

Microbial diversity in acidic anaerobic sediments at the geothermal Caviahue-Copahue system, Argentina

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Abstract. The microbial diversity of sediment in the acidic Agua del Limón hot spring (located in the geothermal Caviahue-Copahue area, Argentina) was investigated using a combination of molecular and cultivation techniques, with particular emphasis on indigenous anaerobic prokaryotes. Bacteria involved in the iron (*Acidithiobacillus ferrooxidans* and *Leptospirillum* spp.) and sulfur (*Acidithiobacillus* spp., *Thermotogales*-like bacteria, *Thiomonas* sp., and *Desulfurella* sp.) cycles were identified in the clone library. Although no obvious sulfate-reducing bacteria were detected by culture-independent techniques, several isolates related to the mesophilic, spore-forming sulfate-reducer “*Desulfobacillus acidavidus*” strain CL4 were isolated on solid media incubated at both 30°C and 40°C. The 16S rRNA gene of another isolate showed 94% similarity to *Desulfotomaculum thermobenzoicum*. Enrichment cultures of sulfate reducers using Copahue samples were also dominated by “*Dsb. acidavidus*” CL4.

Introduction

Interest in microorganisms that inhabit extreme environments has increased enormously in recent years due, among other things, to their potential application in various biotechnological processes. Environments characterized by low pH values, such as volcanic areas, hydrothermal and acid mine drainage-affected areas, are of particular interest when prospecting for microorganisms with potential application in biohydrometallurgical processes [1, 2]. The microbial diversity and ecology of the geothermal volcanic area of Copahue, Argentina has been studied for many years, with the main emphasis being aerobic, acidophilic sulfur- and iron-oxidizing bacteria and archaea [2]. Relatively few studies have investigated the anaerobic zones of this environment, and these have tended to be restricted to neutrophilic sulfate-reducing bacteria (SRB). Here we describe the first approach to analyse the microbial diversity in anaerobic sediments at Copahue, using a combination of molecular and cultivation techniques, focusing on acidophilic/acid-tolerant SRB.

Site description and samples. The geothermal Caviahue-Copahue system is an extreme environment located in the north-west of Neuquén Province, Argentina. This area is characterized by a wide range of temperatures (20-90 °C) and pH (<1 to 8) and elevated concentrations of transition metals [2]. Table 1 shows the physicochemical parameters of the samples taken in February 2012 from five hot springs at Copahue. All samples were characterized by negative E_H values, indicating anaerobic conditions.

Table 1. Physicochemical characteristics of Copahue samples

Site	pH	T [°C]	E _H [mV]
B9 (3) Baño 9	5	67	-290
AL1 Agua del Limón	2	41	-200
LMi Las Maquinitas	5	90	-126
LMa1 Las Máquinas1	4	74.4	-249
LMa2 Las Maquinas2	3	38	-70

Methodology

Microbial community analysis by terminal restriction fragment length polymorphism (T-RFLP). DNA was extracted from Copahue samples using the Ultra – Clean™ Soil DNA Isolation Kit (MoBio Laboratories, USA) according to the manufacturer's instruction. 16S rRNA genes were amplified from the DNA extracts using universal bacterial primers (Cy5-labeled 27F and unlabeled 1387R) for terminal restriction enzyme fragment length polymorphism (T-RFLP) analysis. Amplified gene were purified and then digested with the restriction enzymes HaeIII, CfoI and AluI. The digested DNA was analysed by T-RFLP on a Beckman Coulter 8000 capillary system [3].

Bacterial 16S rRNA gene clone library analysis of sample AL1. Extracted DNA was amplified by PCR using the universal bacterial primers 27F and 1387R. The purified PCR product (pooled from three independent reactions) was ligated using the pGEM-T Easy Vector System (Promega, USA) and the resulting plasmids transformed into *Escherichia coli* strain DH5 α according to the manufacturer's instructions. Positive clones (80) were subjected to RFLP analysis, using the restriction enzymes MspI and CfoI. The plasmid of at least one representative clone for each RFLP group was purified using the Concert Rapid miniprep system (Life Technologies) and sequenced. Sequences were compared with those in the NCBI database using BLAST, and the phylogenetic affiliations of the clones determined by the ARB software package and using the Classifier tool of the Ribosomal Database Project (RDP). The constructed clone library was evaluated by rarefaction analysis using the software aRarefaction 1.3 (Steven Holland, Stratigraphy Lab, University of Georgia).

Acidophilic SRB enrichment cultures. Sediment samples from Copahue hot springs were inoculated into liquid medium (pH ~3.8) previously used to enrich aSRB [4]. This contained 3 mM glycerol, 0.01 % (w/v) yeast extract, 4 mM zinc, 100 μ M FeSO₄, 0.87 g K₂SO₄/L, trace elements and basal salts solution [4]. The enrichments were incubated in sealed anaerobic jars (using the AnaeroGen system; Oxoid, UK) at 30°C. After one month, concentrations of glycerol and sulfate were determined by ion chromatography and the microbial community analysed by T-RFLP.

Isolation and identification of acidophilic SRB. Samples were streak–inoculated onto overlay plates that promote the growth of aSRB [4] and incubated anaerobically at 30 and 40°C. Colonies were purified by repeated single colony isolation. 16S rRNA genes of isolated colonies were amplified and analysed by sequencing and T-RFLP.

Results

Microbial diversity of Copahue samples. Bacterial 16S rRNA gene clone library analysis of sample AL1 revealed the presence of various microorganisms involved in the sulfur cycle (Fig. 1), belonging to the *Beta*-, *Delta*- and *Gammaproteobacteria*. Members of the *Gammaproteobacteria* class were closely related to *At. ferrooxidans*, *At. ferridurans* and *At. thiooxidans* (98-99% sequence similarity).

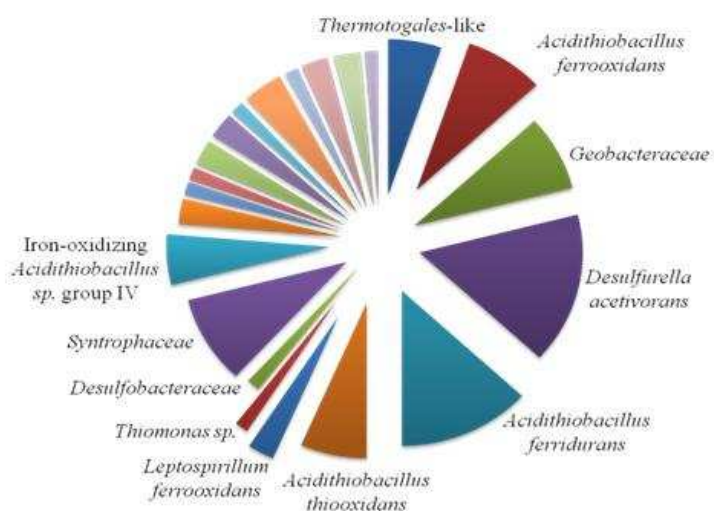


Fig. 1. Bacterial diversity of Caviague-Copahue sediment sample AL1

Desulfurella spp. and unclassified *Deltaproteobacteria* were also found in the clone library. Bacteria of the genus *Desulfurella* include acid-tolerant sulfidogens which have been detected in several acidic anaerobic sediments [5]. Sequences clustering with the phylum *Deltaproteobacteria* were distantly related to the *Syntrophaceae*, *Desulfobacteraceae* and *Geobacteraceae* families. One clone was closely (99% sequence similarity) related to the betaproteobacterium *Thiomonas intermedia* ATCC 15466, a moderate acidophile that oxidizes reduced sulfur [7]. Sequences affiliated with bacteria of the iron cycle were represented by *Leptospirillum* spp. and *At. ferrooxidans*. Four clones could not be assigned to any phylogenetic group and were classified to 35% as *Thermotogales* using the RDP Classifier tool. Rarefaction analysis showed that the curves tended to approach the saturation plateau (data not shown) and a coverage index of 92% was estimated from the number of expected ARDRA patterns vs. the number of clones in the library.

Results of the T-RFLP analysis of four Caviague-Copahue sediment samples are shown in Fig. 2. Similar profiles were obtained for samples LMa2 and AL1 (Fig. 2a); both samples were dominated by *At. ferrooxidans/ferridurans* (253 nt), *Thermotogales*-like bacteria (319 nt) and *Desulfurella acetivorans* (211 nt). Profiles from samples B9(3) and LMi displayed more quantitative variation. The dominant peak (221 nt) in sample LMi could not be assigned to any clones identified in the clone library or any sequence present in the Bangor University database of acidophilic bacteria.

Acidophilic SRB in Copahue sediments. Three isolates were obtained from samples B9(3), LMi and LMa1 on overlay plates incubated at 30°C, all of which were closely related (99% gene sequence identity) to the mesophilic, spore-forming sulfate-reducer “*Desulfobacillus acidavidus*” strain CL4 [4]. Two more isolates were obtained from plates incubated at 40°C. One of these (isolate LMa2_C1) was a spore-forming, slightly curved rod, with 94% similarity to *Desulfotomaculum thermobenzoicum*, representing a possible novel genus and species. The other isolate (AL1_E1) formed unusual “fried egg” colonies and was only 96% related to “*Dsb. acidavidus*” CL4.

Sulfidogenesis at low pH and characterization of enrichment cultures. The major objective of the enrichment cultures was to establish a sulfidogenic microbial community from the environmental samples, which could grow at low pH. Complete consumption of glycerol was observed in all anaerobic enrichment cultures, and sulfate also decreased from 14 mM to 13 mM after 30 days of incubation at 30°C. T-RFLP analysis after 30 days revealed the sulfate-reducer “*Dsb. acidavidus*” strain CL4 as the dominant organism in the cultures.

Discussion

Biomolecular and cultivation-based analysis of Copahue anaerobic sediment AL1 confirmed that it is mostly composed of anaerobic prokaryotes that catalyze the reduction of sulfur (*Desulfurella* spp.

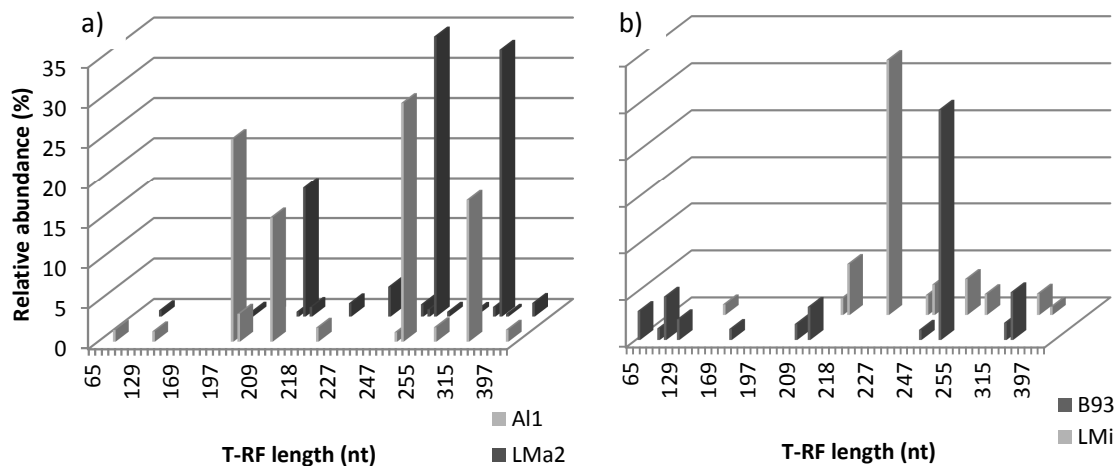


Fig. 2. T-RFLP analysis of sediment samples from Copahue digested with the restriction enzyme HaeIII.

Thermotogales-like-bacteria) or ferric iron (e.g. *At. ferrooxidans/ferridurans*). Although no obvious sulfate-reducers (which are usually present in minor numbers) were detected by molecular techniques, several mesophilic, sulfate-reducing isolates were obtained both directly on plates and from enrichment cultures. These included LMa2_C1 which has 94% sequence similarity to its closest classified relative, *Ds. thermobenzoicum*. Members of the genus *Desulfotomaculum* originate from thermal environmental sites but were also reported in acidic natural environments such as the Tinto River. Some species are able to grow autotrophically, while others grow by fermentation of glucose or other organic compounds [7, 8]. More studies are required to confirm the identity of this new species. Isolate AL1_E1 isolated at 40°C has 96% sequence similarity to “*Dsb. acidavidus*” CL4, which was isolated from an anaerobic microbial mat in an abandoned mine in south-west Spain and was reported to grow at 30°C [4]. Since the volcanic hydrothermal environments such as Copahue are quite different from those in acid mine drainage, isolate AL1_E1 could also be different from the strain previously described. In addition a sulfidogenic microbial community, able to grow at pH 3 and dominated by “*Dsb. acidavidus*” CL4, was established from the hot spring sediments. These acidophilic consortia could be more efficient than pure cultures of SRB in the bioremediation of acidic mine-impacted waters found in Argentina and elsewhere.

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