep-sea thermoacidophilic enrichments. While archaea of the genus *Thermoplasma*, whose phylotypes were detected in enrichments 739 and 816, are known to be true acidophiles (Segerer et al. 1988), bacteria identified in the enrichments 761 and 711 turned out to be moderately acidophilic and acidotolerant organisms belonging to the genera *Thermoanaerobacter* and *Thermoanaerobacterium*. Most of the clones obtained from the enrichment 711 (Fig. 3) were closely related to *T. siderophilus*. However, the only pure isolate 711-75 and the minority of clones were clustering with *Thermoanaerobacter ethanolicus*. The isolate 761-119 belonged to genus *Thermoanaerobacterium*. Minimum pH for the growth of the new isolates was found to be around 3.0, while for other acidotolerant representatives of the genus *Thermoanaerobacterium* it was reported to be around 4.0 (Schink and Zeikus 1983; Liu et al. 1996; Cann et al. 2001), and for some members of genus *Thermoanaerobacter* around 4.5 (Kim et al. 2001). Thus, sharing the main metabolic properties with other members of these genera, they differ from them by higher acidotolerance.

Strain 518-21 isolated from East Pacific Rise was identified as a representative of *T. siderophilus* (99.3% of rDNA sequence similarity). So far, representatives of the *Thermoanaerobacter* genus have never been found in the deep-sea environment (Jeanthon 2000). The pH range for growth of the type strain of *T. siderophilus* isolated from terrestrial hot springs of Kamchatka (Slobodkin et al. 1999) was 4.8–8.2, while our isolate 518-21 had a minimum pH of growth at 3.0. Growth of the type strain was inhibited by 4% NaCl, while the new strain, though not dependent on sea salt, tolerated up to 7% NaCl. Thus, strains of the same species may be highly adapted to different environmental conditions.

It can be concluded that diverse populations of anaerobic prokaryotes able to carry out an efficient process of organic matter decomposition are present in terrestrial and deep-sea hot environments with low pH values. Whereas terrestrial communities contain highly specialized acidophilic archaeal populations (genera *Thermoplasma* and *Acidilobus*), no obligately acidophilic anaerobes were detected in the deep-sea cultures studied here. Contrary to the stable physicochemical conditions that generally prevail in terrestrial environments, the large fluctuations in pH in the turbulent mixing zones at deep-sea vents may not favor the lifestyle of obligately acidophilic organisms. On the basis of our results, acidic and high-temperature conditions occurring at vents may rather support the growth of organisms able to thrive over a broader pH range. We cannot, however, exclude that yet uncultivated strictly acidophilic thermophiles occupy certain niches in the deep-sea hydrothermal ecosystem.

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