Extremophiles (2002) 6:419–425
Digital Object Identifier (DOI) 10.1007/s00792-002-0274-7

ORIGINAL PAPER

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Microbial community in acidic hydrothermal waters of volcanically active White Island, New Zealand

Received: June 15, 2001 / Accepted: March 12, 2002 / Published online: June 13, 2002

Abstract We report the first description of the microbial community in a stream of acidic hydrothermal waters on volcanically active White Island, New Zealand, using both molecular and microbiological methods. α- and β-Proteobacteria, green-sulfur bacteria, and uncultured Firmicutes were identified from the community DNA-based 16s rRNA gene library. The same bacterial groups and the Rhodophyte *Cyanidium caldarium* were represented in enrichment cultures. *C. caldarium*, two Firmicutes and an acidophilic α-Proteobacterium, *Acidiphilium cryptum*, were brought into pure culture. Bacteria cultured from the stream grow at pH ≥ 2, and the *Cyanidium* grows at pH 0.2.

Key words 16S rRNA · *Cyanidium* · *Acidiphilium* · White Island · Hydrothermal · Acid Stream

Introduction

Thermally and chemically extreme habitats in volcanically active areas host diverse microorganisms (Ward et al. 1990; Hugenholtz et al. 1998). Such habitats include submarine hydrothermal vents (Takai and Horikoshi 1999; Sievert et al. 2000), hot springs (Wahlund et al. 1991; Jones et al. 1997; Skirnisdottir et al. 2000), and the craters of active volcanoes (Takano et al. 1997). White Island, an andesitic stratovolcano in the Bay of Plenty, New Zealand (37°31′26″ S, 177°11′5.6″ E) has a subaerial extent of about 3.5 km^2 and hosts an extensive hydrothermal system. Numerous fumaroles and springs discharge through the crater floor complex (Houghton and Nairn 1991). Seawater is not considered a significant component of the reservoir hydrothermal fluids in White Island (Giggenbach 1987). Dissolved volcanic gases in the hydrothermal fluids thus give rise to strongly acidic surface waters. As such, the island represents an ideal location for investigations into extremophilic microorganisms. There are no reports in the literature of bacterial communities associated with hydrothermal environments on White Island.

We have initiated a sampling and mapping program to investigate microbial colonization, biogeochemical processes, and potential novel microbial species on White Island. This report is the first description of microbial communities in Acid Stream waters of White Island using molecular and microbiological methods. We generated 16S rRNA gene libraries from community DNA and enrichment cultures, and further characterized the latter by isolation of pure cultures. Pure cultures were in turn described on the basis of 16S rRNA sequencing and physiological profiles. Microbial communities in White Island hydrothermal waters comprise α- and β-Proteobacteria, Firmicutes and eukaryotic algae.

Communicated by K. Horikoshi

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Materials and methods

Sample collection

Water was collected from the center of a flowing stream of acidic water (Acid Stream, depth 3 cm, width 2 m), directly into sterile 1-l Nalgene bottles and sterile 15-ml centrifuge tubes. Samples were maintained in darkness at ambient

temperature and returned to an IGNS laboratory in Wairakei within 5 h of collection. Water chemistry was determined by methods described in Christenson (2000).

Generation of 16S rRNA library and restriction fragment analysis

Total community genomic DNA was isolated using Instagene matrix (BioRad, Hercules, CA, USA). The universal primers 533F (Burggraf et al. 1997) and 1492R (Lane 1991), and the *Archaea*-specific 23FPL and 1391R (Barns et al. 1994), were used for PCR under the following conditions: initial denaturation, 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 50°C for 60 s and 72°C for 90 s, and a final extension at 72°C for 7 min. PCR amplicons were cloned using the pCR Blunt-TOPO cloning kit (Invitrogen Corporation, Carlsbad, CA, USA). Recombinant plasmid DNA was isolated by alkaline lysis. For restriction fragment analysis, 16S rDNA fragments were further amplified by PCR from each purified plasmid. PCR amplicons were digested with *Hin*P1 I and *Msp* I restriction enzymes after Hugenholtz et al. (1998). Restriction fragment patterns were grouped and representative clones sequenced in a Beckman CEQ 2000 DNA analysis system. The nucleotide sequences were submitted to GenBank at the NCBI. Their phylogenetic affiliations were determined through comparison with sequences in GenBank, using the gapped BLAST search program (Altschul et al. 1997).

Enrichment, isolation and characterization of pure cultures

Acid Stream water (1–5 ml) was aseptically transferred to liquid media, and incubated at 30° and 37°C with illumination, or in darkness at 30°, 45°, and 60°C for up to 1 year (Table 1). The media employed were designed to enrich microbial groups that might be expected to occur in acidic waters of volcanic origin (e.g., TA medium, a low-pH minimal medium), as well as more cosmopolitan marine types (e.g., Marine Broth, Difco). 16S rRNA clone libraries were generated from genomic DNA isolated from microbial communities in different media: in MB (37°C), TA – pH 1.29 (30°C), and TA – pH 3.0 (45°C).

Pure bacterial cultures were prepared through repeated transfers on nutrient media. Purity was determined by light microscopic examination, Gram staining, and cell characteristics. The ability of pure bacterial cultures to utilize single carbon sources was determined on Biolog microplates (Biolog, Hayward, CA, USA). Constitutive enzyme activities were determined in API Zym (bioMerieux Vitek, St Louis, MO, USA) (24 h, 30°C). Pigments in an algal culture were characterized after Bidigare and Trees (2000).

Electron microscopy

Acid Stream water was fixed with electron microscope grade gluteraldehyde in a sterile 15-ml tube (~0.5% final concentration). After 30 min, the gluteraldehyde concentration was increased to ~2%. The sample was stored at 4°C until processed for scanning electron microscopy (SEM) after Kunkel (1984), with modifications as described below.

A 1-ml subsample of an algal culture in TA (pH 1.29) was diluted with an equal volume of sterile filtered phosphate buffer. Half (1 ml) of the diluted sample was filtered through a Nuclepore polycarbonate filter (1 µm pore size) in a Swinnex filter holder. The other 1 ml was filtered through a Durapore membrane filter (0.65 µm). Filters were rinsed 3 times with 0.2 M sodium cacodylate, 3 times with 0.1 M sodium cacodylate and postfixed with 1% OsO_4. Each was rinsed 3 times with sterile distilled water and then dehydrated in an ethanol series. Bacterial cultures were processed for electron microscopy in the same manner, except that Durapore filters (0.45 µm) were used.

Filters were mounted on SEM stubs and sputter-coated with gold : palladium on a Hummer II sputter coater (2 × 1 min at 10 mA). Specimens were observed using a Hitachi S-800 field emission scanning electron microscope with an operating voltage of 15 kV. Images were recorded as digital files.

Table 1. Culture media used in this study and target microorganisms

Medium	Target group
Marine broth (MB) (Difco, Detroit, MI, USA)	Heterotrophic bacteria
Allen medium (pH 2.7, 4.9) (Allen 1959)	Acidophilic algae
Castenholz D (Castenholz 1982)	Cyanobacteria
"A", "A–N" (Kumazawa and Mitsui 1981)	Cyanobacteria
10% ASW (modified from Brown 1982)	Microalgae
TA (Kurosawa et al. 1998) (pH 1.29, 1.57, 2.09, 2.6, 3.00)	Acidophilic *Archaea*
ASY (Miyake et al. 1984) (with 15 g/l NaCl)	Nonsulfur bacteria
Medium 1 (pH 6.8, 7.0) (Eichler and Pfennig1988)	Green and purple sulfur bacteria
Succinate salts (Stanier et al. 1966)	Nitrate reducers

Confocal microscopy

A 1-ml sample of a pure algal culture in TA (pH 1.29) was centrifuged (14,000 *g* for 5 min). The pellet was resuspended in 100 µl of TA medium, and 10 µl of cell suspension were combined on glass slides with 1 µl of 100 µM CellTracker Red (Molecular Probes, Eugene, OR, USA). Samples were observed under a Zeiss LSM 510 confocal microscope with 40× (water immersion) and 63× (oil immersion) objectives with a rhodamine filter.

Results and discussion

Water chemistry

Analyses of Acid Stream water show that the fluids vary temporally in total salinity but are essentially dilute mix-

tures of sulfuric and hydrochloric acids containing varying quantities of dissolved andesite rock and other components (e.g., Fe – 123 mg/l, Al – 70 mg/l, Cl – 2,000–4,400 mg/l, SO_4 – 2,700 mg/l, SiO_2 – 370 mg/l, and pH ~2.5). This acidic chloride–sulfate water forms through condensation of acid magmatic gases into meteoric waters of local derivation (Christenson 2000). The chemical environments under which these fluids form are dynamic, and gas–water–rock reactions lead to a variety of possible compositional pathways for fluid evolution.

Microbial communities

We investigated the structure of the microbial consortium in the Acid Stream of White Island using a polyphasic approach comprising both molecular and microbiological methods. Scanning electron micrographs show the presence of filamentous and coccoid cells (Fig. 1A), and larger, sheathed cells (Fig. 1B).

Characterization of clone libraries from community DNA

A 16S rRNA gene library was generated from community DNA isolated from the Acid Stream community using universal bacterial PCR primers. The 900-ml sample yielded 75–100 µg of DNA. Ninety-six clones produced 14 distinct restriction fragment patterns. A clone representing each restriction pattern was sequenced. Nucleotide sequences were screened in a chimera detection program (http://www.cme.msu.edu/RDP/cgis/chimera). Chimeric forms were eliminated from further analyses. The resulting clone groups affiliated with six phylotypes. Four shared >98% sequence homology with bacterial 16S rRNA sequences from marine and soil environments. Nucleotide sequences of the 16S rDNA fragments in representative clones were submitted to GenBank (Table 2). Low 16S rRNA homologies (<97%) of the two remaining clones, however, do not mean that they are taxonomically unique (see Stackebrandt and Goebel 1994; Stackebrandt and Pukall 1999).

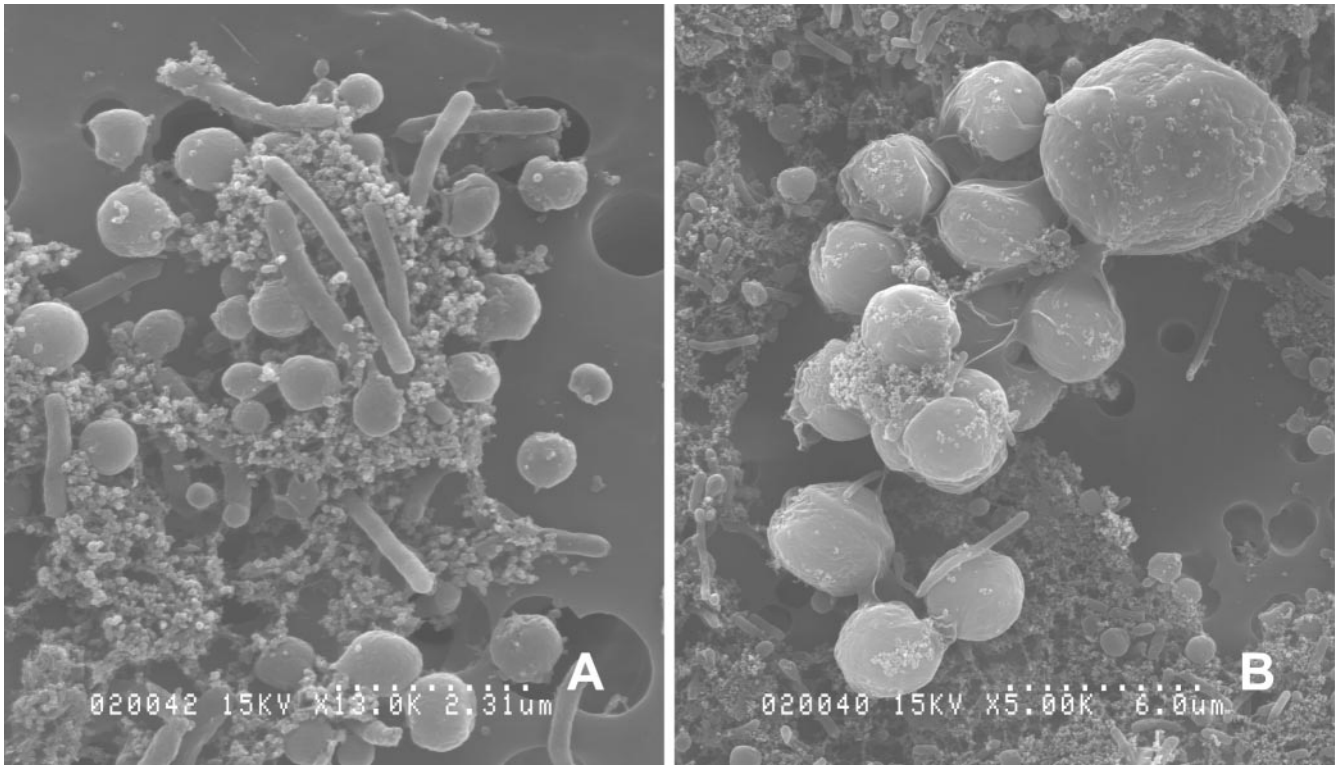

Fig. 1. Electron micrographs of Acid Stream water showing **A** dividing rods and putative cocci, and **B** larger, sheathed spherical cells

Table 2. Base sequence homologies of cloned 16S rDNA inserts amplified from Acid Stream (S) community DNA

Clone	Accession no	Closest match	Homology (%)	Percentage of library
S29	AF356020	*Chlorobium vibrioforme*, green sulfur bacterium	99.15	7.4
S35	AF356021	Uncultured bacterium, Australian caves	96.29	11.1
S45	AF356023	Uncultured Gm+ bacterium, marine sediments, Japan	98.48	70.4
S49	AF356024	*Rhodovulum* sp., marine, purple nonsulfur bacterium	95.78	3.7
S53	AF356025	*Ralstonia solanacearum*, isolated from heavy water	100	3.7
S56	AF356026	Uncultured Gm+ bacterium, marine sediments, Japan	98.85	3.7

Clone libraries from enrichment cultures

The microbial community in Acid Stream water was enriched in different media and growth conditions and characterized by 16S rRNA gene libraries comprising 24 clones each. The library from TA (pH 1.29, 30°C) contained clones that affiliated with only two species (Table 3, "CY"). Different α-Proteobacteria and Firmicute clones were detected in TA medium with a higher pH and incubated at 45°C (Table 3, "RO"). Clones generated from an enrichment culture in MB (37°C) were homologous to β-Proteobacteria and the Firmicute *Ralstonia solanacearum* (Table 3, "BA"). Different media and incubation conditions clearly promoted the establishment of very different communities. No microbial growth was determined in any medium at 60°C.

Characteristics of pure cultures

Nine different growth media with variations in pH, temperature, and illumination were used to isolate pure cultures. Microbial growth indicated by increases in turbidity and the presence of dividing cells was recorded in all media at 30°, 37°, and 45°C, with the exception of succinate salts (for nitrate reducers) and Medium 1 (for green and purple sulfur bacteria). After 10 weeks incubation, the most prominent cell type in TA medium (for acidophiles; pH 2.4, 45°C) was an alga of 2.5–6 μm diameter. This alga divided through fission in multiple planes until some 10–15 daughter cells had dispersed (Fig. 2A). Scanning electron micrographs showed more detailed structure of the mother cell and its progeny. These cells divided within a sheath-like material (Fig. 2B). Confocal microscopy confirmed the SEM observations of a sheath (Fig. 2C). The alga grows in TA supplemented with filtered hydrothermal fluids from a lake in the White Island crater (final pH 0.2). This culture's 16S rRNA sequence shared over 99% identity with a *Cyanidium caldarium* 16S plastid gene (Table 3). Indeed, its pigment profile (chlorophyll *a*, β-carotene, and zeaxanthin) is consistent with a *Cyanidium* designation (data not shown), an alga that can grow at extremely low pH and tolerate harsh chemical conditions (Allen 1959; Yoshimura et al. 1999).

Two bacteria were brought into pure culture after 10 weeks incubation of Acid Stream water in MB (37°C). Physiological and biochemical tests on each, as well as their 16S rRNA sequences, suggested the orange-pigmented Gram-positive rod (NZ2), and a beige-pigmented Gram-variable rod (NZ3) were *Nocardia corynebacteroides* and a *Nocardioides* sp., respectively (Table 3). A Gram-negative strain (NZ6) isolated from TA (pH 2.6) after 1 year at 30°C produced only weak, off-white colonies on solid TA plates at the same pH. The 16S rRNA gene sequence from NZ6 affiliated with that from *Acidiphilium* sp. (Table 3). NZ6 cells in TA medium produced the "blebs" previously described in an *Acidiphilium cryptum* isolate (Küsel et al. 1999). 16S rRNA nucleotide sequences of pure cultures were submitted to GenBank (Table 3). Neither NZ2 nor NZ3 affiliated significantly with species in the Biolog databases. They did, however, use a broad range of substrates (Table 4). In API Zym, NZ2 and NZ3 demonstrated esterase (C_4), esterase lipase (C_8), lipase (C_{14}), leucine and valine arylamidases, acid phosphatase, and α-glucosidase activities. NZ3 additionally expressed alkaline phosphatase and cystine arylamidase activities.

The rooted phylogenetic tree of 16S rRNA sequences generated from community DNA from the Acid Stream and enrichment cultures clearly shows the clones are represented in the collection of pure cultures while cultures of NZ2 and NZ3 had no cloned representatives (Fig. 3). Knowledge of water chemistry and use of specific media are valuable in targeting microorganisms to bring them into pure culture. We did not detect any representative of the *Archaea* in the Acid Stream. In this respect, *Archaea* have also been shown not to dominate at submarine hydrothermal vents (Moyer et al. 1994; Guezennec et al. 1996), a habitat they were once thought to dominate (Huber et al. 2000).

White Island presents a dynamic and challenging environment. An eruption 5 months after our visit covered half

Table 3. Base sequence homologies of cloned 16S rDNA inserts amplified from enrichment cultures. Details of pure cultures are given in the text

	Accession no	Closest match	Homology (%)
MB, pH 7, 37°C			
BA1	AF356009	*Pandoraea andropogonis*, formerly *Pseudomonas woodsii*	93.69
BA4	AF356010	Uncultured Gm+ bacterium, marine sediments, Japan	98.35
BA5	AF356011	*Ralstonia* sp., formerly *Pseudomonas syzygii*	99.33
TA, pH 1.29, 30°C			
CY4	AF356012	*Cyanidium caldarium*, acidophilic Rhodophyte	99.33
CY8	AF356013	*Pandoraea andropogonis*, formerly *Pseudomonas woodsii*	93.74
TA, pH 3, 45°C			
RO3	AF356015	*Acidosphaera rubrifaciens*, acid spring/acid mine waste	93.87
RO5	AF356027	*Rhodovulum* sp., marine, purple nonsulfur bacterium	95.78
RO23	AF356014	Uncultured Gm+ bacterium, marine sediments, Japan	98.24
Pure culture			
NZ2	AF356016	*Nocardia corynebacteroides*	98.58
NZ3	AF356018	*Nocardioides* sp.	94.89
NZ4	AF356019	*Cyanidium caldarium*	99.10
NZ6	AY029220	*Acidiphilium* sp./*A. cryptum*	99.93

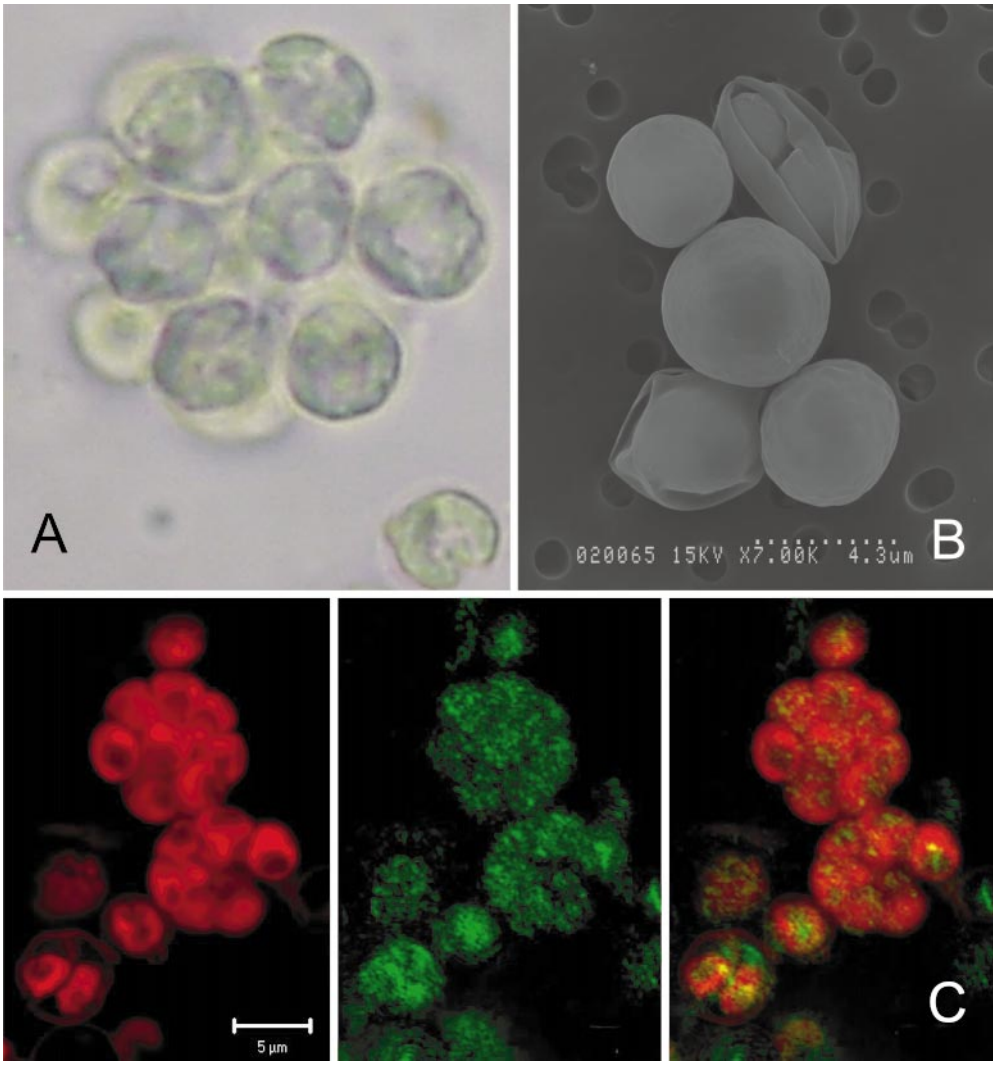

Fig. 2. **A** Light micrograph of a green-pigmented alga purified from Acid Stream water. This group of cells has formed through fission in multiple planes of a single mother cell, and will disperse once 10–15 daughter cells are present. **B** Electron micrograph of a sheathed algal cell from a mixed culture derived from stream water and incubated in TA medium (pH 1.29, 30°C). **C** Confocal micrograph of a dividing algal cell from Acid Stream water in TA medium. The cell *lower left* is surrounded by a sheath (pH 1.29, 30°C)

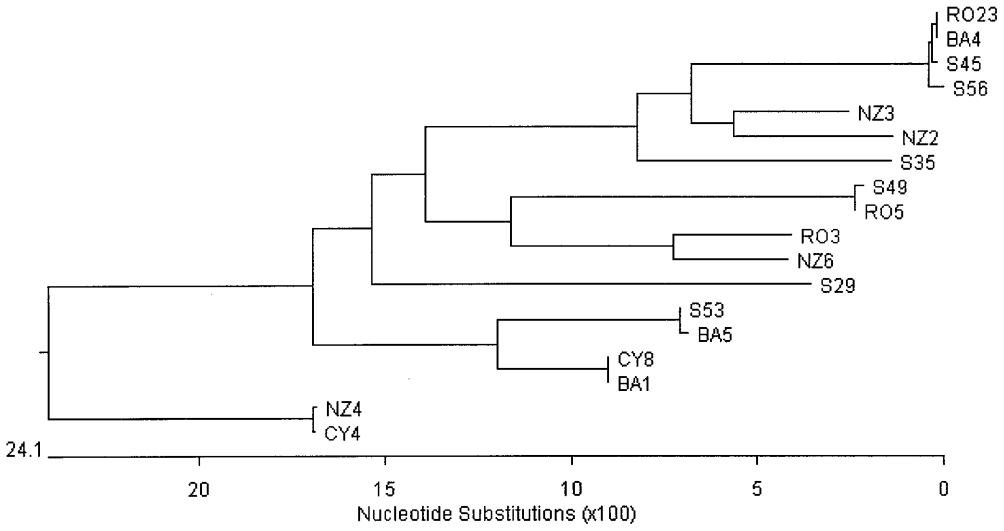

Fig. 3. Evolutionary distance dendrogram of 16S rRNA sequences of representative clones from Acid Stream water, enrichment cultures, and from pure cultures. Pure cultures are prefixed NZ. Sequences were aligned over a maximum of 718 bases in MegAlign. The length of each pair of branches represents the distance between sequence pairs

Table 4. Single carbon source utilization by pure cultures of NZ2 and NZ3 (determined in Biolog)

Substrate	NZ2	NZ3	Substrate	NZ2	NZ3	Substrate	NZ2	NZ3
2,3-Butanediol	+	–	D-Psicose	+	+/–	L-Threonine	+	–
2′-Deoxy adenosine	+	+	D-Raffinose	+	–	Maltose	+	–
2-Aminoethanol	+	–	D-Ribose	+	–	Mannan	+	–
Acetic acid	+	+	D-Sorbitol	+	–	Methyl pyruvate	+	–
Adenosine	+	+	D-Trehalose	+	+	m-Inositol	+	–
α-D-Glucose	+	–	Gentiobiose	+	–	Mono-methyl succinate	+	+
α-Hydroxy butyric acid	+	–	γ-Hydroxy butyric acid	+	–	*N*-Acetyl-D-glucosamine	+	–
α-Keto butyric acid	+	–	Glucose-1-phosphate	+	–	*N*-Acetyl-D-mannosamine	+	–
α-Keto glutaric acid	+	–	Glucose-6-phosphate	+	–	*N*-Acetyl-L-glutamic acid	+	–
α-Methyl D-galactoside	+	–	Glycerol	+	–	*p*-Hydroxy phenyl acetic acid	+	–
β-Cyclodextrin	+	–	Glycogen	+	–	Propionic acid	+	+
β-Hydroxy butyric acid	+	+	Inosine	+	–	Putrescine	+	–
β-Methyl D-galactoside	+	–	Itaconic acid	+	–	Pyruvic acid	+	+
β-Methyl D-glucoside	+	–	Lactamide	+	–	Sebacic acid	+	–
Bromo succinic acid	+	–	L-Alanyl-glycine	+	–	Succinamic acid	+	–
D,L-Carnitine	+	–	L-Asparagine	+	–	Succinic acid	+	–
D-Arabitol	+	–	L-Aspartic acid	+	–	Sucrose	+	+
Dextrin	+	–	L-Glutamic acid	+	–	Thymidine	+	+
D-Fructose	+	+	L-Lactic acid	+	–	Thymidine monophosphate	–	+
D-Gluconic acid	+	+	L-Malic acid	+	–	Tween 40	+	+
D-Glucosaminic acid	+	–	L-Proline	+	–	Tween 80	+	+
D-Lactic acid, methyl ester	+	+	L-Pyroglutamic acid	+	–	Xylitol	+	–
D-Mannitol	+		L-Rhamnose	+	+			
D-Mannose	+		L-Serine	+	–			

of the island with up to 30 cm of ash and pyroclastics. A new eruption crater also formed, and the acidic crater lake was ejected. We will investigate whether any changes in microbial community have taken place in the Acid Stream after the eruption, and also widen our search for Archaea. The island presents an ideal site for investigations into microbial colonization, biogeochemical processes, and for the discovery of novel microbial species and their products.

Acknowledgments We thank Shaobin Hou, Charles W. Riley, and Claude Belisle for helpful comments on the manuscript. We gratefully acknowledge Jamie Foster (Kewalo Marine Laboratory, University of Hawai'i) for assistance with confocal microscopy, and Bob Bidigare, JoAnn Radway, and Abigail Perucho for technical assistance. Karen Britten (IGNS) provided valuable support in the field. This work was supported by the ERC program of the National Science Foundation under award number EEC 9731725.

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