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ORIGINAL PAPER

Microbial life in Champagne Pool, a geothermal spring in Waiotapu, New Zealand

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Abstract Surveys of Champagne Pool, one of New Zealand's largest terrestrial hot springs and rich in arsenic ions and compounds, have been restricted to geological and geochemical descriptions, and a few microbiological studies applying culture-independent methods. In the current investigation, a combination of culture and culture-independent approaches were chosen to determine microbial density and diversity in Champagne Pool. Recovered total DNA and adenosine 5′-triphosphate (ATP) content of spring water revealed relatively low values compared to other geothermal springs within New Zealand and are in good agreement with low cell numbers of $5.6 \pm 0.5 \times 10^6$ cells/ml obtained for Champagne Pool water samples by 4′,6-diamidino-2-phenylindole (DAPI) staining. Denaturing Gradient Gel Electrophoresis (DGGE) and 16S rRNA (small-subunit ribosomal nucleic acid) gene clone library analyses of environmental DNA indicated the abundance of *Sulfurihydrogenibium*, *Sulfolobus*, and *Thermofilum*-like populations in Champagne Pool. From these results, media were selected to target the enrichment of hydrogen-oxidizing and sulfur-dependent microorganisms. Three isolates were successfully obtained having 16S rRNA gene sequences with similarities of ~98% to *Thermoanaerobacter tengcongensis*, 94% to *Sulfurihydrogenibium azorense*, and 99% to *Thermococcus waiotapuensis*, respectively.

Keywords Champagne Pool · Waiotapu · Geothermal · Thermophilic microorganisms · Hydrogen-oxidizer · Sulfate-reducer · Arsenic

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Introduction

The Waiotapu geothermal region covers a surface area of around 18 km^2 in the North Island of New Zealand. It is one of the largest areas of terrestrial thermal activity in New Zealand with a predicted surface heat flow of ~540 MW (Hedenquist 1986a).

The largest geothermal feature within the Waiotapu region is Champagne Pool. The hot spring is ~65 m in diameter (Fig. 1a), has an estimated volume of 50,000 m^3 (Hedenquist and Henley 1985) and was formed over 900 years ago by hydrothermal eruption (Lloyd 1959). The alkali chloride spring water (pH 5.5 at around 75°C) consists of high concentrations of silica and metalloid ion-sulfide complexes (Jones et al. 2001), which form the white terrestrial silica sinter rim that rises 50 cm above the pool surface and the orange subaqueous sediments around the margin of Champagne Pool (Fig. 1b). Champagne Pool fluid is oversaturated with orpiment (As_2S_3), stibnite (Sb_2S_3), and carlinite (Tl_2S) leading to precipitation of those sulfide minerals (Pope et al. 2005). The hot spring discharges about 10 l/s of undiluted geothermal water and around 7 l/s of vapor (Hedenquist and Henley 1985) providing a mean residence time of the order of 34 days. The

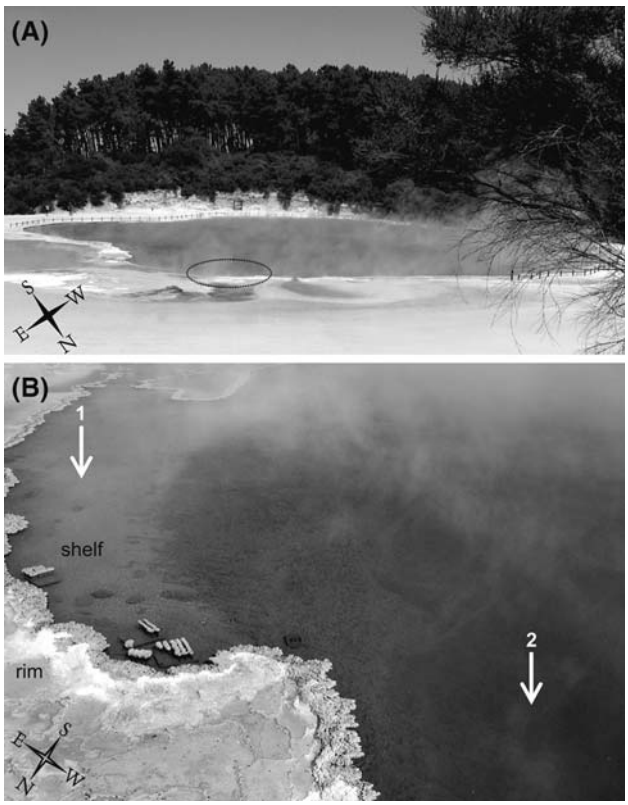

Fig. 1 Champagne Pool hot spring surrounded by a gray–white rim of silica. **a** Sampling area is indicated by a *dotted ellipse*. **b** Sampling site 1 was located within the shallow water area (~10 cm below surface water level) on the subaqueous shelf covered by orange-colored sediment. Site 2 was off the shelf within the deep-water area (maximum depth ~150 m) and samples were obtained from 30 to 100 cm below surface water level

pool name derives from its effervescence due to the copious amounts of gas evolved, mainly 73.0% (v/v) CO_2, 16.2% (v/v) N_2, 6.4% (v/v) CH_4, 2.3% H_2, 1.7% (v/v) H_2S, and traces of O_2 (Jones et al. 2001), which simultaneously serves to mix the water and maintain an even temperature of around 75°C by convection and pH values of ~5.5 by buffering due to CO_2.

The Waiotapu geothermal region has been subject to many geological and geochemical investigations (Giggenbach et al. 1994; Hedenquist and Henley 1985; Hedenquist 1986b; Lloyd 1959; Pope et al. 2005). Only a few studies have recently described microbial activity in Champagne Pool restricted to electron microscopy examinations (Jones et al. 2001; Mountain et al. 2003; Phoenix et al. 2005) and PCR studies (Ellis et al. 2005). Micrographs have shown filamentous, rod- and coccoid structures to be similar to morphologies of *Chloroflexus*, *Thermothrix*, *Thermus*, *Thermoproteus*, *Pyrobaculum* or *Thermofilum* species (Jones et al. 2001) and S-layer patterns to be similar to *Clostridium thermohydrosulfuricum* or *Desulfotomaculum nigrifacans* (Phoenix et al. 2005). Ellis et al. (2005) identified a 16S rRNA gene sequence closely related to *Thermofilum pendens*. However, successful attempts to culture microorganisms from Champagne Pool have not been reported.

Previous studies showed that the standing biomass and microbial diversity in the pool is low (Ellis et al. 2005; Jones et al. 2001) with reasons suggested being: (1) Lack of substrate due to long residence time of spring water. (2) Toxic levels of arsenic or other pool components. (3) Silica deposition precludes bacterial growth due to nucleation. Niederberger (2005) reported a dissolved organic carbon concentration for Champagne Pool water of 700 mg/m^3, which was at least on a par with many other thermal pools, which contained greater biomass. Additionally, there would appear to be ample CO_2 and H_2 for methanogenesis and other reduced inorganics to support autotrophy. Champagne Pool is supersaturated in silica and evaporative cooling (or bacterial nucleation) enhances precipitation. Exposed glass slides build up a silica sinter at a rate of 0.023 mm/day to form mini-stromatolites in 2–3 months due to wave action. The sinter contains fossilized bacteria and has a complex chemistry (Mountain et al. 2003). However, sinter does not form as readily in anaerobic zones and is unlikely to explain the low biomass throughout the pool.

In this investigation, we applied culture and culture-independent approaches to describe microbial density and diversity in Champagne Pool. Microbial density assessed by microscopy, DNA extraction and determination of the adenosine 5′-triphosphate (ATP) content of spring water and colonized slides revealed relatively low values compared to other hot springs in New Zealand (Niederberger 2005). The culture-independent studies involved Denaturing Gradient Gel Electrophoresis (DGGE) analysis and construction of 16S rRNA (small-subunit ribosomal nucleic acid) gene clone libraries of environmental DNA obtained from Champagne Pool. On the basis of these results culture media were selected and designed. Isolates phylogenetically related to strains of the genera *Sulfurihydrogenibium*, *Thermoanaerobacter*, and *Thermococcus* were successfully obtained.

Materials and methods

Sampling and ATP measurements

Water and sediment samples were taken in 2004, 2005, and 2006 from the northeast side of the terrestrial hot spring Champagne Pool in Waiotapu, New Zealand (Fig. 1a). In total, 14 sampling trips to Champagne Pool have been undertaken in February, May, June, July, September, October, and December collecting 2–5 l spring water samples on each trip. Samples were collected from 10, 30,

and 100 cm below water surface level (Fig. 1b), immediately transferred into sterile 2-l glass flasks (Schott, Ketchum, ID, USA) and hermetically closed. By 2 h at the latest the samples were processed in the laboratory.

To estimate biomass in Champagne Pool the content of ATP was determined. Intracellular ATP is closely related to metabolic activity. Therefore, glass slides were immersed in Champagne Pool and incubated for several days. The glass slides provided a surface for potential microbial colonization (Niederberger 2005). ATP measurements were carried out by wiping defined areas of the glass slides using a commercially available Clean Trace swab device (Biotrace, Bridgend, UK). The swab system extracts ATP from microbial cells, and released ATP reacts with luciferin and luciferase reflected by light emission. Light production was measured using a portable ATP meter (Uni-Lite, Biotrace) and recorded as Relative Light Units (RLU). Fresh spring water samples were measured by dispensing a standard volume of sample directly onto a swab device and RLU were determined as described.

DNA extraction

Nucleic acids were extracted by enzymatic digestion (Marmur 1961) and by a fast method based on physical disruption and cationic detergents modified from the protocol of Dempster et al. (1999). Approximately 500 ml spring water was filtered through a cellulose acetate filter (pore size 0.22 μm, diameter 47 mm). The filter was then placed in cetyltrimethylammoniumbromide (CTAB) extraction buffer [100 mM Tris–HCl, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB, 1% (w/v) polyvinylpyrrolidone (PVP) (360,000), pH 8.0] and freshly made 0.4% (w/v) 2-mercaptoethanol and incubated at 100°C for 20 min. Next, nucleic acids were extracted with an equal volume of phenol, followed by extraction with chloroform-isoamyl alcohol [24:1 (v/v)]. To the aqueous phase an equal volume of isopropanol and 0.1 volume of 3 M sodium acetate (pH 5.5) were added. After overnight incubation at –20°C the solution was centrifuged. The pellets were then washed twice in 70% ethanol, air-dried and resuspended in TE buffer (10 mM Tris–HCl, pH 8; 1 mM EDTA).

PCR amplification of 16S rRNA gene and gene cloning experiments

Microbial 16S rRNA genes were amplified in PCR mixtures consisting of PCR buffer (10 mM Tris–HCI, 50 mM KCI, pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTP (dATP, dCTP, dGTP, and dTTP), 1.0 μM of each primer, 5.0% (w/v) dimethyl sulfoxide (DMSO) and 1.0 U of *Taq* polymerase (Roche, Penzberg, Germany). For DGGE-PCR reaction buffer (10 mM Tris–HCI, 50 mM KCI, pH 8.3), 1.25 mM $MgCl_2$, 0.2 mM dNTP (dATP, dCTP, dGTP, and dTTP), 0.2 μM of each primer and 1.25 U of *Taq* polymerase (Roche) were used. Sequences of PCR primers are shown in Table 1. For amplification 20–100 ng of DNA templates were used. The *Taq* polymerase was added to the reaction mixtures after the initial denaturation step to minimize the possibility of nonspecific DNA amplification. PCR conditions using archaeal and bacterial primers were an initial denaturation step at 94°C for 210 s, 33 cycles annealing for 30 s, extension at 72°C for 120 s and denaturation at 94°C for 30 s, followed by a final extension step

Table 1 Target, position, specificity, sequences, and annealing temperature of oligonucleotide primers used for PCR studies

Target	Position[a]	Oligonucleotide primer sequence (5′–3′)	*T* (°C)
Bacterial 16S rRNA gene (Johnson 1994)	27F	AGAGTTTGATCCTGGCTCAG	50[d]
	1522R	AAGGAGGTGATCCA(A/G)CCGCA	
Bacterial 16S rRNA gene (DGGE) (Lane 1991)	338F	**GC**[b]-CTCCTACGGGAGGCAGCAG	65[e]
	519R	ATTACCGCGGCTGCTGG	
Archaeal 16S rRNA gene (Niederberger et al. 2006)	347F	CCAGGCCCTACGGGGCGCA	60
	1335R	GTGTGCAAGGAGCAGGGAC	
Archaeal 16S rRNA gene (DGGE) (Niederberger et al. 2006)	915F	**GC**[c]-AGGAATTGGCGGGGGAGCAC	60
	1335R	TGTGCAAGGAGCAGGGACG	
Nanoarchaeal 16S rRNA gene (Huber et al. 2002)	9F	G(A/G)GTTTGATCCTGGCTCAG	65[e]
	511R	CTTGCCCACCGCTT	

[a] Corresponding 16S rRNA gene sequence position in *Escherichia coli*

[b] GC-clamp: CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCC

[c] GC-clamp: CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC (Ferris et al. 1996)

[d] Annealing temperature was 50°C for 2 cycles and 48°C for 31 cycles

[e] Annealing temperature was decreased from 65 to 54°C in intervals of 0.5°C per cycle

for 360 s. For DGGE-PCRs the initial denaturation was extended to 300 s, annealing and extention steps were applied for 60 s and the total cycle number was 36. The annealing temperatures for each primer set are enlisted in Table 1. Archaea-specific primers often lack specificity for Nanoarchaea. Therefore an additional primer set was used which was specific for Nanoarchaea. PCRs amplifying the nanoarchaeal 16S rRNA gene (primer 9bF and 511mcR) were performed according to Huber et al. (2002).

Amplified 16 rRNA genes were purified by agarose gel electrophoresis and used in cloning experiments with pGEM-T Easy vector as described by the manufacturer (Promega, Madison, WI, USA). Clones containing insert were categorized by enzymatic digestions with *Eco*R I, *Rsa* I and *Bam*H I (Roche) following electrophoresis in 3% agarose gel and TBE buffer (89 mM Tris–borate and 2 mM EDTA, pH 8.3). Representatives of each distinct restriction enzyme fingerprint were selected for sequencing.

DNA sequencing of clone inserts were carried out by the Waikato DNA Sequencing Facility at the University of Waikato, Hamilton, New Zealand based on MegaBACE DNA analysis system (Amersham Biosciences, Piscataway, NJ, USA). In sequencing reactions the oligonucleotide primers M13pUC forward 5′-CCCAGTCACGACGTTGTA AAACG-3′, M13pUC reverse 5′-AGCGGATAACAATT TCACACAGG-3′ and T7 5′-TAATACGACTCACTAT AGGG-3′ were used.

DGGE analysis of 16S rRNA gene fragments

DGGE profiles were obtained by separation of the DGGE-PCR products obtained from environmental DNA in polyacrylamide gels with denaturing gradients (Muyzer et al. 1993) ranging from 40 to 80% and 45 to 65% for bacterial and archaeal samples, respectively (100% denaturant is defined as 7 M urea and 40% (v/v) deionized formamide). Electrophoresis was conducted in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8.0) at constant voltage of 140 V for 5 h using a DCode system (Bio-Rad, Hercules, CA, USA). After electrophoresis, gels were stained with ethidium bromide (0.5 mg/l) for 10 min, washed in distilled water for 5 min and then photographed (AlphaImager System, AlphaInnotech, San Leandro, CA, USA) under UV light with a wavelength of 302 nm.

DNA fragments were excised from polyacrylamide gels, reamplified in DGGE-PCRs and verified by a second DGGE analysis to ensure purity. Representative DNA fragments were sequenced.

Enrichment experiments

Based on the results of DGGE analysis and gene cloning experiments media for hydrogen-oxidizing (modified MSH medium), sulfur-oxidizing (*Sulfolobus* medium) and sulfur-reducing (V24N medium) microorganisms were used in enrichment experiments. Medium V24N (Stetter 1986) and *Sulfolobus* medium (Brock et al. 1972) were prepared as previously described, except that the final pH was adjusted to a value of 5.5. MSH medium (Aguiar et al. 2004) was modified and contained the following components (per litre of anaerobic water): 0.15 g of NaOH, 0.50 g of KCl, 1.36 g of $MgCl_2$ $6H_2O$, 7.00 g of $MgSO_4$ $7H_2O$, 2.00 g of $Na_2S_2O_3$ $5H_2O$, 0.40 g of $CaCl_2$ $2H_2O$, 0.20 g of NH_4Cl, 0.25 g of K_2HPO_4, 1.95 g of MES and trace minerals containing final concentrations per litre medium: 5.00 mg of Na-EDTA $2H_2O$, 1.50 mg of $CoCl_2$ $6H_2O$, 1.00 mg of $MnCl_2$ $4H_2O$, 1.00 mg of $FeSO_4$ $7H_2O$, 1.00 mg of $ZnCl_2$, 0.40 mg of $AlCl_3$ $6H_2O$, 0.30 mg of Na_2WO_4 $2H_2O$, 0.20 mg of CuCl, 0.20 mg of Ni_2SO_4 $6H_2O$, 0.10 mg of $NaSeO_3$, 0.10 mg of H_3BO, 0.10 mg of Na_2MoO_4 $2H_2O$. The pH value of the medium was adjusted to 5.5. Aliquots of 40 and 8 ml were autoclaved under CO_2 atmosphere in 160 ml serum bottles and 30 ml test tubes, respectively. After inoculation using 5% inocula the initial gas phase was exchanged with 79% H_2/16% CO_2/5% O_2 at 170 kPa.

Pure cultures were obtained by dilution series continued over eight tenfold steps. Cells from the highest dilution step were inoculated in medium solidified by 0.6% (w/v) gelrite gellan gum and 0.06% (w/v) $MgSO_4$ $7H_2O$. Individual colonies were picked from the solidified medium for new dilution series. This process was repeated at least twice. The first pure cultures were designated as strains CP.B1, CP.B2, and CP.B3.

Culture experiments were performed with *Bacillus caldotenax* cultures using untreated, N_2 or CO_2 sparged, sterile-filtered (filter pore size of 0.22 μm) and autoclaved (20 min at 121°C under 104 kPa) Champagne Pool water supplemented with Tryptic Soy Broth (TSB; Difco) to final 1× and 0.1× (w/v) concentrations. Lower TSB concentrations were used as higher concentrations might alter spring water components in the culture medium (e.g., precipitation) due to the autoclaving process. *B. caldotenax* was selected due its simple culture requirements and its ability to grow under thermophilic and slightly acidic conditions as found in Champagne Pool. Incubations were performed at 70°C at pH 6.0.

Light and electron microscopy

Microbial cells were observed under a phase-contrast microscope (Olympus BH-2).

Microbial enumeration was performed by staining DNA with 4′,6-diamidino-2-phenylindole (DAPI). Formalin-fixed water samples obtained from Champagne Pool were filtered through black polycarbonate nucleopore filters (Osmonics Inc., Minnetonka, MN, USA, pore size

0.22 μm). Filter-bound cells were stained with DAPI using standard procedures (Porter and Feig 1980; Wetzel and Likens 2000) and observed under a Leica DMR research microscope (excitation filter BP 340–380 nm, dichromic mirror RKP 400 nm, suppression filter LP 425 nm) at 100× objective magnification. Images were taken with an Olympus DP70 digital camera.

For scanning electron microscopy (SEM), cells were captured onto a 0.22 μm filter by filtration and fixed using 2.5% glutaraldehyde. The filter was exposed to four changes of 0.1 M sodium cacodylate buffer, rinsed in water and dehydrated in an increasing concentration series of ethanol, 50, 75 and 90%, respectively, then to four changes of absolute ethanol. The filter was critically point dried, sputtered with platinum and viewed using a Hitachi S-4100 field emission SEM.

Phylogenetic analyses

The partial 16S rRNA gene sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1997) and the sequences were aligned using the ARB software package (Ludwig et al. 2004). The phylogenetic position of the 16S rRNA gene sequences were determined using the PHYLIP package within ARB with analysis of sequences undertaken using DNADIST, DNAML, DNAPARS, FITCH, NEIGHBOR, and SEQBOOT programs (Felsenstein 1993).

Nucleotide sequence accession numbers

16S rRNA gene sequences have been deposited in the GenBank database under accession numbers as follows: EF101533 (DGGE fragment a), EF101534 (DGGE fragment b), EF101535 (DGGE fragment c), EF101536 (DGGE fragment d), EF101537 (OTU Arc03), EF101538 (OTU Bac03), EF101539 (OTU Bac04), EF101540 (OTU Bac12), EF101541 (CP.B3), EF101542 (CP.B1), and DQ989208 (CP.B2).

Results

Physico- and biochemical properties

No seasonal variability is expected for the geothermal site and during sampling from 2004 to 2006 spring water from Champagne Pool displayed only slight fluctuations in temperature (74.2 ± 0.8°C) and pH (5.5 ± 0.1) values. Although some hot springs in the Rotorua area (ca. 30 km away from Champagne Pool) showed significant changes in water levels in the past 3 years, the water level of Champagne Pool remained stable.

Sediment and water samples collected from different depths revealed low concentrations of ATP (Table 2). Inhibitory effects of Champagne Pool water on the ATP assay can be excluded as measurements of ATP standards of defined concentrations in sterile water and in spring water samples showed similar values (data not shown). The highest ATP levels in Champagne Pool were detected in the orange-colored sediment indicating a higher biomass in the sediments than in the water column. Similar results were obtained by measurements on the surface of glass slides that had been immersed in Champagne Pool for 24 days and had been colonized by microorganisms.

Nucleic acid concentrations of up to 6.4 μg/ml could be extracted from mixed spring water and sediment samples. Values were significantly lower than for DNA concentrations obtained from enrichment cultures isolated from Champagne Pool (3.9 mg/ml). To exclude possible inhibition of the applied DNA isolation method by Champagne Pool compounds, DNA from *Escherichia coli* cells was

Table 2 Summary of ATP content determined for Champagne Pool samples

Sample	Depth (m)	RLU	ATP (ng/ml)
25 μl of water sample	0.00	677	1.8
25 μl of sediment suspension	0.10	4,800	12.4
25 μl of water sample	0.10	740	1.9
25 μl of water sample	1.00	875	2.3
Glass slides (1,976 mm^2) immersed in sediment	0.10	8,900	[a]
Glass slides (1,976 mm^2) immersed in water	0.30	2,300	[a]
Glass slides immersed in hot pool KP1 (Kuirau Park, Rotorua, New Zealand)[b]	–	57,826	[a]
Glass slides immersed in hot pool AQ1 (Kuirau Park, Rotorua, New Zealand)[b]	–	>180,000	[a]

ATP values for the hot springs KP1 (75°C, pH 7.5) and AQ1 (95°C, pH 7.5) are listed as reference. Values are given as Relative Light Units (RLU) and ATP concentrations

[a] Data were obtained from slide surfaces and cannot be expressed per volume

[b] Niederberger et al. (2005)

extracted with and without pool sample water. No inhibition of the assay could be detected.

Microscopy

Microscopic observation of water and sediment samples and glass slides incubated in Champagne Pool revealed coccoid, various rod-shaped and predominately filamentous cell structures (Fig. 2). SEM micrographs showed different morphologies of the isolates CP.B1 and CP.B2 (Fig. 5).

Total cell numbers by direct counting of DAPI-stained cells using epifluorescence microscopy were $5.6 \pm 0.5 \times 10^6$ cells/ml.

Molecular analysis

Archaeal and bacterial 16S rRNA genes were successfully amplified by PCR, whereas amplification of nanoarchaeal 16S rRNA genes remained negative. DGGE profiles derived from the archaeal and bacterial PCR products, displayed two (Fig. 3a) and five distinct bands (Fig. 3b), respectively. For four of the excised DNA fragments sequencing data were obtained. Phylogenetic analysis of both the archaeal DNA fragments and two of the bacterial DNA fragments revealed relationship to species of the genera *Sulfolobus*, *Thermofilum*, *Nevskia*, and *Sulfurihydrogenibium* (Table 3, Figs. 3, 4).

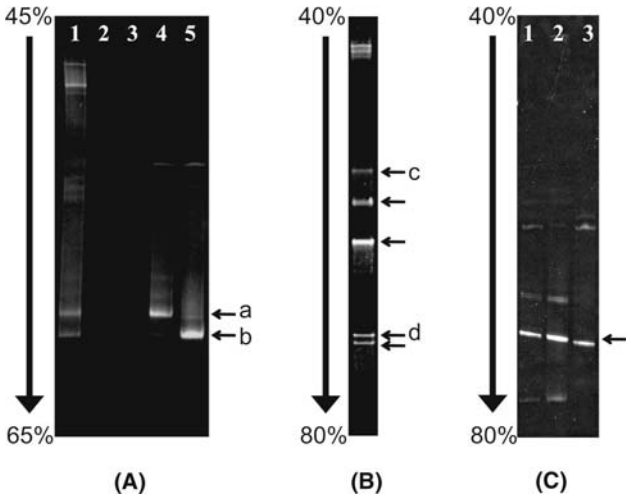

Fig. 3 DGGE profiles of archaeal (**a**) and bacterial (**b**, **c**) 16S rRNA gene fragments amplified by PCR from Champagne Pool water samples in polyacrylamide gels with corresponding denaturing gradients. Bacterial DGGE profiles were obtained in April 2004 (**b**) and October 2006 (**c**). **a** *Lanes*: 1, Champagne Pool; 2, no sample; 3, negative control; 4, excised DNA fragment a; 5, excised DNA fragment b. **c** *Lanes*: 1 and 2, Champagne Pool; 3, Isolate CP.B2. Phylogenetic analysis of DNA fragments revealed phylogenetic relationship to species of the genera *Thermofilum* (a), *Sulfolobus* (b), *Nevskia* (c), and *Sulfurihydrogenibium* (d)

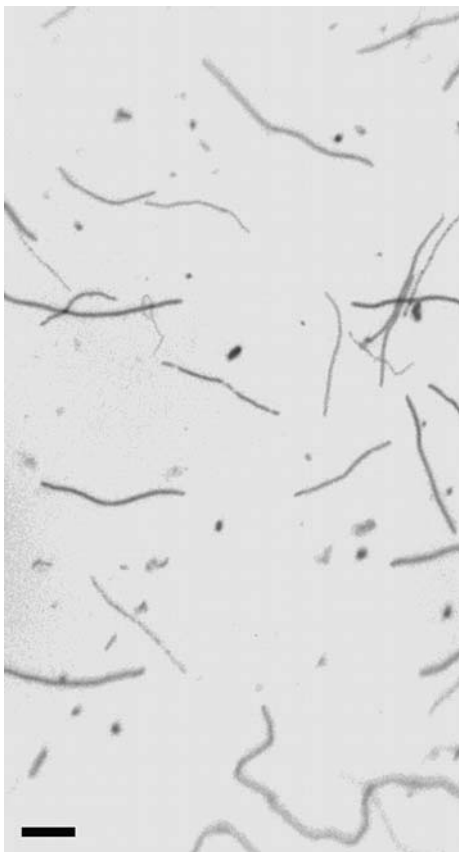

Fig. 2 Inverse image of DAPI-stained Champagne Pool water samples. *Bar* 5 μm

Clone libraries consisting of 40 archaeal and 90 bacterial 16S rRNA genes amplified by PCR were generated. Analysis of the clone libraries indicated species of the genera *Sulfolobus*, *Sulfurihydrogenibium* and *Paracoccus* (Table 3, Fig. 4) to be present in Champagne Pool partially confirming the results of the DGGE analysis. Alignment of the 16S rRNA gene sequences of the most abundant bacterial Operational Taxonomic Units (OTUs) Bac12 and Bac04 with *Sulfurihydrogenibium azorense* revealed a gap of 258 bp between both OTUs indicating that OTUs Bac12 and Bac04 were the same inserts but with different orientation within the cloning vector. Although OTU Bac03 had close database matches to species of *Paracoccus*, the highest sequence similarities were for several uncultured bacteria and a thiosulfate-oxidizing bacterium from a hydrothermal vent. For OTU Bac86 no sequence similarity to known 16S rRNA gene sequences could be established.

Differences between DGGE and clone library results might be due to changes in the microbial community. DGGE and clone library analyses have been conducted separately using DGGE samples obtained from different

Table 3 Summary of 16S rRNA gene sequences identified by culture and culture-independent approaches in samples obtained from Champagne Pool

Sample	Species	(bp)	(%)
DGGE			
Fragment a	*Thermofilum pendens*	330	92
Fragment b	*Sulfolobus acidocaldarius*	411	95
Fragment c	*Nevskia ramose*	146	91
Fragment d	Uncultured *Sulfurihydrogenibium sp.*	64	92
Clone libraries			
OTU Arc03 (100%)[a]	*Sulfolobus tokodaii*	699	94
OTU Bac03 (17%)[b]	*Paracoccus thiocyanatus*	1,426	90
OTU Bac04 (61%)[b]	*Sulfurihydrogenibium azorense*	715	95
OTU Bac12 (20%)[b]	*Sulfurihydrogenibium azorense*	536	95
OTU Bac86 (2%)[b]	No similarity	600	
Cultures			
CP.B3	*Thermococcus waiotapuensis*	962	99
CP.B1	*Thermoanaerobacter tengcongensis*	1,310	98
CP.B2	*Sulfurihydrogenibium azorense*	1,506	94

The lengths of the DNA fragments and the sequence identities to the closest relatives are displayed

[a] Percentage of clones containing archaeal inserts

[b] Percentages of clones containing bacterial inserts

sampling periods. Bacterial DGGE profiles (Fig. 3b, c) varied over time showing fewer bands and changes in band intensities. While DNA fragment d (Fig. 3b) assigned to *Sulfurihydrogenibium*, showed only a relative weak band intensity in April 2004, in October 2006 a DNA fragment of the isolate CP.B2 (closely related to *Sulfurihydrogenibium*) was the most intense band (Fig. 3c). Although band intensities might not reflect the degree of abundance of species in an environment due to PCR biases, the latter DGGE profile is in good agreement with the clone library results, in which *Sulfurihydrogenibium* is the most abundant bacterial species.

According to the results obtained from DGGE analysis and the gene cloning experiments growth media were designed to isolate species closely related to members of the genera *Sulfolobus*, *Thermofilum*, and *Sulfurihydrogenibium*. As *Nevskia* and *Paracoccus* strains are both mesophilic bacteria and the 16S rRNA gene sequences obtained shared low sequence similarities of ~91% to *Nevskia ramosa* and 90% to *Paracoccus thiocyanatus*, medium was not selected to enrich those species.

Enrichment

To enrich hydrogen-oxidizing microorganisms modified MSH medium as described was used. After inoculation with Champagne Pool spring water (inocula size 5%) microbial growth was observed by changes in turbidity within 2 days. Cells appeared to have a slightly curved rod-shaped morphology (Fig. 5b). The first pure culture was designated as strain CP.B2.

In V24N growth medium (Stetter 1986) selected to enrich sulfur-reducing microorganisms different morphologies could be distinguished. A rod-shaped bacterium (isolate CP.B1, Fig. 5a) and a coccoid archeon (strain CP.B3) were successfully isolated.

Enrichment attempts in the *Sulfolobus* medium (Brock et al. 1972) under chemolithothrophic and heterotrophic conditions were not successful.

DNA isolated from the three novel isolates and successive analysis of the amplified 16S rRNA gene revealed a distant phylogenetic relationship of isolate CP.B1 to *Thermoanaerobacter tengcongensis* (Xue et al. 2001), of CP.B2 to *S. azorense* (Aguiar et al. 2004), and of CP.B3 to *Thermococcus waiotapuensis* (Gonzalez et al. 1999) (Table 3). All isolates grew under temperature and pH conditions as found in Champagne Pool (Table 4).

Growth experiments using Champagne Pool water

In growth experiments with *B. caldotenax* cells in untreated and sterile-filtered Champagne Pool water growth could not be detected. However, when the spring water was purged with N_2 or CO_2 or autoclaved prior to inoculation, microbial growth was observed. These results suggest that a volatile component inhibiting microbial life might have been removed from Champagne Pool water during the sparging or autoclaving process.

Discussion

Both archaeal (Fig. 4a) and bacterial representatives (Fig. 4b), but no Nanoarchaea were detected in the terrestrial hot spring, Champagne Pool. While culture-independent methods indicate the abundance of strains phylogenetically related to species of the genera *Sulfurihydrogenibium*, *Nevskia*, *Paracoccus,* *Sulfolobus*, and

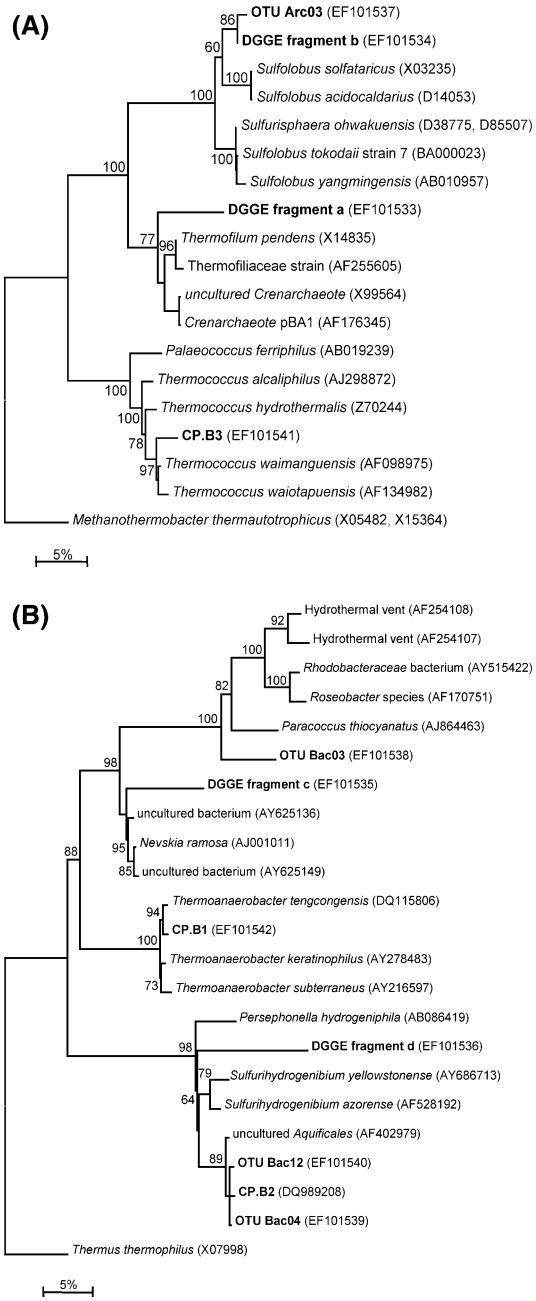

Fig. 4 Phylogenetic trees demonstrating relationships of archaeal (**a**) and bacterial 16S rRNA gene sequences (**b**) obtained from Champagne Pool spring samples. The dendogram was produced with ARB (Neighbor joining) and bootstrap values were determined using PHYLIP. Bootstrap values at the nodes are from 1,000 iterations (only bootstrap values >60% are indicated). The numbers in parentheses are accession numbers. Sequences obtained in this study are indicated in bold. The *scale bars* represents an estimated sequence divergence of 5%

Thermofilum, pure cultures were isolated from Champagne Pool distantly related to *S. azorense* (Aguiar et al. 2004), *T. tengcongensis* (Xue et al. 2001), and *T. waiotapuensis* (Gonzalez et al. 1999). Those findings (Table 3) are in

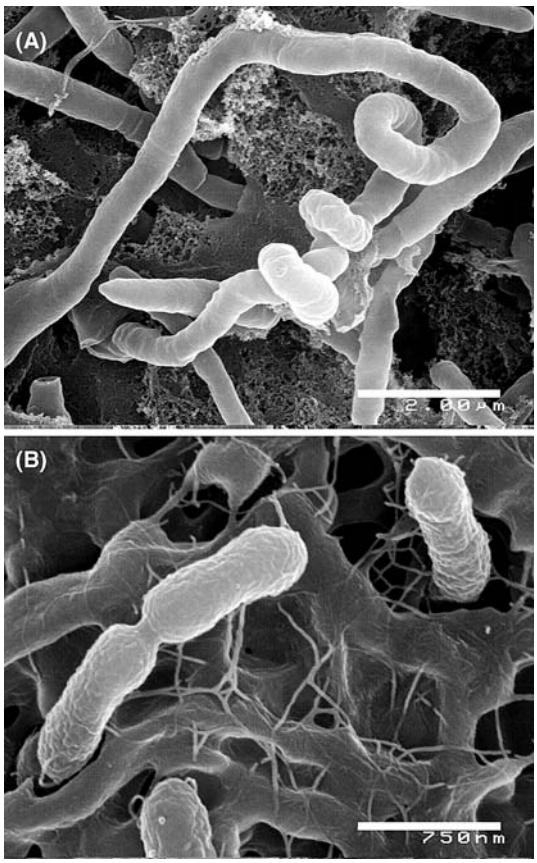

Fig. 5 Scanning electron micrographs of isolate CP.B1 (**a**) and CP.B2. (**b**). **a** *Bar* 2 µm. **b** *Bar* 750 nm

Table 4 Growth temperature and pH ranges for microbial strains isolated from Champagne Pool

Isolate	Energy metabolism	*T* (°C)	PH
CP.B1	Sulfur reduction	45–80 (65)	5.5 (n.d.)
CP.B2	Hydrogen oxidation	45–75 (70)	4.8–5.8 (5.4)
CP.B3	Sulfur reduction	70–85 (n.d.)	45–8.0 (7.0–7.5)

Values in brackets are the optima

n.d. not determined

agreement with the physicochemical properties of Champagne Pool. Dissolved gases in the spring water contain CO_2, H_2, and O_2 in the shallow areas (Giggenbach et al. 1994; Jones et al. 2001) providing chemolithothrophic conditions for hydrogen-oxidizing microorganisms such as *Sulfurihydrogenibium* species. Available sulfur compounds in Champagne Pool are essential for growth of sulfur-oxidizing (*Sulfolobus* species), sulfur-reducing (*Thermoanaerobacter* and *Thermofilum*), and sulfur-dependent (*Thermococcus* species) microorganisms. Other than *Nevskia* and *Paracoccus* all detected microbial representatives are thermophilic. However, the low sequence similarities of ~91% between the DNA fragment c and

N. ramosa, and 90% among the cloned bacterial 16S rRNA genes and *P. thiocyanatus* may indicate that the sequences represent organisms phenotypically different from *Nevskia* and *Paracoccus* strains able to grow under thermophilic conditions. The detection of a *Thermofilum* signature is in agreement with previous reports based on electron microscopy examinations (Jones et al. 2001) and PCR studies (Ellis et al. 2005). However, in microscopic examinations of Champagne Pool water and sediment samples only filamentous, coccoid and rod structures were present, while ''golf club''- and ''lobe''-shaped morphologies, distinct characteristics for members of the genera *Thermofilum* and *Sulfolobus*, respectively, could not be observed.

DGGE profiles, nucleic acid yields, cell counts, and ATP measurements indicate low diversity and biomass in Champagne Pool. ATP determinations (Table 2) showed differences between sediment and water samples, with 6× (suspension) and 4× (glass slides) more ATP in the sediment, suggesting an accumulation of biomass within the sediment. However, the highest values obtained for glass slides incubated in Champagne Pool (8,900 RLU) were much lower than reported for other hot springs (>180,000 RLU) in New Zealand (Niederberger 2005). Those findings are consistent with relatively low cell numbers of $5.6 \pm 0.5 \times 10^6$ cells/ml determined for Champagne Pool water samples by DAPI staining.

This evidence raises the question: what is the limiting factor for microbial diversity and biomass in Champagne Pool besides the thermal conditions in the spring? There may be several factors responsible. Geothermal systems of New Zealand are known to contain high concentrations of metalloids (Hirner et al. 1998). Arsenic ions and compounds are found in Champagne Pool water to a concentration up to 5.6 mg/l (Giggenbach et al. 1994). Arsenic toxicity is mediated by arsenite reacting with thiol functional groups in enzymes and inhibiting their activities and by arsenate through substitution of phosphate (Stolz and Oremland 1999). It has also been shown that trivalent species of arsenic, predominately found in anaerobic environments such as Champagne Pool, were highly inhibitory to methanogenic microorganisms (Sierra-Alvarez et al. 2004). Therefore, only metal ion-tolerant or metal ion-resistant microorganisms might survive in Champagne Pool. In the present study culture experiments of *B. caldotenax* in medium supplemented with Champagne Pool water suggest that the spring water might contain volatile compounds toxic to some microorganisms. Possible candidates might be H_2S and toxic volatile methyl and hydride derivatives of arsenic and antimony, such as AsH_3 (arsine), CH_3AsH_2 (monomethylarsine), $(CH_3)_2AsH$ (dimethylarsine), $(CH_3)_3As$ (trimethylarsine), SbH_3 (stibine), CH_3SbH_2 (monomethylstibine), $(CH_3)_2SbH$ (dimethylstibine), and $(CH_3)_3Sb$ (trimethylstibine). In Champagne Pool geothermal water concentrations of 4.95 ppm AsH_3, 1.90 ppb CH_3AsH_2, 0.10 ppb $(CH_3)_2AsH$, 5 ppb SbH_3, and 0.05 ppb CH_3SbH_2 have been detected (Hirner et al. 1998). Previously published investigations (Jones et al. 1999; Mountain et al. 2003; Phoenix et al. 2005) also considered that microbial cells in Champagne Pool may be subject to biomineralization and bioimmobilisation by rapid silicification and deposition in the sinter.

Champagne Pool seems to be an extreme environment for life forms in terms of its thermal and unique chemical properties. In this study we demonstrated low microbial diversity and biomass in Champagne Pool and were able to isolate three novel strains. The characterization and description of the novel isolates may bring deeper insights into the ecology of the thermal spring.

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