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Short communication

Microscopic examination of acidic hot springs of Waiotapu, North Island, New Zealand

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Abstract Microorganisms other than *Sulfolobus* are reported for the first time in the low pH high temperature springs of Waiotapu, North Island, New Zealand. In one spring of the tourist reserve, rod-shaped organisms with a toga-like wall were observed, in addition to *Sulfolobus*-like spheres. In another acid hot spring, microbial cells were small and intermingled with mineral crystals, making their presence difficult to detect, even by scanning electron microscopy (SEM). Evidence for the existence of microorganisms included: (1) phase contrast microscopic observation; (2) DNA-specific staining with UV microscopy; and (3) SEM examination of natural samples. At pH 2, the highest temperature at which microbes were observed was 77°C and at pH 4.5, it was 87°C. Cells at pH 2, 77°C were small rods encrusted with mineral crystals shown by X-ray microanalysis to consist mainly of silicon and aluminum. At pH 4.5, spherical, rod-shaped, and filamentous cells were seen. Attempts to culture the observed microbes were unsuccessful. PCR, cloning, sequencing, and BLAST analysis identified *Thermofilum pendens* in Champagne Pool, and *Sulfobacillus*, *Thiobacillus*, and several unknown bacteria in Frying Pan Flat pools.

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INTRODUCTION

Microbiological activity in North Island, New Zealand was first investigated by Kaplan (1956) in a survey of temperature, pH, and redox potential (Eh) in the Taupo Volcanic Zone (TVZ). One of the study sites was Waiotapu. Brock & Brock (1971) found microorganisms in neutral pH habitats at the highest temperature, 101°C, but not at high temperatures in acidic springs at Waiotapu. Bohlool (1975) examined the acidic springs at Waiotapu and found *Sulfolobus* and no other microorganisms at temperatures greater than 70°C and below pH 3.

In solfatara areas throughout the world, both spheres and rod-shaped organisms are commonly observed (Hallberg & Johnson 2001). Culture independent studies with polymerase chain reaction (PCR) and 16S rRNA gene analysis have revealed a wide diversity of uncultured Archaea and Bacteria in Obsidian Pool, a neutral pH hydrothermal habitat in Yellowstone National Park (Barns et al. 1994; Hugenholtz et al. 1998). Similar analyses of acidic hot springs on the Caribbean island of Montserrat showed a comparative reduced diversity with a more limited number of species identified by sequence analyses and confirmed by enrichment culture (Atkinson et al. 2000; Burton & Norris 2000). Some of these microorganisms were cultivated from Yellowstone National Park (Johnson et al. 2003; Weiss Bizzoco et al. 2003, unpubl. data).

Our interest in New Zealand springs is whether microorganisms other than *Sulfolobus* exist in high temperature acidic springs and whether they can be cultured at high-temperature and low-pH as were those present in Yellowstone National Park springs (Weiss Bizzoco et al. 2003; Weiss unpubl. data). Cell shape is an issue because only spherical *Sulfolobus* cells were thought to be present in New Zealand acidic hot springs. This study reports the existence of rod-shaped microorganisms in high-temperature

low-pH springs of Waiotapu, previously reported to be devoid of such organisms (Brock & Brock 1971; Bohlool 1975).

MATERIALS AND METHODS

Study area and sampling

The Waiotapu geothermal area lies within the TVZ, North Island, New Zealand (Fig. 1A). The general locations of thermal features are given in Fig. 1B. The hot springs studied were situated in the Waiotapu Tourist Reserve, located 25 km south-southeast of Rotorua. An enlargement (Fig. 1C) shows the two areas sampled, Champagne Pool and Frying Pan Flat. The four springs sampled in Frying Pan Flat were small to medium sized pools adjacent to the walkway.

Samples from pools were collected in sterile 20 ml glass vials and sealed while warm to ensure that dissolved gasses were not lost during cooling. In Champagne Pool, the water column was sampled and in Frying Pan Flat, water samples with sediment were taken. Samples were collected, using a short extension pole with a wing-nut screw-lock clamp to attach the sample vial. All samples were sent international airmail to the laboratory in the United States and arrived within 7 days from the time of sampling.

Temperature in the field was measured with a maximum recording mercury thermometer and confirmed with a second mercury thermometer. The measurements were within a half a degree of each other. The pH was measured at the site with pH strips (Merck, Darmstat, Germany) after the water had cooled to 23°C. A Radiometer pH meter (model PHM 82) was used to measure pH in the laboratory. Field and laboratory measurements did not vary by more than 0.1 pH units.

Laboratory analysis

For DNA staining, 4′, 6-diamidino-2-phenylindole, HCl (DAPI) stain was used at a final concentration of 5 μg ml^{-1} as described in Lindstrom et al. (2002). Microscopic examinations used a Leitz Dialux 20 phase contrast fluorescence microscope equipped with epi-illumination and a DAPI filter block. Image collection and photographs are described in detail in Lindstrom et al. (2002).

Cells were counted in a Petroff-Hausser (P-H) cell counter. When counts were below 2×10^7 cells ml^{-1}, the sample was concentrated by filtration with a 0.22 μm black membrane filter and counted both by

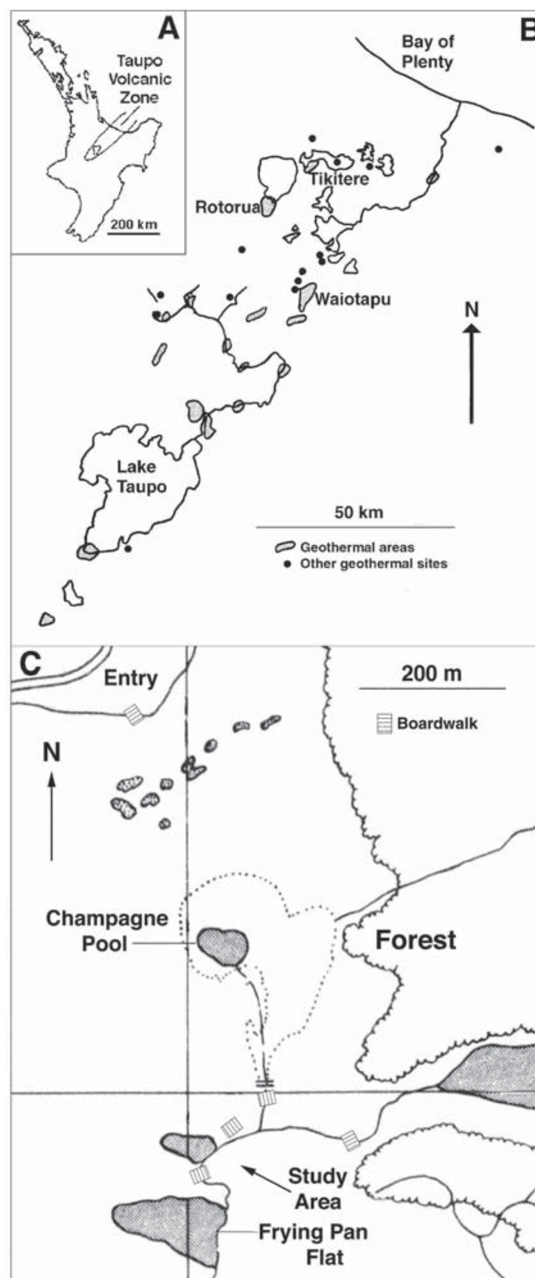

Fig. 1 Location of study sites in the Taupo Volcanic Zone (TVZ), North Island, New Zealand. **A**, TVZ location; **B**, location of Waiotapu, Tikitere and other geothermal sites in the TVZ (adapted from Hedenquist, 1991); **C**, location of sampling sites in Waiotapu Geothermal Reserve, (after Lloyd 1959).

UV-DAPI staining and in a P-H cell counter. Methods for scanning electron microscopy (SEM), X-ray microanalysis, and negative staining have been described previously (Lindstrom et al. 2002; Weiss Bizzoco et al. 2003). Community DNA was extracted and stored at –20ºC until analysis. For SEM, samples were adhered to a 12-mm-diameter cover glass, coated with 0.1% polyethyleneimine, and fixed in 4% glutaraldehyde in 10 m*M* N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) (pH 7) for 1 h (23ºC). Samples were washed three times in HEPES buffer and fixed in 1**%** OsO_4 in HEPES buffer at 23ºC (pH 7) for at least 1 h. Samples were rinsed three times in distilled water, dehydrated in a graded ethanol series, and dried from ethanol by the critical-point method with a Tousimis Samdri 790 drier. Samples were coated with Au-Pd on a Hummer and photographed in a Hitachi 2700 SEM with a Princeton Gamma Tech IMAX X-ray microanalyser. For X-ray microanalysis, samples were filtered on a 0.22 µm Millipore membrane, washed three times with distilled water, transferred to an adhesive carbon conductive tab on an aluminum sample stub (Ted Pella Inc., Redding, CA, United States), and analysed without coating. Controls used were the aluminum sample stub with and without the carbon conductive tab. For negative staining, cells attached to Formvar plastic-coated copper grids were stained with 1% uranyl acetate, dried with filter paper (Whatman no. 1), and examined.

For community DNA extraction, a 2 ml sample was centrifuged at 14 000 g for 5 min. The cell pellet was suspended in 20 m*M* Tris, pH 8, 2 m*M* EDTA, 1.2% Nonidet P40, 20 mg/ml lysozyme before incubation at 37°C for 30 min. The cells were then treated in proteinase K at 70°C for 10 min with purification of DNA on minicolumns (Qiagen DNeasy tissue kit, Chatsworth, CA, United States). An environmental DNA extraction control was processed in parallel to assess the extent of laboratory or sample processing contamination.

For PCR amplification of the 16S rRNA genes, purified DNA was used as a template in a 50 µl reaction volume containing 10 m*M* Tris, pH 8.3, 1.65 m*M* $MgCl_2$, 4 × 200 µm deoxynucleoside triphosphates, 0.02U/µl RedTaq DNA polymerase (Sigma), and either a Bacteria-specific primer (27F 5′-AGAGTTTGATCMTGGCTCAG-3′) and the universal 1492R primer (5′-TACGGYTACCTTGTTACGACTT-3′; M=A or C, Y= C or T) or Archaea-specific primer sets (21Fa 5′-TTCCGGTTGATCCYGCCGGA-3′/915Ra 5′-GTGCTGCCCCGCCAATTCCT-3′ and 333Fa 5′-TCCAGGCCCTACGGG-3′/915Ra) used with each primer at a concentration of 1 µm. Amplification was carried out in thin-walled PCR tubes in a DNA thermal cycler PTC-100 (MJ Research, Watertown, MA, United States) as follows: 94°C for 2 min followed by 25 cycles at 94°C for 1.5 min, 55°C for 45 s, and 72°C for 1.5 min. A final extension was for 20 min at 72°C. PCR products were separated from the reaction mixture using an UltraClean PCR cleanup kit (Mo Bio Laboratories Inc., Solana Beach, CA, United States). Environmental DNA PCR amplified by a *Thermotoga*, Thermotogales- (Harmsen et al. 1997), or Bacteria-specific primer 27F/805R (5′-GACTACCAGGGTATCTAATCC-3′) was cloned with a commercial pGEM®-T cloning kit (Promega, Madison, WI, United States) and sequenced, using *T. maritima* DNA as a positive control. PCR products were sequenced with vector-specific M13 used with an ABI Prism® 3100 capillary electrophoresis DNA sequencer and BigDye™ Terminator. Sequences obtained were compared to existing databases using the BLAST search program (Altschul et al. 1997).

PCR amplified environmental DNA was analysed and visualised in 1% agarose gels (Sigma, St. Louis, MO, United States) buffered with 89 m*M* Tris base, 89 m*M* boric acid, and 2 m*M* ethylenediamine-tetraacetic acid at pH 8. Tracker dye and 500 kb standards were used (Apex, Triangle Park, NC, United States).

Samples from Yellowstone National Park pools, similar in temperature and pH to the New Zealand pools, contained a dominant sequence (Weiss Bizzoco unpubl. data) and were used as positive controls for direct sequencing of PCR amplified environmental samples.

For cultures, Brock's medium (Brock et al. 1972) with (0.074 m*M* ferric citrate) and without iron was used at pH 2 and 3. All cultures were grown in 10 ml of medium in 20 ml screwcap vials as standing cultures at 55 or 70°C in a water bath without aeration. For heterotrophic isolations, 0.1% yeast extract (Difco) was added. For chemolithotrophic cultures, 1% steam sterilised sulphur was mixed periodically during a 4-week incubation. This procedure produced positive isolations in most instances with uncharacterised high-temperature low-pH microorganisms sampled at Yellowstone National Park (Weiss Bizzoco et al. 2003, pers. obs.). All New Zealand samples were cultured upon arrival in the laboratory in the United States, within 7 days from the time of sampling.

RESULTS

General observations

The springs ranged in temperature from 72°C to 87°C and in pH from 2 to 5.5. Table 1 presents the features of the springs and of the microbial populations sampled. Microorganisms were present in all the springs sampled (Table 1).

An attempt to grow cells, both Bacteria and Archaea, from low pH samples (pH 2), using aerobic culture methods, did not produce positive cultures using autotrophic, heterotrophic, or mixed methods with a small amount of yeast extract added to stimulate attachment to sulphur. Such methods routinely produced isolates in samples from Yellowstone National Park (Weiss Bizzoco et al. 2003, unpubl. data). New Zealand samples may have been exposed to conditions such as low temperature before culturing that may have resulted in acidification of the cytoplasm and cell death, or other unknown factors may have prevented growth of cells.

Examination of cells by negative stain electron microscopy also proved to be of little value under the conditions used. This was because few of the cell types seen by light microscopy bound to the grids. With Formvar plastic-coated grids, few cells bound. When a thin layer of polyethyleneimine was applied to plastic-coated grids to increase binding, substantial debris contaminated the sample.

Low pH thermal springs

Champagne Pool was the largest spring sampled. It is a silica-rich spring containing a large amount of chloride, c. 1900 mg/l compared with 36–886 for other acid hot springs, pH 2–5.5, in Waiotapu (Hedenquist 1991; Jones et al. 2001). The spring has a pH of 5.5 and a temperature of c. 75°C. An examination of samples from Champagne Pool revealed both thin filaments and rods. With DAPI staining (Fig. 2) filamentous shapes were particularly evident, as were the differences in diameter and length of rod-shaped cells. The main organisms in Fig. 2 were a long thin filament, and a variety of short, wide rod-shaped and oval cells. At least three different types of cells were present. The fluorescence in DAPI staining was seen as bright spots or uniform throughout the cell, suggesting differences in the arrangement of DNA within the cytoplasm. The cell concentration was relatively low (Table 1), but microorganisms were easily recognised by DAPI staining and phase contrast microscopy.

The water from Champagne Pool flows out on Primrose Terrace, over Bridal Veil Falls, and into Frying Pan Flat where four other thermal springs

Table 1 Microbial distribution in low pH hydrothermal habitats, Waiotapu, North Island, New Zealand.

Location	(°C)	pH	Microscopic description	10^6 cells ml^{-1} ($\bar{x} \pm$ SE), n = 30
Champagne Pool	75	5.5	Rods, thick and thin rods in clusters, filaments, thin filaments embedded in cell aggregates	0.96 ± 0.24
Frying Pan Flat				
(1) Clear pool, slow flow-through, loose gray bottom	87	4.5	Rods, wide rods, short rods, thick rods in small clumps, a few filaments, thin filaments and spherical cells	12 ± 7
(2) Small gray pool alongside walkway	77	4.0	Single and doublet rods, many small clusters of rods, a few thin filaments and *Sulfolobus*-like spheres	17 ± 1
(3) Small clear pool, loose sulphur on bottom	77	2.0	Rods, short rods, doublet rods, a few filaments attached to sulphur crystals, thin filaments, rod-shaped cells in refractile material, a few *Sulfolobus*-like spheres	1.2 ± 0.1
(4) Small clear pool, sulphur on walls	72	2.0	Many single rods, a few long filaments, rods with toga on either end, clustered cells, cells embedded in matrix	8.0 ± 0.9

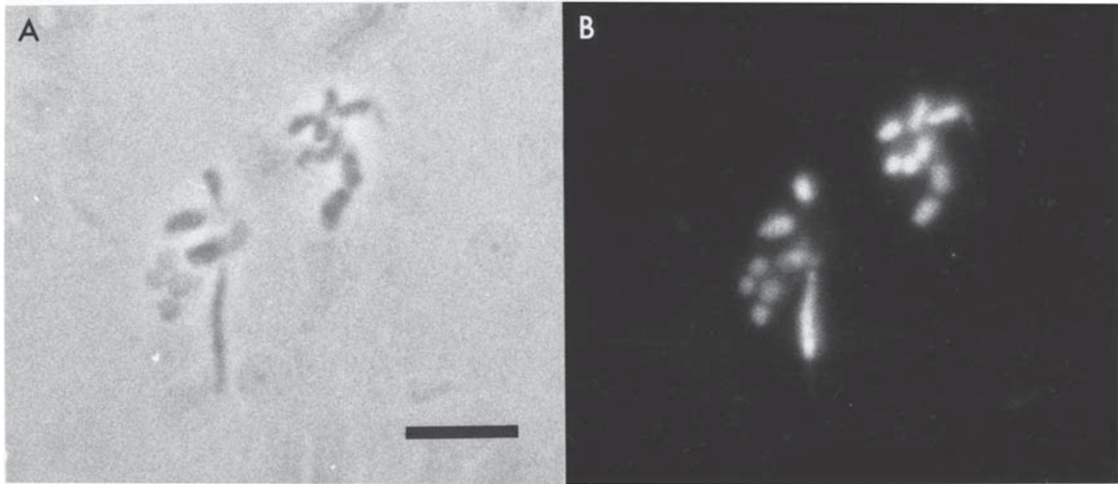

Fig. 2 DAPI-stained cell clusters sampled at Champagne Pool, North Island, New Zealand, 75°C, pH 5.5. Cells appear oval, as short wide rods, and thin filaments. **A**, Phase contrast; **B**, DAPI stain. DNA appears as bright spots or uniform DAPI fluorescence. Scale bar: 5 µm.

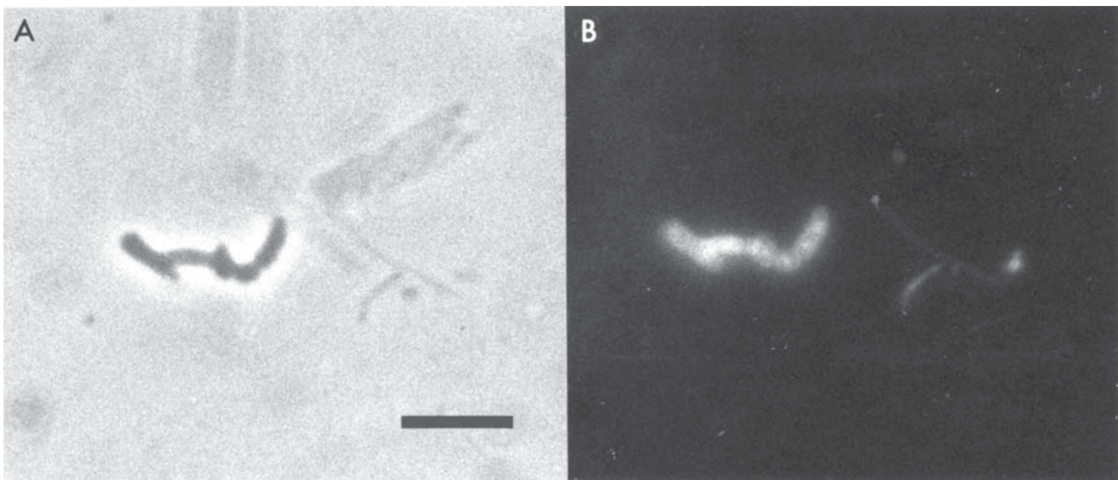

Fig. 3 DAPI-stained rod-shaped cell group with several rod-shaped morphologies, wide rods, thin rods, very thin cells, and a sphere. Cells sampled in pool 1 with slow flowing water, Frying Pan Flat, North Island, New Zealand, 87°C, pH 4.5. **A**, Phase contrast; **B**, DAPI stain. DNA appears as faint uniform DAPI fluorescence on very thin filament, thin filament, and a sphere and bright sites on wide rods. Scale bar: 5 µm.

were sampled. The pool with the highest temperature sampled in this area was pool 1 at 87°C and pH 4.5. This pool (Table 1) had a flow-through water supply. As shown in Fig. 3, cells appeared in a variety of shapes, short wide rods, filaments, and spheres. Cell aggregates were also evident (Weiss Bizzoco pers. obs.) and consisted of dissimilar microorganisms. A sample was also taken from nearby pool 2 (Table 1) at a lower temperature of 77°C in a slightly more acidic habitat of pH 4. DAPI staining revealed bright fluorescent sites on cells in clusters (Weiss Bizzoco pers. obs.). In pool 3 at the same temperature, but at a lower pH of 2, DAPI stained cells were associated with a matrix mass. Examination at higher magnification by SEM showed mineral-like crystals (Weiss Bizzoco pers. obs.). Examination of the crystals by X-ray microanalysis, using a Millipore-filtered non-coated sample attached to a carbon conductive tab and analysed without the membrane filter, showed major peaks for aluminium (Al) and

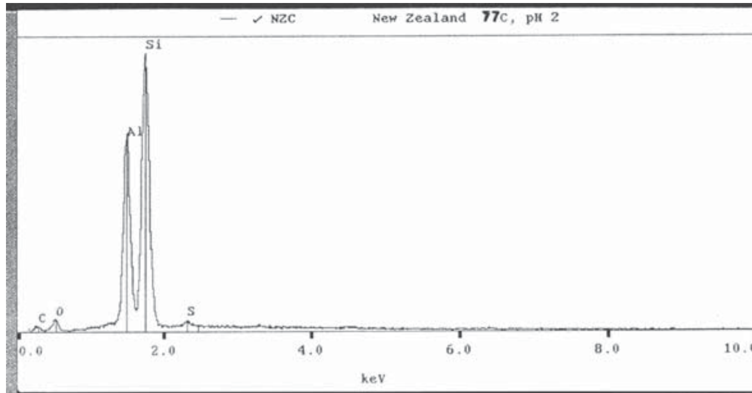

Fig. 4 X-ray spectrum of crystals from pool 3, Frying Pan Flat, North Island, New Zealand, 77°C. pH 2. Non-coated Millipore filtered sample mounted on a carbon conductive tab without the Millipore membrane. Major peaks are aluminum (Al) and silicon (Si); minor peaks are carbon (C), oxygen (O) and sulphur (S).

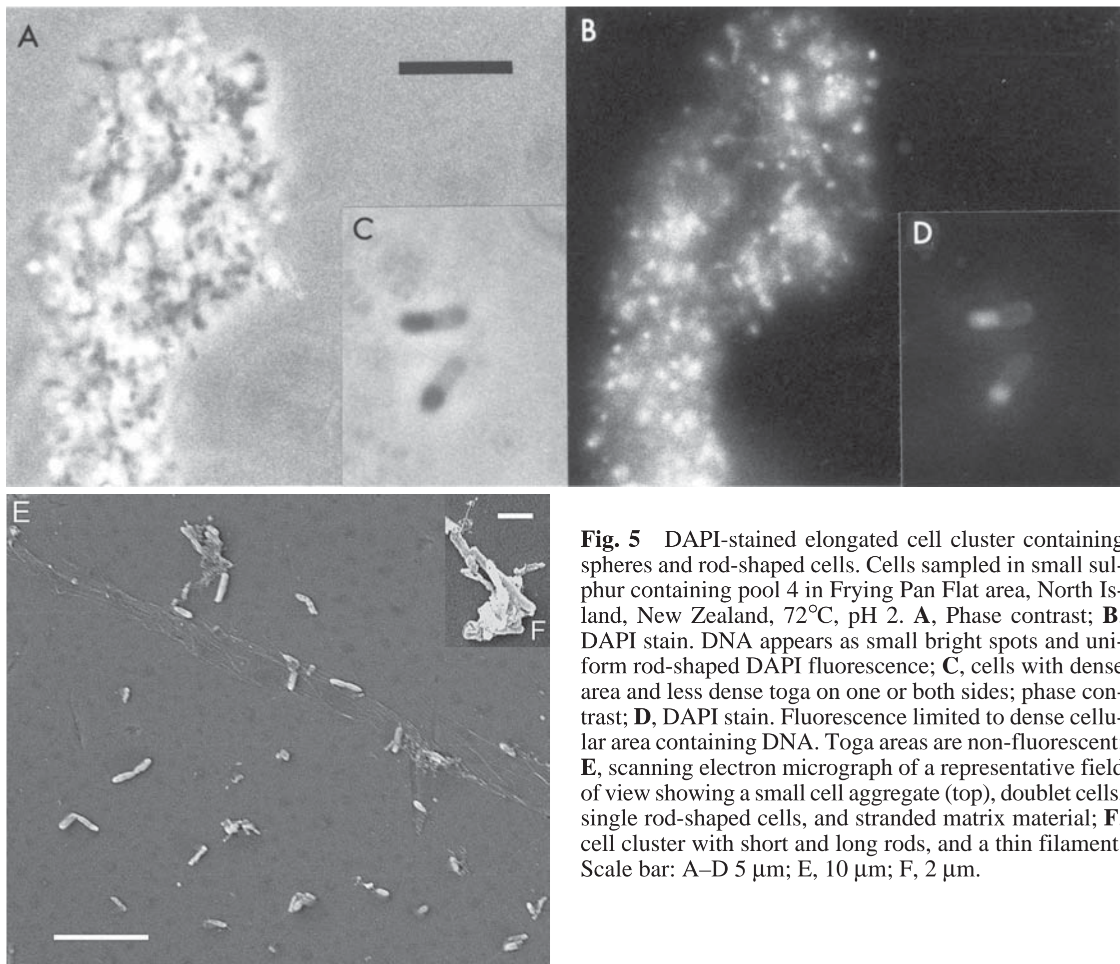

Fig. 5 DAPI-stained elongated cell cluster containing spheres and rod-shaped cells. Cells sampled in small sulphur containing pool 4 in Frying Pan Flat area, North Island, New Zealand, 72°C, pH 2. **A**, Phase contrast; **B**, DAPI stain. DNA appears as small bright spots and uniform rod-shaped DAPI fluorescence; **C**, cells with dense area and less dense toga on one or both sides; phase contrast; **D**, DAPI stain. Fluorescence limited to dense cellular area containing DNA. Toga areas are non-fluorescent; **E**, scanning electron micrograph of a representative field of view showing a small cell aggregate (top), doublet cells, single rod-shaped cells, and stranded matrix material; **F**, cell cluster with short and long rods, and a thin filament. Scale bar: A–D 5 µm; E, 10 µm; F, 2 µm.

silicon (Si) (Fig. 4). The controls for the sample stub with the carbon conductive tab showed a single carbon (C) peak, and no Al (Weiss Bizzoco pers. obs.).

Another spring (pool 4) at a lower temperature, 72°C, at pH 2 had both rods and *Sulfolobus*-like spheres. Fig. 5A,B shows a large DAPI-stained cluster of cells with a spherical appearance and a few

fluorescent rods. Other cells in this spring had an unusual morphology that resembled *Thermotoga* with a phase dense area flanked on one side by a less dense toga (Fig. 5C). DAPI fluorescence was seen only in the DNA containing cytoplasmic area (Fig. 5D). The toga was not fluorescent. It represents an elongated cell wall that lacks cytoplasm. The toga was present on one or both ends and when present on both, it was of unequal length.

When samples from this spring were fixed, coated, and examined by SEM, as in Fig. 5E, individual cells were seen. Both rod-shaped cells and occasional filaments (10–13 µm) were evident. A cluster of short rods also appeared (Fig. 5F). Typically rods were c. 0.5 µm × 1–7 µm. They appeared as single cells, doublet cells (end-to-end pairs), or two adherent individual cells.

Sequence based analysis

Direct sequencing of environmental DNA yielded c. 600 base pair fragments of the16S rRNA gene, suggesting the possible presence of a significant or dominant organism (or copy number) in a given spring (Table 2).

PCR with domain specific 16S rDNA Bacteria- and Archaea-specific primers detected both Bacteria and Archaea in all the Waiotapu springs examined. To evaluate this approach—DNA extraction of

Table 2 Identified Bacteria and Archaea using direct PCR amplification and sequencing. (Preliminary GenBank organism, genus identification based on percentage relatedness of 16S rDNA sequence comparisons with the database, and >97% identity indicates a high degree of similarity; Contaminant, represents non-thermophilic microorganism.) (ND, Not determined.)

Sample (sampling temp. and pH)	PCR primers		Preliminary GenBank organism (% identity)	Contaminant[3]
	Bacteria [1]	Archaea [2]		
Negative controls				
Sterile water[4]: environmental extraction	–	–	–	–
PCR control	–	–	–	–
Positive controls				
PCR: *Halobacterium* DNA	–	+	*Halobacterium* (100)	–
YNP Thermoacidophile DNA		+	–	
Source spring, 79°C, pH 2.2	+	–	Unknown bacterium[5]	–
Environmental extraction controls, YNP Sylvan springs[6]				
84°C, pH 2	+	–	*Alicyclobacillus* (98), *Sulfobacillus* (91)	–
80.5°C, pH 1.9	+	–	*Alicyclobacillus* (97)	–
80°C, pH 5.5	ND	+	–[7]	–
Environmental extraction, New Zealand				
87°C, pH 4.5	+	+	–[7]	–
77°C, pH 4	+	+	–[7]	–
77°C, pH 2	+	+	–[7]	–
72°C, pH 2	+	+	–[7]	–
Champagne Pool (75°C, pH 5.5)	+	+	*Thermofilum pendens* (95), Thermofiliaceae (95), Uncultured crenarchaeote pBA1 (94), *Thermocladium* (94), Crenarchaeotal sp. clone pJP 33 (93), *Vulcanisaeta* (93), Uncultured archaeon GBa2r048 (93)	–

[1]Primers used 27F/1492R.
[2]Primers used 21Fa/915Ra and 333Fa/915Ra.
[3]Organisms considered to be contaminants.
[4]Water autoclaved and filtered through 0.22 µm Millipore membrane.
[5]Bacterial DNA extracted from uncharacterised Yellowstone National Park organism (Weiss Bizzoco et al. 2003).
[6]Upper terrace, 80.5°C, pH 1.9; 84°C, pH 2; 80°C, pH 5.5.
[7]No identity established.

environmental samples, PCR, sequencing, and BLAST analysis—Yellowstone National Park acidic geothermal samples served as positive controls. These samples contained both known and unknown phylotypes that have been enriched and isolated, and their phylogenetic identities have been analysed (Johnson et al. 2003; Weiss Bizzoco unpubl. data). The DNA extraction procedure has also been used successfully to clone multiple sequences from a single site, Amphitheater Springs, Yellowstone National Park (Weiss Bizzoco et al. 2003, unpubl. data).

Using agarose electrophoresis, all of the New Zealand samples were positive for bacterial DNA (Weiss Bizzoco pers. obs.). With BLAST analysis and cloning, there were no contaminants (Table 2). Of the five samples subjected to PCR and sequencing with two different Archaea-specific primer sets, 21Fa/915Ra and 333Fa/915Ra, only 333Fa/915Ra yielded a positive response after PCR and sequencing. BLAST analysis of the Champagne Pool sample showed 95% identity with *Thermofilum pendens*. Because one spring in the Sylvan Springs complex had a temperature and pH similar to that of Champagne Pool, 80°C versus 75–76°C (both were pH 5.5), DNA extracted from this spring and PCR amplified with Archaea-specific primers was sequenced for comparative purposes. A dominant sequence was detected, but no GenBank identity was established.

Because the sheathed rod in Fig. 5C,D resembled *Thermotoga*, an attempt was made to obtain DNA evidence supporting this observation. Environmental DNA PCR-amplified with Bacteria-, *Thermotoga*-, and Thermotogales-specific primers, cloned and sequenced did not confirm the presence of *Thermotoga*. A phylogenetic tree prepared from the resulting sequence analysis is given in Fig. 6.

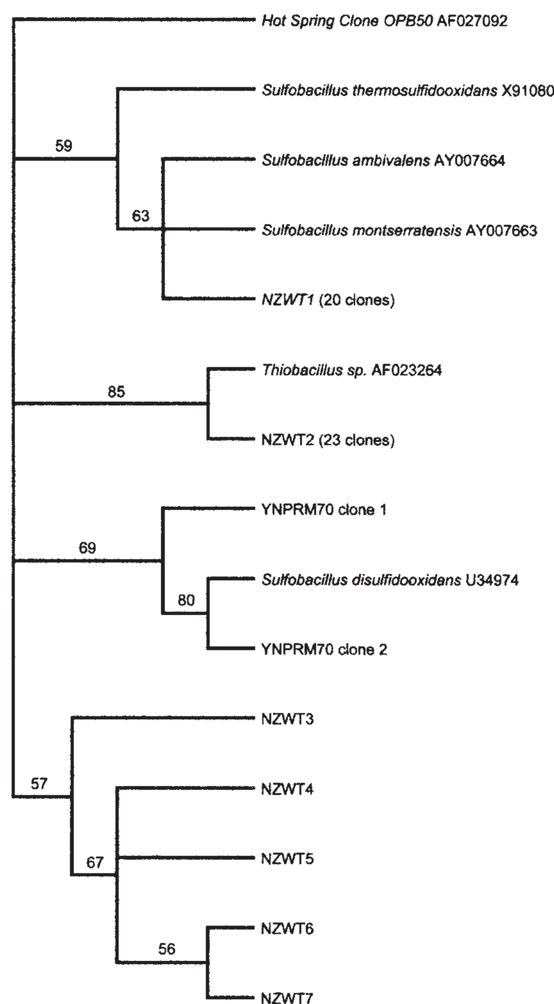

Fig. 6 Phylogenetic tree based on cloning experiments using environmental DNA. Maximum likelihood analysis was based on 16S rRNA gene sequences; bootstrap probability values obtained after 1000 replicate samplings are given at branch points as percentages. OPB50, uncultured clone Obsidian Pool, Yellowstone National Park; YNPRM70 clones 1 and 2, uncultured clones Roaring Mountain, Yellowstone National Park, 70°C, pH 2.2, NZWT clones 1–7, uncultured clones Waiotapu, New Zealand.

DISCUSSION

The present observations extend the range of microorganisms in these extreme habitats (>70°C, <pH 3) beyond *Sulfolobus* (Bohlool 1975), and differ from earlier studies in three ways. First, DNA staining with DAPI was used to detect organisms; second, DNA-based methods were used to identify new microorganisms; and third, SEM of natural samples was used to examine microbial morphologies. SEM of natural samples was essential for recognising the distinctive rod-shaped morphology of cells, particularly for documenting smaller cells intermixed with crystals and debris. Clustered cells, the predominant form seen by DAPI staining, were easier to detect by UV microscopy because of increased localised fluorescence associated with increased numbers of cells.

Observations of rod-shaped and filamentous microorganisms in Champagne Pool were in agreement with the results of previous investigators

who found filamentous bacteria and rods in the waters of Champagne Pool (Brock & Brock 1971; Bohlool 1975). Filtered waters revealed filaments 6–18 µm long upon examination in the SEM (Summers & Boerema 2001). The microbes were preserved by silicification of living organisms. The resulting stromatolites contained a variety of both rod-shaped and coccoid organisms; one isolate, a coccoid organism has been obtained from this spring (González et al. 1999). In keeping with this finding, the sinter deposits were shown to contain a variety of silicified filaments and coccoid microbes (Jones et al. 2001). Slide immersions accumulated filamentous bacteria shown by thin section electron microscopy to be covered with a silica matrix that was orange in colour (Mountain et al. 2001). A low diversity of thermophilic anaerobic microbes was identified on the orange shelf around Champagne Pool. Filaments were usually <0.5–1 µm in diameter and thought to contribute to sinter formation by increasing the surface area available for silica deposition (Jones et al. 2001). Many of these organisms may be cyanobacteria (blue green bacteria) that commonly have an upper temperature limit for growth of 75°C and a lower temperature optimum of c. 65°C.

The present study found at least one morphologically unusual organism. At the light microscope level it resembled *Thermotoga* in having an extended cell wall toga, but was present in a habitat at a lower pH than might be expected for a member of the Thermotogales. Other organisms seen represented a variety of shapes and sizes of both rod and coccoid cells. Patel et al. (1989) observed a marine species of *Thermotoga* in Fiji, although continental species have also been found in low ionic strength hot springs in Africa (Windberger et al. 1989). Patel et al. (1985) also isolated *Fervidobacterium*, a member of the Thermotogales and a strict anaerobe, from New Zealand hot springs at Rotorua and Waimangu from mildly acidic springs, pH 4.9 and 5.5, at both low and high temperatures. Morphologically it resembles *Thermotoga*, but at one end of the cell it has a single terminal sphere or balloon. The toga can also be a large sphere, encasing several cells as part of the cell cycle.

The Waiotapu cell toga resembles the cell in shape and one end of the phase light extended wall structure is usually longer than the other. *Fervidobacterium* was not seen or isolated in samples from Waiotapu (Patel et al. 1985, 1989), but was present in nearby thermal areas of Waimangu. *Thermotoga*-like cells at Waiotapu were seen at pH 2, 72°C (Fig. 5C,D), but not at pH 2, 77°C in the immediate vicinity. Such an organism has not yet been seen in other New Zealand springs or in Fiji. It is not clear what properties, other than temperature, influence the distribution of this species or *Fervidobacterium*.

The several genera of Thermotogales as well as a number of uncultured clones (GenBank) indicate that Thermotogales are widely dispersed (Burton & Norris 2000). Although DNA evidence supporting its presence in New Zealand springs was not obtained, the organism seen in Fig. 5 could be a member of the Thermotogales that did not respond to either of the *Thermotoga*/Thermotogales-specific primer sequences used to amplify 16S rDNA. This result could be the result of a low copy number, DNA extraction difficulties, absence of *Thermotoga* DNA, primer design, or other unknown reasons.

Estimates of cell concentration may be low. Counting cells includes the possibility of missing very small cells such as those seen by SEM. With DAPI staining, the small size raises the question of whether a given DAPI-fluorescent particle represented a cell. In addition, a fixed volume placed on a filter seems like a logical approach for counting low cell concentrations, but several cells embedded in crystals seen by SEM may give a confusing image when viewed by DAPI staining. Specifically, cells that appear in a small aggregate might normally be undercounted if individual cells could not be clearly observed. Bacterial cell counters might exclude larger cell masses, seen by light microscopy (Fig. 5A,B), as well as cells associated with sulphur crystals or other types of large particles.

Although the higher contrast provided by DAPI staining and SEM allowed us to observe microbes in natural samples, this did not constitute proof that these microorganisms are growing *in situ*. They could be nonviable xenic species that entered the spring by underground waters, or other routes. The samples may have been exposed to conditions such as low temperature before culturing that may have resulted in acidification of the cytoplasm and cell death, or other unknown factors may have prevented growth of cells. The inability to grow cultures of organisms from the two very low-pH high-temperature springs, possibly owing to decreased viability during sample transport, nutritional requirements, or other factors such as anaerobic growth requirements, can be contrasted with the results of DAPI staining. With DAPI staining, some cells showed brightly fluorescent nucleoids suggesting exponential growth whereas others showed overall fluorescence characteristics of stationary phase cells (Poplawski & Bernander 1997).

All of the sites sampled contained cell aggregates. Several lines of evidence suggest that these aggregates may contribute to the survival of organisms in the thermal pools. First, three of the low-pH springs examined had large cell aggregates (>500 cells) in the sediment (Fig. 5; Table 1); the fourth had smaller aggregates. Second, similar aggregates appear in the water column sample of Champagne Pool (Table 1), suggesting that they do not form in the sediment. Third, some organisms in the aggregates of Champagne Pool appeared as long, thin protruding filaments resembling *Thermofilum pendens* (Table 2), an organism that has an obligate requirement for a lipid fraction from *Thermoproteus tenax* (Zillig et al. 1983). *T. pendens* would thus benefit from an association with a large cell aggregate that meets this requirement. Fourth, Jones et al. (2001) reported that the shelf surrounding Champagne Pool contains silicified filaments that resemble *Thermofilum* and *Thermoproteus* (Zillig et al. 1983). Finally, the existence of aggregated microbial cells is not limited to New Zealand pools. In Yellowstone National Park, mixing pools characteristically contained similar aggregates of cells (Weiss Bizzoco pers. obs.), suggesting that such collections of cells may be widespread and contribute to survival of these organisms.

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