Extremophiles (2002) 6:201–207
Digital Object Identifier (DOI) 10.1007/s007920100242

ORIGINAL PAPER

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Acidophiles of saline water at thermal vents of Vulcano, Italy

Received: April 12, 2001 / Accepted: August 15, 2001 / Published online: January 22, 2002

Abstract DNA was extracted from samples taken from close to acidic hydrothermal vents on shore of the Aeolian Island of Vulcano (Italy). RNA gene sequences were amplified by PCR, cloned, and sequenced. A sequence with an origin in samples at 35° and 45°C corresponded to that of a novel *Acidithiobacillus* species that was isolated from water close to the vents. Novel, iron-oxidizing mesophilic acidophiles were isolated through enrichment cultures with ferrous iron but were not represented in the clone banks of environmental rDNA. These acidophiles were related to *Thiobacillus prosperus*, which was isolated previously from Vulcano. The archaeal sequences that comprised a clone bank representing a high-temperature sample (75°C) corresponded to those of *Acidianus brierleyi* and of thermophiles previously isolated from Vulcano, *Thermoplasma volcanium* and *Acidianus infernus*.

Key words Acidophiles · Vulcano · Hydrothermal vents · 16S rDNA

Introduction

A variety of mesophilic and thermophilic bacteria and thermophilic Archaea have been isolated from the hydrothermal vents of Vulcano, one of the Aeolian Islands in the Tyrrhenian Sea north of Sicily (Gugliandolo et al. 1999). Water close to these submarine vents is generally acidified to about pH 5 or 6 (Gugliandolo and Maugeri 1998). The acidity has been attributed to dissolved volcanic gases (principally CO_2), hydrolysis of SO_2, and oxidation of H_2S on mixing with surface water (Sedwick and Stüben 1996). Acidophiles previously isolated from Vulcano include mesophilic, sulfur-oxidizing *Thiobacillus*-like strains (Gugliandolo and Maugeri 1993), mesophilic, ferrous iron- and mineral sulfide-oxidizing *Thiobacillus prosperus* (Huber and Stetter 1989), and thermophilic *Acidianus infernus* (Segerer et al. 1986) and *Thermoplasma* species (Segerer et al. 1988). The descriptions of these organisms indicated that their tolerance to salt differed between particular isolates of species. Although most could grow in seawater, optimum growth in all cases was at lower salt concentrations or, in the case of *Thiobacillus prosperus*, without salt. Acidophiles are generally inhibited by chloride ions that accumulate intracellularly in response to positive membrane potentials, the consequent reduction of which leads to denaturing acidification of the cytoplasm (Alexander et al. 1987; McLaggan et al. 1990). The thermal waters of Vulcano are slightly less saline than seawater, which has been attributed to seawater mixing with low-salinity groundwater and to its alteration through interaction at high temperature with subsurface minerals (Gugliandolo et al. 1999; Sedwick and Stüben 1996).

This report describes the extraction and analysis of 16S rDNA from the most acidic sites at Vulcano. This study was intended to indicate potentially significant components of the in situ microbial communities without bias of selective strain isolation procedures. Enrichment culture of indigenous sulfur- and iron-oxidizing acidophiles allowed comparison of the readily isolated strains with those predicted by environmental rDNA analysis.

Materials and methods

Sample sites and sample collection

Characteristics of submarine hydrothermal vents and beach fumaroles of Porto di Levante, Vulcano, have been described previously (Gugliandolo et al. 1999; Sedwick and Stüben 1996). Onshore and adjacent to the beach of the East Bay (Baia di Levante), the principal acidic waters com-

Communicated by W.D. Grant

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Table 1. Characteristics of water-sediment samples

Sample site	Temperature (°C)	pH	Ferrous iron (mg l^{-1})
Large, shallow pool adjacent to beach	35	2.5	>500
Volcanic sand/gravel (water-saturated)	45	3.5	10
Hot water-filled vent	75	2	>500

prised a thermal pool popular with bathers and a larger, rectangular pool. Three samples were taken (September 1999) within a few meters of each other near one corner of the rectangular pool where there were some small hot vents and some gaseous effervescence through warm sediment (Table 1). Water temperatures at sample sites were measured and pH values were estimated with indicator papers (range, pH 1–6; Merck, Lutterworth, UK). Subsequent measurement of the pH of samples with a probe in the laboratory at room temperature gave similar values. Approximate concentrations of chloride and ferrous iron were estimated on site using paper test strips (Merckoquant; Merck). Sediments or vent walls were scraped during sampling so that water samples contained approximately 50% v/v solids. Samples were kept at about 20°C for almost 2 days before they were used to establish enrichment cultures at various temperatures or before they were frozen at –80°C before DNA extraction.

DNA extraction

The extraction procedure for environmental samples was based on that used by Barns et al. (1994) with hot spring samples from Yellowstone National Park: 10 ml of each sample containing suspended solids was mixed with 10 ml extraction buffer before treatments with lysozyme, proteinase K, lysis buffer, and freeze/thaw cycles. Extractions with phenol and phenol-chloroform-isoamyl alcohol were followed by precipitation and washing of DNA. The polyvinylpolypyrrolidone treatment step in the original method was omitted.

16S rDNA cloning, sequencing, and analysis

Amplifications of 16S rDNA sequences were performed with a Perkin-Elmer (Norwalk, CT, USA) DNA Thermal Cycler: 480 and 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min. Reaction mixtures contained 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µmol each dATP, deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP), a universal prokaryotic reverse primer (R1492, 5′-tacggytaccttgttacgactt-3′), and either a eubacterial (F27, 5′-AGAGTTTGATCMTGGCTCAG-3′) or archaeal (F25, TCYGGTTGATCCYGCCRG-3′) forward primer (each primer at 100 pmol). Polymerase chain reaction (PCR) products were cloned using *Taq* polymerase and the TOPO TA Cloning Kit (Version J) vector (pCR 2.1-TOPO) and host *Escherichia coli* strain (Invitrogen, Groningen, The Netherlands). Clone banks for each original sample were made and clone types grouped on the basis of restriction fragment-length polymorphism (RFLP) identities following *Eco*RI/*Sau*3AI and *Eco*RI/*Rsa*I double digests. Cloned rRNA genes of subgroup representatives were sequenced using Applied Biosystems Sequencers (Warrington, UK), models 373A and 377. The GenBank database was searched for similar sequences using BLAST programs (Altschul et al. 1997). The PILEUP program of GCG (Genetics Computer Group, University of Wisconsin, Madison) was used for an initial alignment of sequences. Evolutionary distances were calculated with the Jukes–Cantor method in the DNADIST program, a phylogenetic tree was inferred with a neighbor-joining method (NEIGHBOR), and bootstrap values were determined using the SEQBOOT program, all in the PHYLIP package, version 3.57 (Felsenstein 1995).

The 16S rDNA sequences of clone types V1 (accession number AF339743), V3 (AF339744), and V5 (AF339745) were deposited with GenBank. The 16S rRNA gene of *Thermoplasma volcanium* DSM 4299 was also amplified, cloned, and sequenced (unpublished work; N.P. Burton and P.R. Norris), and submitted to GenBank with accession number AF339746.

Enrichment culture media and conditions

All enrichment cultures were grown in a mineral salts medium containing (in g l^{-1}) $(NH_4)_2SO_4$ (0.2), $MgSO_4 \cdot 7H_2O$ (0.4), K_2HPO_4 (0.1), and $FeSO_4 \cdot 7H_2O$ (0.01). This medium was adjusted with H_2SO_4 to the following pH values and supplemented separately with the following substrates: pH 1.7, ferrous iron (50 mM; 13.9 g $FeSO_4 \cdot 7H_2O$ l^{-1}); pH 3, sulfur flowers (5 g l^{-1}); pH 3, potassium tetrathionate (2 mM); pH 2, yeast extract (0.01% w/v). Tetrathionate (0.5 mM) was also added to ferrous iron-containing medium for enrichment of autotrophic bacteria because some species require a source of reduced sulfur for their growth on iron (Norris and Barr 1985). Cultures were supplemented with various concentrations of salt (NaCl) and were incubated at different temperatures as indicated in the text.

Ferrous iron and tetrathionate-containing media (pH 2) were solidified with Phytagel (Sigma-Aldrich, Poole, UK) at a final concentration of 0.4% (w/v) for growth of single colonies of iron- and sulfur-oxidizing acidophiles.

Results

Description of sample sites

The acidity of water at some of the onshore thermal vents (Table 1) was much greater than that generally associated with the submarine hydrothermal vents of Vulcano. Three

samples were collected for DNA extraction and to establish enrichment cultures of acidophiles (see Table 1).

There was no evidence of thermal sources in most of the area covered by the rectangular pool. The sample (35°C) from this pool was warmer than the bulk of the water and sediment because it was taken near the few thermal vents that were present.

A warmer sample was taken from water-saturated, hard-packed volcanic sand and gravel adjacent to the rectangular pool. This area had standing surface water in places but only to a depth of less than a centimeter and was subjected to continuous gas venting. The temperature ranged from about 40° to 45°C at the surface but increased to 55° to 60°C at a depth of a few centimeters.

The final sample was of dark gray, turbid fluid (75°C) from a hydrothermal vent. It was vigorously gassed but did not overflow its well.

The 35°C pool sample: predicted community types

The 16S rDNA clone bank that was established from the acidic pool sample comprised only two clone types as revealed by RFLP analysis and subsequent sequencing of cloned inserts (Fig. 1A). Clone type V1 is proposed to represent a novel species of *Acidithiobacillus*, a genus recently created for acidophilic strains of *Thiobacillus* (Kelly and Wood 2000). This genus is phylogenetically separated from the thiobacilli and from other acidophiles in the Proteobacteria (Fig. 2). An acidophile with a 16S rDNA sequence identical to that of clone type V1 was readily isolated after enrichment culture with sulfur or tetrathionate as substrate and revealed to be a salt-tolerant and slightly thermotolerant mesophile. Its optimum temperature for growth was about 40°C, between those of *Acidithiobacillus thiooxidans* (about 30°C) and *Acidithiobacillus caldus* (about 45°C). An organism with an identical 16S rDNA sequence to that of clone type V2 was also isolated after enrichment culture with sulfur. Its 16S rDNA sequence was almost identical to that of *A. thiooxidans* (see Fig. 1A). Initial RFLP analysis indicated two additional clone types had been obtained, each only once, but sequencing revealed them to contain chimeras of clone type V1 and V2 sequences.

The 45°C water-saturated, volcanic sand sample: predicted community types

Clone type V1, found in the 35°C sample, was also prevalent in the bacterial rRNA gene clone bank (Fig. 1B) representing organisms in the heated, saturated ground that was adjacent to the large acidic pool. The lower end of the temperature range at the surface of the sampled area (just over 40°C) was close to the maximum temperature for growth (about 42°C) of the organism that was isolated and predicted to be the source of the clone type V1 sequence (see above).

Clone type V3 corresponded to an organism whose closest relative appeared to be the iron-oxidizing, salt-tolerant acidophile *Thiobacillus prosperus*, which was discovered previously in the same area (Huber and Stetter 1989). The description of *T. prosperus* (Huber and Stetter 1989) referred to other physiologically similar iron-oxidizing acidophiles that might merit separate species or genus status in view of very low DNA–DNA hybridization values with *T. prosperus*. Clone type V3 could correspond to one of this

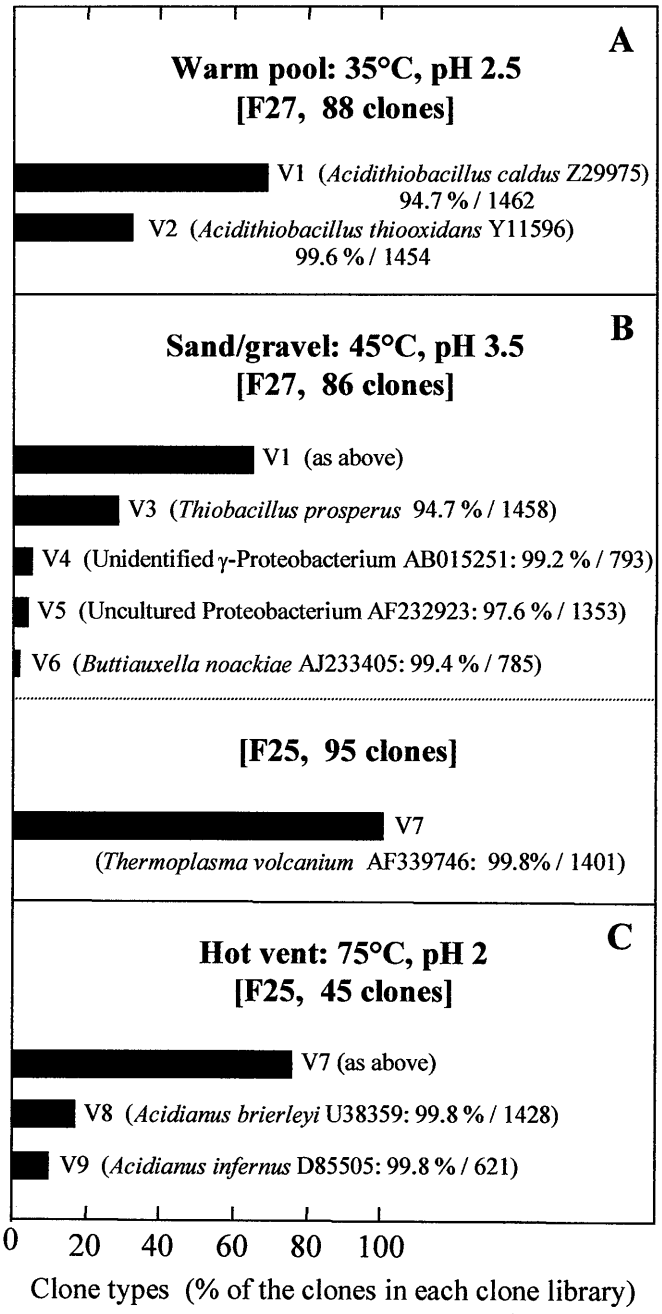

Fig. 1A–C. Analysis of 16S rDNA clone libraries from Vulcano samples. Clone types (*V numbers*) from three sample sites (**A–C**) are shown as a percentage of the total clones in each of four libraries. The forward primers used in the polymerase chain reaction (PCR) and the number of clones for which restriction fragment-length polymorphisms (RFLPs) were examined are given. Database sequences with the highest percentage identities to Vulcano clone sequences are given with source organism names and the number of nucleotides over which the comparisons were made (*in parentheses*)

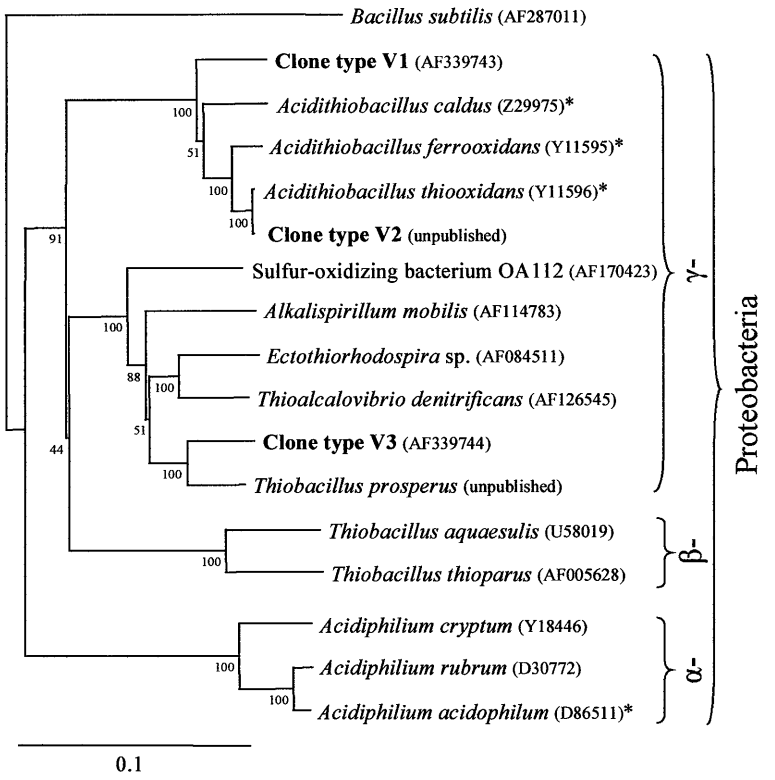

Fig. 2. Phylogenetic tree of 16S rRNA gene sequences (from alignment of 1,427 nucleotide positions) showing Vulcano clones that were related to *Acidithiobacillus* species and to *Thiobacillus prosperus*, with *Bacillus subtilis* as an outgroup. An *asterisk* indicates organisms that were known as *Thiobacillus* species when the sequences were deposited with the database. *Numbers* at branch nodes, bootstrap values based on 100 resamplings; *bar*, substitutions per nucleotide

group. Its 16S rDNA sequence shared almost 95% identity with that of *T. prosperus* (Fig. 1B) and 90%–91% identity with those of the other bacteria most related by rRNA gene sequence: these were a sulfur-oxidizing strain (OAII2) from an unidentified shallow water hydrothermal vent and alkaliphilic sulfur oxidizers such as *Alkalispirillum mobilis* and *Thioalcalovibrio denitrificans* (Fig. 2).

Clone type V4 corresponded to unidentified γ-Proteobacteria isolated from deep-sea sediments (Fig. 1B) (Li et al. 1999) and to several *Pseudomonas* species, but not to any strains associated with thermophily or acidophily. Similarly, clone type V6 corresponded to several *Buttiauxella* species (Fig. 1B), members of the *Enterobacteriaceae* that have been isolated from unpolluted water and soil and frequently from snails and slugs (Müller et al. 1996). These types, with V6 obtained as only a single clone, might not represent strains indigenous to the acidic sample sites. However, clone type V5 might represent an uncultured thermophile that inhabits sulfur-rich geothermal environments. The only closely related database sequence is one amplified from a hot, acidic pool on the Caribbean island of Montserrat (see Fig. 1B) (Burton and Norris 2000). The next most related sequences are from thermophilic sulfur-reducing bacteria of the *Thermosipho* and *Fervidobacterium* genera, but the differences between them and clone type V5 might indicate different bacterial divisions.

A single clone type, V7, comprised the clone bank established from the warm volcanic sand using the archaeal forward primer F25 (see Fig. 1B). The sequence corresponded to that of *Thermoplasma volcanium*, which was isolated previously from Vulcano and other geothermal environments by Segerer et al. (1988), who found three different DNA–DNA homology groups of the organism with some marked geographically defined distribution. The comparison of the clone type V7 sequence (see Fig. 1B) was with that of the type strain of *T. volcanium* (DSM 4299), which was isolated from Vulcano.

Hot water vent (75°C): predicted community types

No bacterial 16S rRNA genes were amplified from the 75°C sample using the forward primer F27. Clone type V7, corresponding to *T. volcanium* (see earlier), was obtained using the archaeal forward primer F25 (see Fig. 1C). The sample temperature was higher than the maximum temperature of about 67°C that was reported to allow growth of *Thermoplasma volcanium* (Segerer et al. 1988). The rDNA might have been extracted from organisms released into the hot fluid from or through the slightly cooler vent wall. Clones corresponding to thermophilic Archaea that grow at the sample temperature were also obtained (Fig. 1C). Clone type V8 corresponded to *Acidianus brierleyi*. Clone type V9 corresponded to *Acidianus infernus*, which was previously isolated from Vulcano (Segerer et al. 1986). One of nine clones initially identified as a V8 clone type on the basis of its RFLP was found by sequencing to be a chimera, comprising primarily the *A. brierleyi* sequence but with some sequence from *A. infernus* from the 3′-terminus.

Table 2. Identity of clone types from laboratory enrichment cultures of acidophiles at 70°C

Substrate	Cloned sequences	Corresponding species	16S rDNA sequence identity
Sulfur	23	*Sulfolobus metallicus* (13 clones)	99.7%
		Acidianus brierleyi (10)	99.4%
Pyrite	29	*S. metallicus* (28)	
		A. brierleyi (1)	

16S rRNA gene sequence identities of the clone types were compared to those of the corresponding species over 548 5′-terminal nucleotides

Enrichment culture of acidophiles: mesophilic and moderately thermophilic iron- or sulfur-oxidizing bacteria

A 35°C sample from the rectangular pool was used to establish enrichment cultures at 30°C with sulfur or tetrathionate as substrates in the presence of salt (3% w/v NaCl). Isolation of single colonies from these cultures on solid medium provided strains corresponding to clone types V1 and V2, as noted. Incubation of the 35° or 45°C samples with sulfur in the presence of salt at 50°C did not produce bacterial growth, but *Acidithiobacillus caldus* was isolated following enrichment culture in the absence of salt at this temperature. Previous attempts to isolate thermophilic sulfur-oxidizing bacteria from Vulcano in the presence of salt also failed (Gugliandolo and Maugeri 1993).

Enrichment culture with ferrous iron as substrate followed by single colony isolation on solid medium produced two iron-oxidizing mesophilic acidophiles from the 35°C pool sample. These bacteria were most closely related phylogenetically to *Thiobacillus prosperus* but differed from it in their obligate requirement for salt for growth (data not shown). Neither corresponded to clone type V3 but shared approximately 95% 16S rRNA gene identity with it and with *T. prosperus* (data not shown).

Enrichment culture of the warm volcanic sand sample with ferrous iron and yeast extract at 50°C yielded moderately thermophilic ferrous iron-oxidizing bacteria. Two isolates, which were obtained in pure culture, resembled *Sulfobacillus* species and shared a high similarity of their 16S rRNA gene sequence with that of *Sulfobacillus thermosulfidooxidans* (99.5% identity over the 5′-terminal 400 nucleotides). Their growth was partially inhibited by 2% w/v NaCl, indicating a similar salt tolerance to that of *S. thermosulfidooxidans* isolates from terrestrial and freshwater geothermal, acidic environments (data not shown).

Enrichment culture of acidophiles: thermophilic Archaea

Enrichment cultures were established with samples from the hot vent (75°C) using (separately) sulfur, pyrite, and yeast extract as substrates at 70°C. Good growth could not be maintained with any of these substrates in the presence of salt (3% w/v NaCl) close to its seawater concentration. In the absence of salt, autotrophic growth was readily established with sulfur and pyrite as substrates. 16S rDNA clone banks were made after lysis of cells harvested from the second serial culture with these substrates. Two RFLP clone types were found. Representative clones were sequenced (Table 2): one corresponded to *Sulfolobus metallicus*, with no other close matches in the database (all below 90% identity), and the other corresponded closely only to *Acidianus brierleyi*. This enrichment culture clone showed a single nucleotide difference from clone type V8 (see Fig. 1) over the 5′-terminal 548 nucleotides. The principal differences between these Vulcano sequences and that of the *A. brierleyi* type strain were contained in a region between helical stalks with -CCAAAA- in V8 and -TTAAAG- in *A. brierleyi*, corresponding to nucleotides 193–198 in *Escherichia coli*.

The selection by enrichment culture at 70°C for *A. brierleyi* over *A. infernus* with sulfur as substrate (see Table 2), in contrast to the indication of both in the environmental sample (see Fig. 1C), could have reflected their optimum temperatures for growth of about 75°C and 90°C, respectively (Segerer et al. 1986). Similarly, previous observations have shown that autotrophic growth of *S. metallicus* on pyrite is much more rapid than that of *A. brierleyi* at 70°C (Norris and Owen 1993, in which *A. brierleyi* was compared to *Sulfolobus* strain BC, which was later identified as *S. metallicus*). *S. metallicus* appeared to dominate the enrichment culture with the mineral substrate at 70°C (see Table 2), but *A. brierleyi* would have been favored over *S. metallicus* at the temperature (75°C) of the environmental sample (Norris and Owen 1993).

Slow but reproducible growth was obtained in enrichment cultures with yeast extract as the sole substrate in the absence of salt at 70°C, but the *Sulfolobus*-like organisms in the culture were not identified. The growth rate of the culture was similar to that obtained with *A. brierleyi* growing heterotrophically and was much slower than that of obligately heterotrophic *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus* (data not shown).

Discussion

This analysis has confirmed some of the expected microbial diversity in a salt-rich, acidic environment. The prevalence of certain clone types might have indicated organisms that were major components of the communities, but the results are only qualitative in the absence of more extensive sampling and in the light of various analytical biases, including sample-handling procedures and potentially differential cell lysis and gene amplification (Wintzingerode et al. 1997).

A great diversity of microorganisms, including many novel phylotypes, was revealed by rRNA-based analysis of samples from hot springs with pH values too high for proliferation of acidophiles (Barns et al. 1994; Hugenholtz et al. 1998). A similar analysis of strongly acidic, nonsaline, hot springs on the island of Montserrat also indicated some novel phylotypes (Burton and Norris 2000). The diversity appeared to be less in the acid springs, but the acidophile populations might have been dominated by relatively few strains so that minority components were not represented in small clone banks of environmental rDNA. Three clone types (V1, V3, and V5) from acidic Vulcano samples did not correspond closely to described species. The bacterial clone type (V1) corresponded to a proposed novel species of *Acidithiobacillus*. This organism might well have been isolated previously from Vulcano without being fully characterized (Gugliandolo and Maugeri 1993). The sequences of clone types V3 and V5 could be targeted with specific fluorescent probes and enrichment culture conditions designed for organisms of the suggested physiologies, aerobic iron-oxidizers and anaerobic sulfur-reducers, respectively.

The prevalence of clone type V1 in the clone libraries from the lower-temperature samples and ready enrichment of the corresponding organism with demonstrated salt tolerance suggest an organism well suited to the environment. In contrast, *Acidithiobacillus caldus*, with less salt tolerance, did not feature in clone banks and was only readily isolated by enrichment culture in the absence of salt. Other acidophiles that lacked genuine salt tolerance, such as *Sulfobacillus thermosulfidooxidans*, also dominated enrichment cultures without salt but were not represented among the environmental rDNA clones. However, *Sulfobacillus* species were also readily found in enrichment cultures from a freshwater geothermal site but again were not represented in rDNA clone banks (Burton and Norris 2000). Any influence of sporulation by these organisms as a factor in their apparently easier revelation by enrichment culture than by direct rDNA extraction is unknown.

The Archaea that were represented in the clone banks (*Thermoplasma volcanium*, *A. infernus*, *A. brierleyi*) have shown optimum growth in the absence of salt or at least at concentrations below that of seawater, but it is possible that they have slightly more salt tolerance than other acidophiles of similar physiology. For example, *T. volcanium* grows with 2% w/v salt and some strains grow slowly with 4% w/v salt (Segerer et al. 1988) whereas the related *Picrophilus* species are inhibited by about 1% w/v NaCl (Schleper et al. 1995). Some isolates of *A. infernus* show a broad tolerance of salt with growth between 0.1% and 4% w/v, which was independent of their marine or freshwater origins (Segerer et al. 1986). The absence of *S. metallicus* from the environmental rDNA clone bank might reflect the temperature of the hottest sample rather than its salinity. Its salt tolerance, with optimum growth below 0.75% w/v salt but some tolerance up to 3% (Huber and Stetter 1991), does not appear greatly different to that of *A. brierleyi* or *A. infernus*, while the *Acidianus* species grow at higher temperatures. However, other unknown factors could influence the natural populations since *T. volcanium* has a lower maximum temperature for growth than *S. metallicus* but rDNA from the *Thermoplasma* rather than from the *Sulfolobus* was amplified from the hot sample. There was no evidence for novel species of salt-tolerant thermoacidophilic Archaea from the environmental gene analysis or from enrichment cultures.

Acknowledgments We gratefully acknowledge the support of a Biotechnology and Biological Research Council studentship.

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