The Microbial Ecology and Colonisation of Surfaces in Selected Hot Springs – a Molecular- and Culture-based Approach

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Abstract

Traditionally, culture-based approaches have been used to investigate the microbial ecology of thermal habitats. The emergence of alternate molecular approaches based on DNA sequences has shown that terrestrial thermal environments have a rich diversity of uncultured prokaryotes. To the best of our knowledge these latter approaches have not previously been applied to New Zealand thermal environments and this became the central aim of this study. The major objective of the study was to target the metabolically active members of the hot pool community by applying a suite of molecular and microscopical techniques to those species colonising surfaces incubated *in situ* in the hot pools.

Two neutral pH pools were investigated in some detail; pool KP1 at 75°C and pool AQ1 at 95°C. Surfaces in pool KP1 were colonised by a high diversity of bacteria (archaea were not detected), as evidenced by complex denaturing gradient gel electrophoresis (DGGE) profiles, and many different species were obtained in enrichment culture. Although colonisation rates were greater in pool AQ1, this was entirely due to only two observed morphotypes and was reflected in DGGE profiles that reflected only two dominant archaeal species. A 16S rDNA clone library of the AQ1 pool water and DGGE profiles of both pool water and colonised slides were dominated by rods of the species *Pyrobaculum*. Slides incubated deep within AQ1 were also colonised by cocci, the closest cultured relative being *Aeropyrum pernix*, a marine hyperthermophile. Two pure cultures were obtained from pool AQ1, *Pyrobaculum* sp. (AQ1.S2) and a novel crenarchaeaotal coccus, (AQ1.S1^T). Isolations of the latter organism were also obtained from two other pools in New Zealand. These were obtained as obligate co-cultures with *Pyrobaculum* rods and could not be isolated into pure culture. A full description of this novel genus of organisms has been accepted for publication in the International Journal of Systematic and Evolutionary Microbiology, with the isolate from pool AQ1 named as the type strain *Ignisphaera aggregans* AQ1.S1^T.

The techniques used to follow colonisation were then applied to a survey of high-temperature pools of New Zealand (>80°C, pH 5.5-9.7), White Island (72-94°C, pH 1.5-3.8) and Yellowstone National Park (75-92.5°C, pH 5.0-8.0). For New Zealand, bacteria were detected by DGGE on colonised slides in pools below 88°C, and archaea, only in pools above 83°C. Bacterial colonisers with dominant signatures included those of *Thermodesulfobacteria* and *Aquificales* species, though interestingly, isolations of these organisms were not obtained. The archaeal colonisers were pH dependent: pools with a pH of 6.0-7.0 had a dominant *Thermofilum* signature; pools above pH 7.0 had both a *Pyrobaculum* sp. and a relative of the Desulfurococcales; and the acidic pools (including White Island) were dominated by *Sulfolobus* sp. Pool water DGGE profiles of the New Zealand pools differed from slide colonisation profiles in some respects, e.g. *Thermococcus* sp. were only detected in pool water. In contrast to the New Zealand results, the Yellowstone National Park slides were dominated by bacteria, with bacterial signatures detected in high-temperature pools (87-92.5°C); representative DGGE bands were sequenced and found to be related to the Aquificales. Nanoarchaeal 16S rDNA PCR amplicons were also obtained from pool water for most of the New Zealand pools (closest relative of AQ1 amplicon being a nanoarchaeal clone from the Uzon Caldera), including the *Pyrobaculum* sp. AQ1.S2 culture. Attempts were also made to isolate marine nanoarchaea from nascent hydrothermal vents situated on the East Pacific Rise; however, these were unsuccessful.

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Abbreviations

ARDRA	amplified rDNA restriction analysis
ATP	adenosine triphosphate
BLASTn	basic local alignment search tool (nucleotide)
bp	base pair
BPB	bromophenol blue
BSA	bovine serum albumin
CAPS	3'-cyclohexylamino-1-propanesulfonic acid
CHES	2-(N-cyclohexylamino)ethanesulfonic acid
CMD	Castenholtz medium
CTAB	hexadecyltrimethylammonium bromide
DAPI	4', 6-diamidino-2-phenylindole
DGGE	denaturing gradient gel electrophoresis
DIC	differential interference contrast
DMSO	dimethyl sulfoxide
dNTP	deoxy-nucleoside triphosphates (dATP, dCTP, dGTP and dTTP)
DOC	dissolved organic carbon
DSM(Z)	Deutsche Sammlung von Mikroorganismen (und Zellkulturen)
EDTA	ethylene diamine tetra-acetic acid
EPR	East Pacific Rise
ERMA	Environmental Risk Management Authority
FISH	fluorescent *in situ* hybridisation
g	gravitational force
GFC	glass fibre cartridge
GIT	guanidine isothiocyanate
GLB	gel-loading buffer
GOPA	Greater Obsidian Pool Area
GPS	global positioning system

IGNS	Institute of Geological and Nuclear Sciences
IJSEM	International Journal of Systematic and Evolutionary Microbiology
IPTG	isopropyl β-D-thiogalactoside
ITS-PCR	intergenic transcribed spacer-polymerase chain reaction
kb	kilobase
LB	Luria broth
MES	2-morpholinoethanesulfonic acid
Milli-Q	Millipore Corporation
MOPS	3-morpholino-2-hydroxypropanesulfonic acid
MPN	most probable number
MSM	mineral salts medium
N. A.	not applicable
NCBI	National Centre of Biotechnology Information
N. D.	not determined
NIWA	National Institute of Water and Atmospheric Research
nm	nanometers
NZ	New Zealand
NZMG	New Zealand map grid
OD_{600}	optical density at 600 nm
OPR	Operon Technologies
ORF	open reading frame
POM	particulate organic matter
PVP	polyvinyl-pyrrolidone
RAPD	randomly amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RLU	relative light units
RO	reverse osmosis
SARST	serial analysis of ribosomal sequence tags
SB	sodium borate
SDS	sodium dodecyl sulphate

SEC	size exclusion chromatography
SEM	scanning electron microscopy
SS	suspended solids
SSCP	single strand-conformation-polymorphism
TAE	tris-acetic acid EDTA buffer
TBE	tris-borate EDTA buffer
TE	tris EDTA buffer
TG	thermophile group
TGGE	temperature gradient gel electrophoresis
T-RFLP	terminal-restriction fragment length polymorphism
TRUCC	Thermophile Research Unit Culture Collection
TSA/S	tryptic soy agar and starch
UV	ultra violet
V	volts
v/v	volume per volume
WHOI	Woods Hole Oceanographic Institute
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YNP	Yellowstone National Park

Chapter 1 : Introduction

1.1 Thermal environments

Thermal environments are created by four distinct processes: solar heating; combustion processes; radioactive decay and geothermal activity (Brock, 1978). These are also various human-influenced thermal environments, which are typically linked to combustion processes and include hot water heaters, cooling towers, and heated water wastes from geothermal/nuclear power stations (Brock, 1978; Mountain *et al.*, 2003).

Solar heating has the capability to elevate temperatures of shallow water bodies up to 40°C and temperatures of up to 60°C have been measured within solar heated soil, desert surfaces and black anthracite wastes (Brock, 1978). However, solar heating effects are transitory, being dependent on the length of day or time exposed to sunlight. Heat generation by combustion comprises both biological and non-biological processes. Biological processes include heat generated by the microbial degradation of organics, for example, temperatures within compost can reach in excess of 60°C (Carpenter-Boggs *et al.*, 1998). Non-biological combustion processes include smouldering coal refuse piles which can attain temperatures of up to 80°C (Bohlool and Brock, 1974) whereas, burning coal refuse piles reach temperatures of up to 140°C (Brock, 1978). Radioactive wastes can also produce high temperatures, for example, soil temperatures of around 70°C have been measured 18 meters beneath a leaking radioactive tank (Brim *et al.*, 2003).

Geothermal heating is a direct result of plate tectonic movements which is manifest at the Earths surface as hydrothermal venting. The heat generated within hydrothermal systems is from three processes: 1 - spreading centres at tectonic plate boundaries, where magma rises and cools producing new lithosphere; 2 - subduction zones where one tectonic plate descends below another; 3 – 'hot spots' which are defined as the heat expressed at the Earth's surface from a mantle plume or a column of hot bouyant rock rising into mantle beneath a lithospheric plate (Reysenbach and Cady, 2001). All three processes release heat to the Earth's crust, increasing the temperature of surrounding water. In turn, this heated water reacts with surrounding rock which alters the chemistry of the water. The water is then forced to the surface as a superheated, highly reduced liquid, rich in dissolved volcanic gases and minerals (Reysenbach and Cady, 2001). As a consequence, marine hydrothermal venting is manifest as shallow hot water vents or deep sea hot sediment/seaps and 'black smoker' chimneys, and terrestrial hydrothermal venting is visible as hot springs, geysers, mud pots, solfataras and steam fumaroles (Huber *et al.*, 2000c).

1.1.1 The hot spring as an environment and habitat

The chemistry of a hot pool can vary due to a number of factors such as the temperature, rock contact, and source and ground water interactions/mixing. Therefore, each pool can be regarded as a chemical reactor with variable input of groundwater, volcanic gases and hydrothermal fluid and outputs of vapour and liquid (Henley, 1996).

Most thermal waters fall into three common types, those containing large amounts of calcium carbonate in solution, acid waters that are rich in sulfates or alkaline waters with high chloride levels (Williams and McBirney, 1979). Calcium carbonate-rich waters are confined to areas with limestone or dolomite geology, where hydrothermal water rises through limestone beds. As the water reaches the surface and runs off, CO_2 is lost and the water cools and as a consequence, acidity increases and the carbonates precipitate at the rims. For example, the terraces of Mammoth Springs in Yellowstone National Park (YNP) are precipitates composed of carbonate-rich travertine (Williams and McBirney, 1979). Acid waters are rich in acid sulphates due to the oxidation of H_2S (present in volcanic emissions) to H_2SO_4, although direct HCl input may also influence the pH (Brock, 1978). Acid waters are typically rich in volatile components such as B, CO_2, H_2S and NH_3 and differing levels of Mg, Fe, Ca, Na and Si depending on surrounding geology. Volatile metals are also common in acid waters, such as Hg, Pb, Bi, Sn, and As (Williams and McBirney, 1979). Alkaline waters are lower in volatile components but rich in elements such as Na, K, Cl, SO_4, HCO_3 and CO_3, and large amounts of silica may be present. As a result of these three common chemical types, pH levels of hot pools are typically bimodal in distribution, within the ranges of pH 2-4 and 7-9 (Brock, 1978; Williams and McBirney, 1979). As mentioned previously, the low pH values of the water in acid pools is created by the oxidation of H_2S to H_2SO_4, whereas the pH values of neutral to slightly alkaline pools are typically influenced by the buffering capacities of carbonate (carbonic acid, pKa 6.37) or silica (silicate, pKa 9.7) (Brock, 1978).

Temperature is also an important factor that influences various properties of hot pool waters. For example, as temperature increases the density, viscosity, surface tension, heat capacity, refractive index, pH and oxygen solubility decrease. In contrast, the vapour pressure, ionization and organic/inorganic solubilities increase (Brock, 1978). For example, as water cools the solubilities of carbonate and silica (common anions of hot spring water) decrease and precipitate (Williams and McBirney, 1979). Also, at 90°C, oxygen dissolves in water at less than 2% than levels at 20°C (Brock, 1978).

Volcanic gases typically present within hot springs include CO_2, H_2, CH_4 and lower levels of CO, N_2 and O_2 (Williams and McBirney, 1979; Rinehart, 1980). These gases can provide the energy and carbon source for autotrophic growth. Similarly, low levels of dissolved organics within pool water can provide carbon sources for heterotrophic organisms with sulfides/sulfur compounds and inorganic ions being available as electron donors/acceptors for chemolithotrophs and heterotrophs (Brock, 1978).

1.1.2 Approaches used to investigate the microbial ecology of geothermal habitats

Microbiological investigation of thermal habitats has largely been by the traditional methods of selective culture and microscopic observation (Barns *et al.*, 1996b;

Stetter, 1998a). The former method in particular has resulted in the isolation and characterisation of many new genera and species of hyperthermophiles (Stetter, 1996), which has fed an explosion of interest in these relatively new environments. The microorganisms isolated from thermal habitats are described in detail in Section 1.1.3, below.

Extremophile microbiology is now an established discipline in its own right, but the description of new isolates based on the traditional method of selective culture is now yielding diminishing returns. More often variants of described species are being obtained rather than dramatically different physiological or phylogenetic types (Huber *et al.*, 2000c). The application of culture-independent, molecular approaches has re-invigorated the field of microbial ecology and it is now generally believed that selective culture, at best, achieves the isolation of no more than approximately 1% of the total species present in even well-studied environments (Amann *et al.*, 1995; Ward *et al.*, 1990). Both culture-dependent and culture-independent techniques are typically used in microbial ecology studies and are discussed in Sections 1.1.2.1 and 1.1.2.2, respectively, including the advantages and limitations of each approach.

1.1.2.1 Culture-dependent approaches

Culture-dependent approaches involve taking a sample from an environment and attempting to enrich for organisms using artificial growth media. However, as mentioned previously, application of molecular-based methods have shown that many un-cultivable organisms exist in even well-studied environments. Therefore, culture approaches may only detect a minor proportion of the microbial members of an environment. Moreover, growth on media typically involves enrichment bias, i.e. the development of a community structure in growth media that is not representative of the original community (Dunbar *et al.*, 1997; Ferris *et al.*, 1996a; Santegods *et al.*, 1996). For example, growth in nutrient rich media may select for organisms adapted to high nutrient availability, i.e. 'the endemic weed species', which will out-compete the other members of the community, hindering isolation attempts. However, new isolation strategies and tools are being utilised in the enrichment and isolation of novel hyperthermophiles, as reviewed by Huber *et al.*, (2000c). One of these techniques involves the use of optical tweezers, which can 'trap' a single cell from enrichment by the use of a laser microscope; the cell can subsequently be deposited into growth medium for cultivation efforts. Although culturing approaches are challenging, laborious and may only detect a small proportion of the microbial community, it is an extremely important part of microbial ecology as the properties and functions of microbial species can be investigated. The ability to culture an organism in pure culture is still the "gold standard' in microbiology, so the description of an organism by solely molecular means is not regarded as complete until its culture can be described.

1.1.2.2 Culture-independent approaches

Most culture-independent techniques commonly involve the extraction of total environmental DNA and utilisation of the polymerase chain reaction (PCR) to target specific genetic markers. The resulting amplified DNA sequence information is used to indicate the presence of a microbe in the original community. These genetic-based methods allow detection of microorganisms without the requirement for culturing, thus providing a quicker, and ostensibly less-biased method of community analysis. Typically, 16S rDNA is used as both a genetic marker in the PCR-based techniques and a chronometer for the evolution of the microorganisms (Madigan *et al.*, 2000). Examples of culture-independent, genetic- and PCR-based methods typically used in microbial ecology are listed and briefly discussed below.

a) Clone libraries: this approach typically involves PCR amplification of the 16S rRNA gene from all microbes in an environment. The resulting PCR amplicons are cloned into a vector and transformed into a bacterial host. Therefore, each bacterial transformant contains a single copy of 16S rDNA, representative of a single microorganism from the original environment. The cloned amplicons can then be individually sequenced to provide the identity of the microbes. However, to save on sequencing costs, the diversity of the cloned amplicons are typically grouped into operational taxonomic units (OTUs). This is undertaken by screening the clones with restriction endonucleases and placing clones with similar restriction fragment length polymorphism profiles (RFLPs) into a single OTU. Therefore, a single representative clone of each OTU is sequenced (Barns *et al.*, 1994; Barns *et al.*, 1996a). The advantage of this technique is that a large proportion of the microbes in a community can be easily identified. However, a limitation of this approach is the lengthy time and the cost involved with cloning and DNA sequencing.

b) Denaturing Gradient Gel Electrophoresis (DGGE): DGGE involves the PCR amplification of a DNA region common to a group of microorganisms under investigation (typically the 16S rRNA gene). The PCR involves using a primer containing a 5' terminal GC-clamp (typically a 30-40 bp GC-rich nucleotide sequence). The resulting PCR amplicons are then exposed to electrophoresis through a polyacrylamide gel containing a gradient of denaturants (urea and formamide). The double-stranded amplicons are consequently separated based on their melting properties (which reflects the nucleotide sequence) under a gradient of increasing denaturant concentration. Movement in the gel will essentially cease at a concentration where the double strands (apart from the GC-clamp) are largely melted (separated) producing a band in the gel. Both strands of the amplicon remain attached at the GC-clamp (Muyzer, 1993). Therefore, the amplicons from a mixed microbial community produce a community-specific banding pattern. Thus, DGGE analysis of an environmental sample can provide a 'fingerprint' of a microbial community, where each band theoretically represents a single microbial member of the original community. However, some studies have shown there may not be a direct correlation, due to multiple ribosomal operons within an organism's genome (Klappenbach *et al.*, 2001). An advantage of this technique is that it is rapid and profiles from differing samples can easily be compared, in addition, DNA bands can be removed from the gel and sequenced to identify the microbial members. However, a limitation of DGGE is that minor members of a microbial community may not be detected (Jackson *et al.*, 2001a). Related to this technique is temperature gradient gel electrophoresis (TGGE), where a temperature gradient is used to denature the PCR amplicons, as opposed to a urea/formamide gradient in DGGE (Schwieger and Tebbe, 1998). In yet another technique, single strand-conformation-polymorphism (SSCP) is similar to DGGE and TGGE and involves separating PCR amplicons (under non-denaturing conditions) due to the amplicons folding into differing secondary structures which consequently influence their electrophoretic mobility (Schwieger and Tebbe, 1998).

c) Terminal-Restriction Fragment Length Polymorphisms (T-RFLP): this technique involves the PCR amplification of a DNA sequence common to the group of microbes under study from total environmental DNA. The PCR involves the use of a PCR primer tagged with a fluorescent label. The PCR amplicons are then digested by selected restriction endonucleases and the resulting restriction fragments separated by an automated DNA sequencer. Only the terminal-restriction fragments are detected by the automated sequencer (running in GENESCAN mode) due to their fluorescent label and the nucleotide lengths of the terminal-fragments are accurately measured and represented as a peak on the digital output of the machine. Therefore, this approach provides a sensitive and rapid technique for assessing amplification product diversity within a community and comparative distribution against other communities. However, this technique does not identify the microorganisms within the original community, but, terminal-fragment lengths can be tentatively assigned to microbes due to assumed lengths as calculated from ribosomal sequence databases (Marsh, 1999; Liu *et al.*, 1997). Related to T-RFLP is amplified rDNA restriction analysis (ARDRA). ARDRA analysis also involves restriction endonuclease digestion of amplified rDNA from microorganisms in an environment; however, the primers are not fluorescently labelled. The resulting restriction fragments are separated by standard electrophoresis and the digestion patterns are used as a fingerprint of a microbial community, therefore, patterns can be compared between differing samples. However, depending on the enzymes used, profiles are typically complex and it can be difficult to assign a restriction fragment to a particular organism (Liu *et al.*, 1997).

d) Real-time PCR: this method is based on the PCR amplification of a gene of interest from an environmental sample using a fluorescent reporter dye. The function of the reporter dye is to allow the detection and measurement of the amount of PCR product formed by the PCR amplification. From comparison to standard curves, the amount of template (and therefore, the approximate number of microbes) can be estimated in the original sample. Moreover, the specificity of the PCR can be modified by using different primer nucleotide sequences, which target sequences specific for different categories of microorganisms in an environment (Rueckert *et al.*, 2005). The advantage of this technique is its rapid detection time.

e) Intergenic Transcribed Spacer PCR (ITS-PCR): this PCR-based analysis involves the PCR amplification of the region between the 16S-23S rRNA genes within a microorganisms genome, i.e. the internal transcribed spacer (ITS) regions. ITS regions within microbial genomes are hyper-variable, typically discriminating below the species level. Therefore, ITS-PCR from a mixed bacterial community may provide a specific set of PCR amplicons which can be profiled by electrophoresis (Janse *et al.*, 2004). However, ITS-PCR profiles may be complex and typically it is difficult to assign DNA bands from an ITS-PCR profile to organisms in the original environment.

f) Serial Analysis of Ribosomal Sequence Tags (SARST): this method is an emerging microbial ecological tool. It was first described by Neufield *et al.*, (2004) and has been successfully used by Kysela *et al.*, (2005) in a hydrothermal environment. SARST involves the extraction of total DNA from an environment, and the subsequent PCR amplification of a short (~ 40 bp), hyper-variable region of the 16S rRNA gene common to all the microbes under investigation. Through enzymatic manipulations, the amplicons are ligated together with specific terminal sequences linking the amplicons. The ligated sequences are subsequently cloned and sequenced. Therefore, the strength of this technique is that many organisms are identified from a single sequencing reaction, although the short lengths of the amplicons provide limited phylogenetic information (Neufield *et al.*, 2004; Kysela *et al.*, 2005).

g) Meta-genomics: this involves extracting total environmental DNA from an environment, shearing the DNA to an appropriate size and cloning the fragments into a vector and transforming into a bacterial host. The cloned DNA can then be screening using various approaches, i.e. targeting certain genetic markers or random sequencing. A disadvantage of this approach is the time and costly nature of the technique and the large sequencing effort required. The meta-genomic approach is reviewed by Handelsman (2004).

Although, these PCR-based methods have greatly improved our understanding of microbial diversity, there are limitations in these approaches. For example, some studies have shown that there may be differential amplification of 16S rRNA templates from a mixed community, i.e. PCR bias (Reysenbach *et al.*, 1992; Suzuki and Giovanonni, 1996). Another limitation of PCR-based microbial ecology is the formation of recombinant, or chimeric sequences during PCR amplification. This is thought to occur by the annealing of a non-completed amplicon to a foreign DNA sequence which is subsequently copied to completion (Hugenholtz and Huber, 2003). Therefore, the amplicon consists of two distinct partial DNA sequences, which together would be associated to a non-extant organism. This has been shown to occur in mixed bacterial communities by Wang and Wang, (1997) and chimeric 16S rDNA sequences are accumulating in public databases (Hugenholtz and Huber, 2003). However, software has been developed to help detect such chimeric sequences (Maidak *et al.*, 2001). Related to this are possible DNA extraction biases, whereby, DNA is extracted from members of a microbial community in differing efficiencies (LaMontagne *et al.*, 2002). Therefore, the PCR of the resulting environmental DNA template will not be representative of the original community.

Other, non-PCR-based, culture independent methods exist which can enumerate and identify microorganisms in environment samples. DNA stains such as acridine orange and DAPI (4', 6-diamidino-2-phenylindole) bind to DNA and can subsequently be viewed using fluorescence microscopy (Madigan *et al.*, 2000). Therefore, cells can be distinguished from other material in an environmental sample. This approach can be extended by the use of commercially available dyes which can distinguish between viable and non-viable cells, due to the differences of membrane integrity between live and dead cells (Strathmann *et al.*, 2000). Although, these techniques provide total cell numbers in an environment, they do not give the identity of the cells. However, another staining-based technique, namely, fluorescent *in situ* hybridisation (FISH) can provide the identity of a cell (Nercessian *et al.*, 2004). This method is reviewed by Amann *et al.*, (1995) and involves using fluorescently labelled nucleic acid probes that bind to regions of homologous nucleotide sequence within a microorganism's cell, which can be subsequently viewed due to their fluorescent label. Typically, 16S rRNA-targeting oligonucleotides are utilised, and their degree of specificity can be controlled by changing the nucleotide sequence of the probe. Therefore, phylogenetic groups of organisms can be targeted or a single species (Rusch and Amend, 2004). A literature review on the molecular-based microbial ecology of terrestrial geothermal environments is presented in Section 1.2, below.

1.1.3 Microorganisms associated with thermal environments

Various microorganisms have been isolated from diverse thermal environments and have been proven to thrive under these extreme conditions (Stetter, 1998a; Madigan *et al.*, 2000). Microbes adapted to growth at high-temperatures are defined as being either, thermophilic, with optimum growth temperatures above 45°C or hyperthermophilic, defined as having optimum growth temperatures above 80°C (Madigan *et al.*, 2000) and typically cannot grow below approximately 60°C. A diverse range of thermophiles are represented in various phyla of the prokaryotes, as reviewed in detail by Kristjansson, (1991) and Brock, (1986). However, the majority of the work described in this thesis is undertaken on pools with temperatures above

80°C, therefore, thermophiles are not discussed in further detail, with the focus of this section being on hyperthermophiles. Both bacterial and archaeal domains of the prokaryotes contain cultured hyperthermophilic representatives (Stetter, 1996), which represent the deepest rooted branches within the evolutionary tree of life (Figure 1.1). This suggests that the common ancestor of life may have been a hyperthermophile, which if true, means that the emergence of life on Earth began in a hydrothermal setting (Shock, 1996).

Isolated and characterised hyperthermophiles have been reviewed by Stetter, (1996) and a more up to date compilation is represented in the following discussion (Sections 1.1.3.1 and 1.1.3.2). Euzéby provides an updated list of prokaryotic names with standing in nomenclature (http://www.bacterio.cict.fr/index.html), however, microbial taxonomy is a continuous work in progress and no listing will remain complete for long.

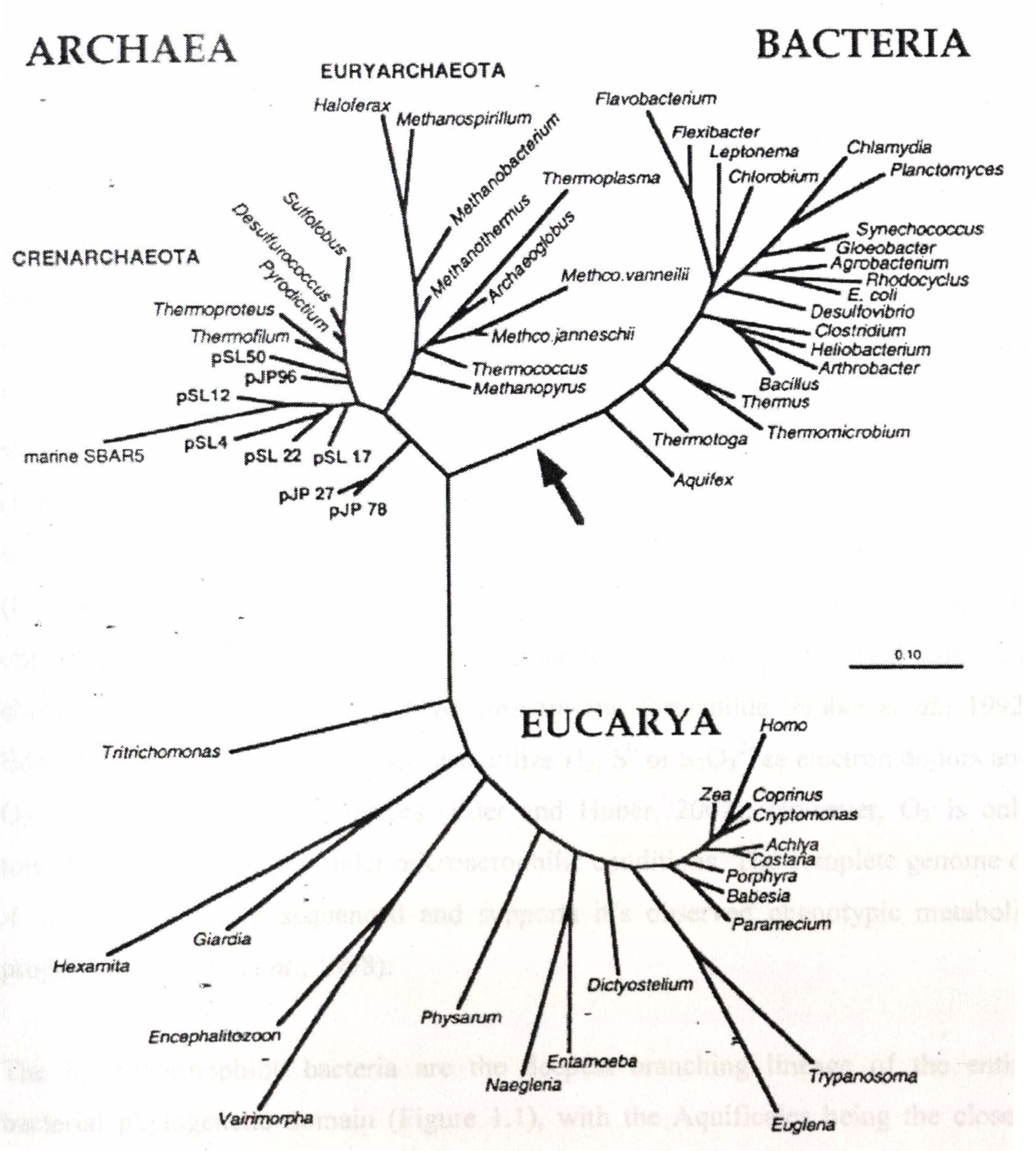


Figure 1.1 Un-rooted phylogenetic tree. The inferred root of the tree is indicated by the arrow. (reproduced from Barns *et al.*, 1996b).

1.1.3.1 Hyperthermophilic bacteria

Only the earliest branching phylogenetic groups of the bacterial domain (see Figure 1.1) contain members that exhibit hyperthermophilic growth. These phylogenetic groups cluster into two assemblages: the Aquifiacae, consisting of the order Aquificales and the Thermotogas (see Figure 1.1), consisting of the orders

Thermotogales and Thermodesulfobacteriales (Madigan *et al.*, 2000; Cole *et al.*, 2005). Each order has between 1 and 6 genera of organisms including hyperthermophilic members as discussed individually for each order below.

The Aquificales (currently containing a total of 5 cultured genera) includes the rod-shaped hyperthermophilic members of the genus *Aquifex* (*A. aeolicus* and *A. pyrophilus*) and genus *Thermocrinis* (*T. ruber* and *T. albus*). *Aquifex* species have been isolated from submarine hydrothermal systems and are the most thermophilic of all bacteria with maximum growth temperatures around 95°C, and optima at ~ 85°C (Eder and Huber, 2002). *Thermocrinis* species have been isolated from terrestrial hot springs and have maximum growth temperatures around 89°C and optima at ~ 80°C (Eder and Huber, 2002). Species of *Aquifex* and *Thermocrinis* are obligately chemolithotrophic and autotrophic neutrophiles, with only *T. ruber* showing chemoorganoheterotrophic growth on formate and formamide (Huber *et al.*, 1992; Eder and Huber, 2002). Theses species utilize H_2, S^0 or $S_2O_3^{2-}$ as electron donors and O_2 or NO_3^- as electron acceptors (Eder and Huber, 2002). However, O_2 is only tolerated by these species under microaerophilic conditions. The complete genome of *A. aeolicus* has been sequenced and supports it's observed phenotypic metabolic properties (Deckert *et al.*, 1998).

The hyperthermophilic bacteria are the deepest branching lineage of the entire bacterial phylogenetic domain (Figure 1.1), with the Aquificales being the closest bacterial group to the evolutionary root. Therefore, both the early evolutionary lineage and autotrophic metabolism (also characteristic to various hyperthermophilic archaea as discussed in Section 1.1.3.2 below) of the Aquificales suggests that H_2 was a key electron donor in early Earth conditions (Madigan *et al.*, 2000). Likewise, H_2-based metabolism is thought to be the major type of metabolism in high-temperature hot springs of Yellowstone National Park, (WY, USA), because, culture-independent microbial ecology studies has revealed a dominance of Aquificales in these habitats (Spear *et al.*, 2005; Blank *et al.*, 2002). This is discussed in further detail in the literature review in Section 1.2, below.

The order Thermotogales consists of five genera and includes the genus *Thermotoga* which contains hyperthermophilic members. The genus *Thermotoga* is currently represented by a total of nine cultured species isolated from diverse thermal habitats including oil producing wells, an anaerobic reactor and solfataric springs with the type species (*T. maritima*) being isolated from geothermally heated marine sediment (Stetter and Huber, 1986). Collectively, these organisms are all neutrophilic, fermentative, chemoorganotrophs, with optimal growth temperatures ranging from 65 to 80°C and maxima at approximately 70 to 90°C. The cell morphology of *Thermotoga* is rod-shaped and typified by having a sheath-like 'toga' (Stetter and Huber, 1986; Madigan *et al.*, 2000). The *Fervidobacterium* genus is also included in the order Thermotogales and although not true hyperthermophiles, two species, namely, *F. nodosum* (Patel *et al.*, 1985) and *F. islandicum* (Huber *et al.*, 1990) have demonstrated growth at a maximum temperature of 80°C. Phenotypically, *Fervidobacterium* species are similar to *Thermotoga* species, as they are anaerobic, fermentative, chemoorganotrophic neutrophiles. However, their morphology is distinct, with cells containing a terminal 'spheroid'.

The order Thermodesulfobacteriales (the second order of the deep branching Thermotoga group), does not contain true hyperthermophiles. The order is represented by 4 species in a single genus, *Thermodesulfobacterium*, which have been isolated from terrestrial geothermally heated hot springs. Collectively, growth temperatures of *Thermodesulfobacterium* species range from approximately 50 to 80°C and all exhibit a metabolism linked to sulfate reduction (Madigan *et al.*, 2000).

1.1.3.2 Hyperthermophilic archaea

The archaeal domain bifurcates into two dominant kingdoms; the Euryarchaeaota and the Crenarchaeaota (see Figure 1.1). However, two new kingdoms of archaea have also been identified, which are close to the theoretical evolutionary root of phylogenetic trees, the Korarchaea (see Figure 1.1) and the Nanoarchaea. All four kingdoms will be discussed individually below, with the focus being on cultured representatives. The metabolism of the archaea are only discussed briefly; an extensive review on the metabolic reactions undertaken by both thermophilic and hyperthermophilic microorganisms is provided by Amend and Shock, (2001). Only the hyperthermophiles relevant to the thesis will be discussed in detail, however, all cultured genera of hyperthermophilic archaea and their corresponding phenotypic characteristics are listed in Table 1.1.

The cultured representatives of the euryarchaeaota are physiological diverse, and include methanogens, halophiles, acidophiles and hyperthermophiles, therefore, most representatives are indigenous to extreme environments (Madigan *et al.*, 2000). The euryarchaeota can be split into phenotypic and phylogenetic groups of organisms including, two major groups of methanogens and halophiles. However, only the methanogens contain cultured hyperthermophilic members. Methanogens produce CH_4 as end product of metabolism, typically using autotrophic, CO_2-/H_2-based metabolism, with only two genera of the over 60 cultured species of methanogens undertaking hyperthermophilic growth, namely, *Methanocaldococcus* (formerly named *Methanococcus*) and *Methanothermus.* Both have been isolated from hydrothermal environments and are included in Table 1.1, below. A total of three other phylogenetic groups make up the euryarchaeota, namely the classes of Thermoplasma, Thermococci and Methanopyri. The cultured members of the Thermoplasma consist of two genera (*Thermoplasma* and *Picrophilus*) which are chemoorganotrophic, thermophilic, extreme acidophiles; however, they are not true hyperthermophiles (Johnson, 1998).

The final two classes of the euryarchaeota (Thermococci and Methanopyri) consist of hyperthermophilic microorganisms. The Thermococci includes two families: Archaeoglobaceae (genera of *Archaeoglobus* and *Ferroglobus*) and the Thermococceae (genera of *Pyrococcus* and *Thermococcus*), whereas, the Methanopyri has only one cultured representative: *Methanopyrus kandleri.* It is a rod-shaped hyperthermophilic methanogen isolated from hydrothermal vent sediments and has one of the shortest branch lengths in the phylogenetic tree of life, suggesting it has evolved the least from the earliest life forms, i.e. its habitat has remained constant since life evolved (Madigan *et al.*, 2000; Kurr *et al.*, 1991). The members of the Archaeoglobaceae family (*Archaeoglobus* and *Ferroglobus* sp.) have only been isolated from hydrothermal marine habitats (Madigan *et al.*, 2000; Stetter, 1996). However, the Thermococceae family contains hyperthermophiles isolated from both marine and terrestrial hydrothermal habitats. *Thermococcus* species are all coccoid, hyperthermophilic, obligate anaerobes and chemoorganotrophs using S^0 as an electron acceptor and have been isolated from both terrestrial and submarine thermal habitats vents (Stetter, 1998a). Cultured *Pyrococcus* species are similar phenotypically to the Thermococci; however, species of *Pyrococcus* have only been isolated from submarine hydrothermal vents (Stetter, 1998a).

The crenarchaeotal kingdom is phylogenetically distinct from the euryarchaeota and all cultured crenarchaeota have been isolated from hydrothermal settings. Therefore, most characterised and published representatives demonstrate hyperthermophilic growth with the exceptions undertaking thermophilic growth (see Table 1.1). However, through culture-independent molecular-based ecological studies, crenarchaeota have been shown to inhabit many colder environments such as soils and plant roots and constitute a major component of the biomass of ocean plankton (Buckley *et al.*, 1998; Simon *et al.*, 2000; Schleper *et al.*, 2005; Karner *et al.*, 2001).

According to 16S rRNA gene analysis and phenotypic characteristics, the cultured representatives of the crenarchaeota group into three orders, the Thermoproteales, Sulfolobales and Desulfurococcales (Burggraf *et al.*, 1997b), as indicated in the 16S rDNA-based phylogenetic tree in Figure 5.3. The cultured genera of each order are described individually below.

The Thermoproteales are rod-shaped organisms that can be differentiated in two groups based on phylogeny, the Thermoproteaceae (genera of *Caldivirga*, *Pyrobaculum*, *Thermocladium*, *Vulcanisaeta* and *Thermoproteus*) and Thermofilaceae (genera of *Thermofilum*). All cultured members are neutrophilic or slightly acidophilic and have been isolated from terrestrial hot spring environments, with only *Pyrobaculum* and *Thermoproteus* species having also been isolated from submarine hydrothermal habitats. Typically, the Thermoproteales are strictly anaerobic, heterotrophic or chemolithoautotrophic reducers of sulfur or thiosulfate (Stetter, 1996; Stetter, 1998a; Itoh *et al.*, 2002). However, both *Caldivirga* and *Thermocladium* can grow under microaerophilic conditions, and some species of *Pyrobaculum* can undertake aerobic respiration (Völkl *et al.*, 1993; Amo *et al.*, 2002; Sako *et al.*, 2001).

The cultured representatives of the Sulfolobales are all coccoid-shaped, hyperthermophilic, sulfur-dependent, acidophiles consisting of the genera, *Acidianus*, *Metallosphaera*, *Sulfurisphaera*, *Stygiolobus* and *Sulfolobus*. *Sulfolobus* species are aerobes, which can grow autotrophically, heterotrophically or facultatively heterotrophically with some species undertaking the oxidation of sulfidic ores (Stetter, 1996). *Metallosphaera* species are phenotypically similar to *Sulfolobus*, but, form a separate group due to phylogenetic differences. In contrast, species of the genera *Sulfurisphaera* and *Acidianus* are facultative anaerobes, with *Sulfurisphaera* undertaking heterotrophic growth using S^0 as an electron donor, and *Acidianus* undertaking S^0 reduction with H_2 (Stetter, 1996). Similarly, *Stygiolobus* grows by S^0 reduction with H_2, but is an obligate anaerobe (Stetter, 1996).

The cultured representatives of the Desulfurococcales are all coccoid to disc-shaped, neutrophilic or weakly acidophilic and strict anaerobes with a fermentative metabolism or anaerobic respiration, with the exception of *Aeropyrum* species, which are strictly aerobic heterotrophs (Nakagawa *et al.*, 2004). Phenotypic and 16S rRNA gene phylogenetic analysis divides the members of the Desulfurococcales into two families, the Pyrodictiaceae and the Desulfurococcaceae. The Pyrodictiaceae form a distinct group (genera of *Pyrodictium*, *Hyperthermus* and *Pyrolobus*) within the 16S rRNA phylogenetic trees, whereas, the Desulfurococcaceae consist of a diverse range of cultured representatives (Burggraf *et al.*, 1997b). These two families can also be distinguished phenotypically by growth temperatures, where the Pyrodictiaceae have optimal temperatures above 100°C, and the Desulfurococcaceae are characterised by maximal growth temperatures up to 100°C.

The Desulfurococcaceae include the genera of, *Aeropyrum*, *Desulfurococcus*, *Ignicoccus*, *Staphylothermus*, *Stetteria*, *Sulfophobococcus*, *Thermodiscus*, *Thermosphaera* and the genera of the 'Acidilobus' group, namely *Acidilobus*, *Caldisphaera* and strain NC12 ("*Caldococcus noboribetus*" which has not been validly characterised or named; Aoshima *et al.*, 1996). The 'Acidilobus' group are a distinct cluster within 16S rRNA gene-based phylogenetic trees and are also different to the other members of the Desulfurococcales, due to their acidic pH growth optima (3.0-4.0), in contrast to the weakly acidophilic/neutrophilic growth optima (pH 5.5-7.5) of the other Desulfurococcales genera (Itoh *et al.*, 2003). The members of the 'Acidilobus' group are heterotrophic, being able to undertake both fermentative and S^0-based heterotrophic metabolism. Likewise, the other genera of the Desulfurococcales are typically heterotrophic sulfur respirers and some species can also ferment complex carbon substrates (Stetter, 1998a). However, exceptions include *Ignicoccus* sp. which are obligate chemolithoautotrophs (H_2 and S^0) and *Stetteria* which is a H_2-dependent mixotroph using S^0 or thiosulfate (Huber *et al.*, 2000c; Jochimsen *et al.*, 1997). Moreover, S^0 inhibits the growth for, *Sulfophobococcus zilligii* (Hensel *et al.*, 1997), *Aeropyrum camini* (Nakagawa *et al.*, 2004) and *Thermosphaera aggregans* (Huber *et al.*, 1998).

The cultured species of the Pyrodictiaceae (genera of *Pyrodictium*, *Hyperthermus* and *Pyrolobus*) have all been isolated from marine hydrothermal environments and have growth temperature optima above 100°C. In fact, *Pyrolobus fumarii* held the record of the highest growth temperature of any organism at 113°C (Blöchl *et al.*, 1997). However, in 2003, a coccus-shaped microorganism, (strain 121), was isolated from a hydrothermal vent that grew at temperatures between 85 and 121°C using Fe^{3+} as an electron acceptor (Kashefi and Lovley, 2003). Strain 121 was most closely related to *Pyrodictium occultum*, however, strain 121 has not yet been fully characterised. *Pyrodictium* species are sulfur dependent chemolithoautotrophs or heterotrophs (Stetter, 1998a). In contrast, *Hyperthermus* is a strictly heterotrophic consumer undertaking fermentation or sulfur-based respiration (Stetter, 1996) and *Pyrolobus* is a chemolithoautotroph whose growth is inhibited by elemental sulfur (Blöchl *et al.*, 1997).

Table 1.1 Validly published hyperthermophilic archaea.

King-dom	Phenotypic group/Order	Genus	Type species (No. of species)	^{A}M/T	^{B}T. opt (°C) (T. range.)	^{B}pH opt. (pH range)	Morph. (all sp.)	Reference
Eury.	Methanogen assemblage	*Methanocaldococcus*	*M. jannaschii* (5)	M	85 (50-86)	6.0 (5.2-7.0)	Cocci	Jones *et al.*, (1983)
		Methanothermus	*M. fervidus* (2)	M	83 (65-97)	6.5 (4.0-7.0)	Rods	Stetter *et al.*, (1982)
	Archaeoglobales	*Archaeoglobus*	*A. fulgidus* (3)	M	83 (60-95)	(5.5-7.5)	Irregular cocci	Stetter, (1998b)
		Ferroglobus	*F. placidus* (1)	M	85 (65-95)	7.0 (6.0-8.5)	Cocci	Hafenbradl *et al.*, (1996)
	Thermococcales	*Thermococcus*	*T. celer* (24)	M/T	87 (75-93)	(5.7-7.2)	Cocci	Zillig *et al.*, (1983b)
		Pyrococcus	*P. furiosus* (4)	M	100 (70-105)	7.0 (5.0-9.0)	Cocci	Fiala and Stetter, (1986)
	Methanopyrales	*Methanopyrus*	*M. kandleri* (1)	M	98 (84-110)	6.5 (5.5-7.0)	Rods in chains	Kurr *et al.*, (1991)
Cren.	Thermoproteales	*Caldivirga*	*C. maquilingensis* (1)	T	85 (75-90)	3.7-4.2 (2.7-4.2)	Rods	Itoh *et al.*, (1999)
		Pyrobaculum	*P. islandicum* (5)	M/T	100 (74-103)	6.0 (5.0-7.0)	Rods	Huber *et al.*, (1987)
		Thermocladium	*T. modesties* (1)	T	75 (45-82)	4.0 (2.6-5.9)	Rods	Itoh *et al.*, (1998b)
		Thermofilum	*T. pendens* (2)	T	88 (70-95)	5.0-6.0 (2.8-6.7)	Slender rods	Zillig *et al.*, (1983a)
		Thermoproteus	*T. tenax* (3)	M/T	88 (70-97)	5.0 (2.5-6.0)	Rods	Zillig *et al.*, (1982)
		Vulcanisaeta	*V. distributa* (2)	T	85-90 (70-92)	4.5-5.0 (3.5-5.6)	Rods	Itoh *et al.*, (2002)
	Sulfolobales	*Acidianus*	*A. infernos* (3)	T	90 (65-96)	2.0 (1.0.5-5)	Lobed cocci	Segerer *et al.*, (1986)
		Metallosphaera	*M. sedula* (3)	T	75 (50-80)	2.0 (1.0-4.0)	Cocci	Huber *et al.*, (1989)
		Stygiolobus	*S. azoricus* (1)	T	80 (57-89)	2.5-3.0 (1.0-5.5)	Lobed cocci	Segerer *et al.*, (1991)
		Sulfolobus	*S. acidocaldarius* (8)	T	70 (55-85)	2.5 (2.0-4.0)	Lobed cocci	Jan *et al.*, (1999)
		Sulfurisphaera	*S. ohwakuensis* (1)	T	84 (63-92)	2.0 (1.0-5.0)	Cocci	Kurosawa *et al.*, (1998)
	Desulfurococcales	*Aeropyrum*	*A. pernix* (2)	M	90-95 (70-100)	7.0 (5.0-9.0)	Cocci	Sako *et al.*, (1996)
		Desulfurococcus	*D. mucosus* (4)	T	85 (55-97)	6.0 (4.5-7.0)	Cocci	Zillig *et al.*, (1983c)
		Ignicoccus	*I. islandicus* (2)	M	90 (70-98)	5.8 (3.8-6.5)	Cocci	Huber *et al.*, (2000a)
		Staphylothermus	*S. marinus* (2)	M	92 (65-98)	6.5 (4.5-8.5)	Cocci in aggregates	Fiala *et al.*, (1986)
		Stetteria	*S. hydrogenophila* (1)	M	95 (70-102)	6.0 (4.5-7.0)	Disc/cocci	Jochimsen *et al.*, (1997)
		Sulfophobococcus	*S. zilligii* (1)	T	85 (70-95)	7.5 (6.5-8.5)	Cocci	Hensel *et al.*, (1997)
		Thermodiscus	*T. maritimus* (1)	M	88 (75-98)	6.5 (5.0-7.0)	Disc	Stetter, (2001)
		Thermosphaera	*T. aggregans* (1)	T	85 (65-90)	6.5 (5.0-7.0)	Cocci in aggregates	Huber *et al.*, (1998)
		Acidilobus	*A. aceticus* (1)	T	85 (60-92)	3.8 (2.0-6.0)	Cocci	Prokofeva *et al.*, (2000)
		Caldisphaera	*C. lagunensis* (1)	T	70-75 (45-80)	3.5-4.0 (2.3-5.4)	Cocci	Itoh *et al.*, (2003)
		Pyrolobus	*P. fumarii* (1)	M	106 (90-113)	5.5 (4.0-6.5)	Cocci	Blöchl *et al.*, (1997)
		Pyrodictium	*P. occultum* (3)	M	105 (82-110)	5.5 (5.0-7.0)	Disc + fibres	Stetter *et al.*, (1983)
		Hyperthermus	*H. butylicus* (1)	M	75-108 (85-107)	7.0	Irregular cocci	Zillig *et al.*, (1990)

^{A}Habitat: marine (M) or terrestrial (T) from which all representatives of each genus were originally isolated. ^{B}Phenotypic characteristics of type species only.

The korarchaeota were originally detected in Obsidian Pool, Yellowstone National Park (Barns *et al.*, 1996a) by 16S rRNA gene cloning and were suggested to be a novel kingdom of the archaea and the deepest branching of all archaea, and therefore, may possess novel biological processes characteristic of ancient microbes. Since this discovery, a continuous laboratory culture (85°C) has been demonstrated to contain korarchaeotal cells by 16S rDNA probing (Burggraf *et al.*, 1997a). Korarchaea have also been detected by other molecular-based 16S rRNA gene studies in terrestrial hot springs of Iceland (Hjorleifsdottir *et al.*, 2001) and Yellowstone National Park, Calcite Spring, (Reysenbach *et al.*, 2000a) and marine hydrothermal environments by Nercessian *et al.*, (2003) and Marteinsson *et al.*, (2001). However, still, much is unknown about the korarchaeota's phenotypic properties.

The nanoarchaeota are a recent addition to the archaeal domain. Small (400 nm in diameter) cocci were identified to be growing attached to 'host' *Ignicoccus* cells by Huber *et al.*, (2002) that were originally isolated from submarine hydrothermal systems. The 'small' cocci were identified as separate cells by molecular probes and the 16S rRNA gene was sequenced from the 'small' cocci and placed the cells within a new phylum and the isolate was named *Nanoarchaeum equitans*. Following these studies the genome of *Nanoarchaeum equitans* has been sequenced (Waters *et al.*, 2003) and its lipids characterised (Jahn *et al.*, 2004) which has revealed that *Nanoarchaeum equitans* has the smallest genome of any organism sequenced apart from viruses (~ 480,000 bp) and lacks the genes encoding for enzymes involved in lipid, cofactor, nucleic or amino acid biosynthesis. Therefore, it was concluded that *Nanoarchaeum equitans* is completely dependent on its host and may in fact be parasitic towards its *Ignicoccus* host and constitute a novel kingdom. Further 16S rRNA gene-based ecological studies have shown that nanoarchaea are present in other submarine hydrothermal (70-98°C) environments and terrestrial locations such as Yellowstone National Park and the Uzon Caldera, Kamchatka (Hohn *et al.*, 2002). A study by McCliment *et al.*, (in press) showed that nanoarchaeal signatures were the first to be detected colonising nascent developing submarine hydrothermal chimneys. However, the phylogenetic placement of nanoarchaea is still uncertain. For example, a current phylogenetic study has concluded that the nanoarchaea may in fact constitute a fast evolving lineage of the euryarchaotea (closely related to Thermococci) rather than a separate novel kingdom (Brochier *et al.*, 2005).

1.2 The molecular-based microbial ecology of terrestrial hot springs

Culture-independent, molecular-based analyses into the microbial ecology of terrestrial geothermal environments emerged in the early 1990s, with most of the work being focused on hot pools situated in Yellowstone National Park (YNP). One of the first studies was undertaken by Ward *et al.*, (1990) which described and successfully utilised a 16S rRNA cloning approach to identify an uncultured bacterial signature of a cyanobacterial mat (50-55°C) obtained from Octopus Spring, YNP. The signature was only distantly related to cultured microorganisms, and therefore, it was concluded that continued molecular-based analysis of natural communities would lead to the discovery of many more novel uncultured microorganisms. Subsequent 16S rDNA-based investigations of many samples from both YNP and hot pools from other regions have confirmed this prediction. For example, a similar study to that of Ward *et al.*, (1990) revealed a diverse range of novel uncultured phototrophic microbes such as cyanobacteria and *Chloroflexus*-like bacteria present in microbial mats from YNP hot springs (including Octopus Spring) over similar temperature (48-65°C) and pH (6.2-9.1) ranges (Ruff-Roberts *et al.*, 1994).

In addition, the emergence of DGGE as a useful molecular tool for the ecological study of microbes in natural environments quickly led to its application in thermal environments. As was the focus for initial studies of thermal environments, samples of microbial mats of up to 70°C and near-neutral pH were typically used for DGGE analysis. For example, Ferris *et al.*, (1996a) used DGGE fingerprinting of both environmental samples and culture enrichments from Octopus Spring (48-72°C) and showed discrepancies between the microbes detected between molecular- and culturing- approaches. Similarly, culturing bias was observed using DGGE-based analysis of cultures and environmental samples (~50°C) obtained from Octopus

Spring cyanobacterial mats by Santegods *et al.*, (1996). DGGE was thus thought to provide a less biased profile of microbial communities and subsequent studies were undertaken on purely environmental samples. For example, both Ferris *et al.* (1997) and Ferris and Ward, (1997) used DGGE to characterise cyanobacterial mats of Octopus Spring, 58-62°C and 53-75°C, respectively. Likewise, investigation into the community structure of microbial mats of several other near-neutral hot pools of similar temperature (~ 35-70°C) in YNP were undertaken by Van der Meer *et al.*, (2000), Boomer *et al.*, (2002) and Nübel *et al.*, (2002). Collectively, these and the earlier studies revealed that microbial mats from hot pools in YNP at temperatures below ~ 65°C and pH 6-9 are dominated by thermophilic, phototrophic cyanobacteria (typically *Synechococcus* sp.) and green-sulfur or -non-sulfur bacteria (typically *Chloroflexus* sp.).

A pioneering culture-independent, molecular-based (16S rDNA cloning) study of the microbial community from the sediment of a higher temperature pool (93°C at source), 'Jims Black Pool' (now named Obsidian Pool) of YNP revealed a large diversity of novel uncultured microbial signatures (Barns *et al.*, 1994). However, in this study PCR primers were developed to detect both archaea and eukaryotes. Eukaryotes were not detected by PCR and only archaeal amplicons were obtained. Members of both the crenarchaeaota and euryarchaeota were detected with the majority of the signatures being distantly related to cultured crenarchaeota (members of all three orders). Further phylogenetic analysis of the 16S rDNA sequences was undertaken and two deep-rooting signatures (clones pJP78 and pJP27) were subsequently recognised to be members of a proposed novel archaeal kingdom, the korarchaeota (Barns *et al.*, 1996a). A similar study was undertaken on sediment from Obsidian Pool (75-95°C) by Hugenholtz *et al.*, (1998), however, in this study bacteria were also targeted by employing bacterial-specific PCR primers. In direct contrast to the results obtained by Barns *et al.*, (1994) archaea were not detected by universal primers and the clone library contained a rich diversity of bacterial signatures with 54 distinct bacterial sequence types/clusters which were dominated by Aquificales-related signatures (27% of 312 clones).

Other near-neutral, high-temperature hot pools of Yellowstone National Park have proven to be dominated by Aquificales-related bacteria. For example, Reysenbach *et al.*, (1994) produced a 16S rDNA clone library from the hyperthermophilic pink filament community (84-88°C) of Octopus Spring outflow and concluded it was dominated by three phylotypes, EM3 (Thermotogales-related sp.), EM17 (Aquificales-related sp.) and EM19 (a deep-rooting bacterial signature). Archaea were not detected by universal prokaryotic PCR primers and *in situ* hybridisation with nucleotide probes attributed the Aquificales, EM17 phylotype to the pink filaments. An analogous study of the black filamentous community of Calcite Spring (83°C, pH 7.6) in Yellowstone National Park was also undertaken by Reysenbach *et al.*, (2000a). A total of five phylotypes were identified by 16S rDNA cloning consisting of a single bacterial and four archaeal phylotypes. Three of the archaeal phylotypes grouped within the creanarchaeota and a single archaeal phylotype was closely related to the korarchaeal clone, pJP27 obtained by Barns *et al.*, (1996a). The bacterial signature was related to the Aquificales clade and probes specific to this clone were used to prove that this phylotype dominated (>95%) the *in situ* black filamentous community.

Similarly, Blank *et al.*, (2002) investigated the microbial communities associated with surfaces, including geyserite, sediment and incubated glass slides, from seven slightly alkaline (7.9-8.9), high-temperature (70-98°C) hot pools of YNP. Archaea were not detected in any of the samples by PCR, although lipid analysis showed the presence of archaeal lipids in some pools. The surfaces from all pools were dominated by both *Aquificales*- (specificially the Thermocrinis clade) and *Thermotogales*-related (closest relative being clone EM3; Reysenbach *et al.*, 1994) signatures. Also, a recent comprehensive 16S rDNA cloning study by Spear *et al.*, (2005) of five YNP pools (63-100°C) has shown that all pools were dominated by members of the Aquificales clade. A total of ~ 2,500 clones were screened by restriction endonucleases and ~ 400 sequenced. Collectively, Aquificales-related signatures typically made up >63% of the bacterial signatures and crenarchaeotal-related members dominated archaeal libraries; however, euryarchaeota and korarchaeota were also detected. Due to the observed Aquificales dominance by this and other studies, Spear *et al.*, (2005) concluded that the primary mode of metabolism in high-temperature, near-neutral ecosystems of YNP is the oxidation of H_2.

Microbial communities in pools of high temperature and near-neutral pH in Iceland and Japan also show Aquificales dominance. Skirnisdottir *et al.*, (2000) produced a 16S rDNA clone library from a sulfur mat from an Icelandic hot spring (60-80°C, pH 6.7). Both archaeal and bacterial signatures were detected, with Aquificales-related clones making up 69% of bacterial phylotypes (171 clones), and korarchaeota (77% of 145 clones) dominating the archaeal library. Hjorleifsdottir *et al.*, (2001) also detected bacterial and archaeal signatures in a pink-grayish filament community from an 85-88°C, pH 6.9 Icelandic hot pool. An Aquificales phylotype dominated the bacterial clones (87% of 68 clones) and members of the crenarchaeota (*Pyrobaculum* sp. dominance) and korarchaeota were present in the archaeal library. Similarly, Takacs *et al.*, (2001) has shown that the blue filamentous community (79-83°C, pH 8.8) of an Icelandic spring consists of a mono-culture of an organism with the closest relatives being both the Aquificales clones of SRI48, from the study by Skirnisdottir *et al.*, (2000) and EM17, from the study of the white filamentous community by Reysenbach *et al.*, (1994). Likewise, two neutral-to-slightly alkaline hot pools at 52-75°C and 72-80°C located in Japan have been shown to be dominated by Aquificales-related signatures by 16S rDNA-based DGGE, cloning and *in situ* hybridisation approaches (Yamamoto *et al.*, 1998; Nakagawa and Fukui, 2003).

Investigation into the microbial communities of high-temperature (approximately >80°C) acidic hot pools (pH <5.0) has also been described by a number of other studies. A survey of the microbial communities in thermal habitats on the Caribbean Island of Monsterrat, included two pools of high temperature and acidic pH; 78°C, pH 1.5 and 98°C, pH 3.0 (Burton and Norris, 2000; Atkinson *et al.*, 2000). Both archaea and bacteria were detected in the 78°C pool, with *Moorella* sp. dominating the bacterial signatures and *Ferroplasma* sp. dominating the archaeal clones. Only archaea were detected in the higher temperature pool (98°C) with 100% of the clones closely related to *Acidianus infernus*. Similarly, signatures closely related to Acidianus dominated an archaeal clone library from a high temperature (80°C) acidic (pH 3.0) hot pool located in a solfataric field in Naples, Italy (Kvist *et al.*, 2005). However, unexpectedly, a high abundance (42% of 201 clones) of non-thermophilic crenarchaeota was also detected and other members of hyperthermophilic, acidophilic crenarchaeota, such as the Sulfolobales were not detected. In contrast, an archaeal clone library from an acidic (pH 2.8) hot spring (84-93°C), located in Japan, was dominated by hyperthermophilic, acidophilic crenarchaeota (80% of 60 clones were *Sulfurisphaera* sp.) and both korarchaeota (related to pJP27; Barns *et al.*, 1996a) and euryarchaeota (Thermoplasmales-related) were present in low abundance in the archaeal library (Takai and Sako, 1999).

The microbial communities of lower-temperature (<70°C) acidic pools have also been described. The microbial diversity of an acidic (~ pH 2.5) hydrothermal water stream situated on the volcanically active White Island, New Zealand, was investigated by Donachie *et al.*, (2002). The temperature of the stream was not stated, however, enrichments were incubated at up to 60°C. Only bacteria were detected by 16S rDNA cloning, which revealed a diverse range of bacterial phylotypes residing in the acidic waters including, α- and β-Proteobacteria and green-sulfur bacteria. Similarly, a microbial community of an algal mat from an acidic stream (pH ~2.7, 46-48°C) located in YNP was documented by Ferris *et al.*, (2003). DGGE analysis indicated five major DGGE bands associated with the community. Each band was subsequently sequenced and revealed the presence of species involved in dissimilative sulfate or sulfur reduction including *Hydrogenobacter* and *Desulphurella*. Jackson *et al.*, (2001b) detected similar species of bacteria by DGGE and cloning analysis of another acidic (pH 3.1) hot pool (58-62°C) of YNP. Furthermore, the presence of archaea were also investigated, revealing six archaeal phylotypes, with five phylotypes being closely related to uncultured crenarchaeota clones from both marine and terrestrial habitats (Takai and Sako, 1999) and a single phylotype grouped within the acidophilic Thermoplasmales phylogenetic clade.

As discussed previously, Donachie *et al.*, (2002) reported on the molecular-based, microbial community of a thermal stream on White Island, however, there are few reported investigations into the microbial ecology of hot pools in New Zealand, with the majority of the work being culture-based (Patel *et al.*, 1985; Patel *et al.*, 1987; Hudson *et al.*, 1987; Slobodkin *et al.*, 1997; Hudson *et al.*, 1989; González *et al.*, 1999; Miroshnichenko *et al.*, 1994; Rainey *et al.*, 1994). There are various reports in the literature on silicification development and growth of sinters within New Zealand's thermal environments (Jones and Renaut, 1996; Jones *et al.*, 1999; Jones *et al.*, 2004; Mountain *et al.*, 2003). However, Mountain *et al.*, (2003) also described culturing attempts from water collected from a waste water drain of a geothermal power station, 49-62°C and pH 8.5. A high diversity of bacteria were enriched for which were identified to be species of *Thermus*, *Bacillus*, *Meiothermus*, *Tepidomonas*, *Thermomonas*, *Porphyrobacter*, *Thermonema*, *Hydrogenophilus* and other uncultured bacteria. In addition, archaeal lipids were detected in silica forming on glass slides incubated in the waste water stream. Similarly, Pancost *et al.*, (2005) used lipid analysis of sinters from four New Zealand hot springs (58-82°C) to provide an insight into the diversity of both bacteria and archaea present in silica sinters. New Zealand hot pools have also been included in a study investigating the relatedness between geographical distinct microbial populations of hot spring communities. Papke *et al.*, (2003) used ITS and 16S rDNA PCR-based methods to investigate the genetic diversity of cyanobacterial populations of New Zealand, North American, Japanese and Italian hot pools. It was concluded that geographical isolation rather than the chemical parameters of the pools was responsible for the evolutionary distances been populations. Furthermore, Whitaker *et al.*, (2003) concluded that barriers to dispersal drove genetic divergence between populations of *Sulfolobus* species from hot pools of Kamchatka, North America and Iceland.

Finally, an emerging area of molecular-based analysis of thermal environments is in the area of virus populations of hot pools. Breitbart *et al.*, (2004) has described the first study of phage community dynamics in Californian near-neutral hot springs (73-84°C) and showed that phage-mediated microbial mortality was significant and is an important feature of microbial food webs in thermal habitats.

1.3 Aims, approach and outline of Thesis

As discussed in the literature review, molecular-based analyses into the microbial ecology of hot springs have been undertaken on various geothermal sites with most of the focus being directed at Yellowstone National Park, USA (Barns *et al.*, 1994; Barns *et al.*, 1996a; Hugenholtz *et al.*, 1998; Reysenbach *et al.*, 2000a; Spear *et al.*, 2005; Reysenbach *et al.*, 1994), Iceland (Takacs *et al.*, 2001; Marteinsson *et al.*, 2001; Hjorleifsdottir *et al.*, 2001; Skirinsdottir *et al.*, 2000) and Japan (Nakagawa and Fukui, 2003; Takai and Sako, 1999; Yamamoto *et al.*, 1998). In contrast, reports of molecular-based analysis of terrestrial New Zealand geothermal sites are scarce in the literature with culture-based analysis being more common. Therefore, one of the central aims of the research was to assess the microbial ecology of selected high temperature terrestrial environments in New Zealand using a molecular-based approach, complemented by traditional culture-based methodologies. This would provide a better understanding of the microbial ecology of New Zealand hot pools and allow comparison with similar environments in other thermal regions of the world.

Molecular-based analyses may give significance to many species which are present in an environment in low numbers and may not be metabolically active, and therefore relatively unimportant. However, in hot pools with a run-off of pool water to cooler locations the growth of thermophilic bacteria will be prevented, but by adsorbing to inert particles or detritus in the pool environment the bacteria can remain in an environment, thus supporting growth (Wimpenny *et al.*, 2000). Based on these concepts, the ecological study of the environments was undertaken incorporating the use of surfaces (glass microscope slides), facilitating microbial colonisation and consequent analysis of biofilms developing on the slides in the hot pool habitats. Moreover, many hot springs are supersaturated in silicates, and as these precipitate on cooling, they provide an ideal surface for bacterial attachment (Brock, 1978). Therefore, glass microscope slides present an analogous surface with the added benefit that colonising bacteria can be observed by either phase-contrast or scanning electron microscopy (Mountain *et al.*, 2003).

The merit of this approach is that colonisation requires organisms to be metabolically active, therefore, dead or dormant cells will be avoided, as will those adapted to rapid growth in artificial culture media – the so called 'endemic weed species'. Thus, the major objective of the study was to target the metabolically active cells of the hot pools by applying a suite of molecular and microscopical techniques to the developing biofilm on the *in situ* incubated surfaces. Rather than asking the question"who's there", which is the result of the grab sample approach, the investigation of biofilms seeks to determine "who's important and active".

The approach of this study was to place glass slides in hot pools for varying periods of time, essentially until the slide itself is covered with a silicate deposit curtailing observation. The numbers and rate of colonisation were to be microscopically catalogued by phase-contrast microscopy and the use of fluorescent dyes such as DAPI (Madigan *et al.*, 2000) allowing cells to be observed under fluorescence. Also, the biomass on the incubated slides was to be estimated by ATP assay (Atkinson *et al.*, 2000). The use of ATP assays not only provides an estimate of biomass but also an indication of metabolically active cells colonising the surface, since any ATP in a cell is quickly hydrolysed following death. Additionally, colonising bacteria would be scraped off incubated slides and used for DNA extraction for 16S rRNA gene-based, DGGE analysis and sequencing of bands within DGGE profiles. Theoretically, these latter approaches would allow the diversity present on the slides to be assessed, which may correlate with the different morphologies observed microscopically. In addition, the sequencing of 16S rDNA signatures on colonised slides would identify the colonising microbes, and media could then be developed in attempt to enrich and isolate these microbes. Moreover, the microbes involved in colonisation would be compared to the microbes residing in the pool water, with the molecular-based analyses complemented with traditional microbiological methods of selective culturing and microscopy.

Initially, three New Zealand hot pools were selected for the study of biofilm development on incubated surfaces and the development of methodology. These results form the basis of Chapter 3. These techniques were then applied to a colonisation survey in a number of New Zealand high-temperature hot pools (including White Island), with comparison to an analogous survey undertaken on hot pools in Yellowstone National Park, (WY, USA) as outlined in Chapter 4. From the initial study described in Chapter 3, a novel coccus-shaped archaea was isolated into pure culture and fully characterised as described in Chapter 5. Finally, an invitation to join the Extreme 2004 expedition to the East Pacific Rise (EPR) led to the study of samples from hydrothermal vents as discussed in Chapter 6. A final conclusion in Chapter 7 outlines the overall significance and future considerations of the work.

Chapter 2 : Materials and Methods

2.1 Analysis of geothermal environments

2.1.1 Access to geothermal environments and samples

Permission to access and sample the geothermal hot pools in Rotorua was obtained from Mr. Peter Brownridge, the Geothermal Inspector of Rotorua District Council. Access to Champagne Pool, Waiotapu, and Tokaanu Thermal Park was possible due to collaboration with Dr. Bruce Mountain, Institute of Geological and Nuclear Sciences (IGNS), Wairakei Research Centre, Taupo, New Zealand. Sampling of hot pools in Yellowstone National Park, WY, USA was undertaken in July 2004 on Permit # 5434 under the supervision of Prof. Everett Shock and Dr. Melanie Holland with the Arizona State University, Phoenix, AZ, USA. Samples from hydrothermal vents situated on the East Pacific Rise were collected using the *Alvin* submersible on the Extreme 2004 voyage on the *Atlantis* (Woods Hole Oceanographic Institute, Woods Hole, MA, USA) by invitation from Prof. Craig Cary (University of Waikato, Hamilton, NZ and University of Delaware, DE, USA). Samples from White Island, New Zealand were collected by Prof. Hugh Morgan (University of Waikato, Hamilton, NZ).

2.1.2 Global positions and images of hot springs.

Images of New Zealand and Yellowstone National Park (WY, USA) hot springs involved in the thesis are presented in Appendices A and B, respectively. The global satellite positions of the New Zealand hot pools are presented in Appendix A.

2.1.3 Incubation of surfaces within hot pools

Glass, Teflon and stainless steel slides were immersed in toluene for 5 minutes, the excess toluene drained and slides rinsed in 70% ethanol. Both surfaces of the slide were then wiped with tissue paper and rinsed in Milli-Q water. The slides were autoclaved and kept dry and sterile prior to *in situ* incubation in a hot pool. Slides were immersed in hot pools by the use of either: plastic slide holders or glass staining racks. The plastic slide holders were suspended in the water from a convenient anchor point using plastic-coated wire and had the base removed facilitating water flow across the surface of the slide. Glass slide staining racks were used when immersion onto the sediment surface of the pool was required. On some occasions, slides were incubated within the sediment layer by directly inserting slides into the sediment of a hot spring.

2.1.4 Adenosine triphosphate (ATP) assay

Determination of ATP concentration was used as an estimate of the amount of biomass developing on slides that had been incubated in hot pools. ATP levels were estimated by a reaction involving the luciferase catalysis of luciferin and ATP to produce light. The light released can be measured as relative light units (RLU) and is directly related to the amount of ATP (and indirectly, biomass) present in the sample. Slides were removed from the hot pool, rinsed in Milli-Q water or 0.9% saline solution in a Coplin jar and the biofilm of a slide assayed by swabbing the surface with an ATP swab (Clean-Trace, Rapid Cleanliness Test, Biotrace, Brigend, U.K.). Swabs are impregnated with a lytic agent which releases ATP from intact cells. The swab was then gently mixed for approximately 30 seconds in the swab buffer and the RLU determined on a portable ATP meter (Unilite direct ATP testing system, Biotrace, Bridgend, U.K.). Variations between ATP readings were checked by taking subsequent readings (typically twice) on a swab following the initial 30 second reading. Background ATP levels were determined by dispensing a standard amount of pool water directly onto an ATP swab and determining the RLU as described above.

2.1.5 Protein determination

Protein determination was carried out on cleaned (10% hydrochloric acid) glass cover slips incubated upright in the hot pools by the use of bulldog clips. Following, *in situ* incubation, the cover slips were removed, dried and placed in cleaned (10% hydrochloric acid) universal bottles. In the laboratory the cover slips were crushed (using a sterile pipette tip) and 1 ml of Milli-Q water dispensed into the universal. Protein on the cover slip was solubilised by autoclaving for 15 minutes at 121°C. Duplicate aliquots of the water were quantified for protein using a micro-assay Bradford test (Dunn, 1989) using standards of up to and including 20 µl of 1 mg/ml bovine serum albumin (BSA).

2.1.6 Microscopy of colonised slides

2.1.6.1 Phase-contrast microscopy

Slides were removed from the hot pool and transported back to the laboratory immersed in pool water. At the laboratory, slides were rinsed in sterile Milli-Q water or 0.9% saline solution and viewed using a 100X phase-contrast objective lens on an Olympus BH-2 microscope (Olympus, Japan). Images were recorded using an Olympus SC35, type 12 camera (Olympus, BH-2, Japan). Colonisation on slides incubated in hot pools of Yellowstone National Park was documented by differential interference contrast (DIC) using a 543 nm neon laser, using a 63X oil objective (Nikon FXA microscope, Tokyo, Japan) and as described above.

2.1.6.2 DAPI (4', 6-diamidino-2-phenylindole) staining

DAPI selectively binds to DNA and can be observed due to its fluorescence under UV light. Slides were removed from the hot pool and transported back to the laboratory immersed in pool water. Slides were then rinsed in Milli-Q water or 0.9% saline solution and fixed in 3.7% formaldehyde for approximately 3 to 4 hours followed by another 0.9% saline rinse step. The slides were then dehydrated for 3 minutes each in successive 50, 80 and 95% ethanol washes. The slides were then stored at room temperature until staining. Approximately 0.5 ml of DAPI staining solution (1 mg/ml DAPI, 1% glutaraldehyde diluted in Milli-Q water) was dispensed onto the slide surface. After 5 minutes of staining the slide was rinsed in Milli-Q water and subsequently by 70% ethanol by gentle pipetting to remove non-specific staining. The slides were viewed using a Leica DMR research microscope (Leica Corporation, Germany) using a Leica filter, block A, with a 100X magnification (excitation filter, BP 340-380 nm; dichromic mirror, RKP 400 nm; suppression filter, LP 425 nm). Digital images were taken using an AxioCam HRc (Carl Zeiss AG., Oberkochen, Germany).

2.1.7 Analysis of hot pool water

2.1.7.1 Temperature, pH and chemical composition

The *in situ* temperature and pH of the New Zealand pools were measured using a mercury thermometer and pH strips (Merck KGaA, Darmstadt, Germany). Pool water from sample sites was also taken back to the laboratory and the pH determined using a pH meter (Jenway 4330, Essex, England). Yellowstone National Park hot pool sites were measured for temperature and pH using a digital temperature probe and pH strips (Merck KGaA, Darmstadt, Germany). Pool chemistry on water samples was determined at the Institute of Geological and Nuclear Sciences (IGNS), Wairakei Research Centre, Taupo, New Zealand.

2.1.7.2 Carbon content

Pool water was collected in acid cleaned (10% hydrochloric acid) glassware and filtered through a pre-combusted GFC filter (6 hours at 450°C). The dissolved organic carbon (DOC) content of the filtrate was then determined at the National Institute of Water and Atmospheric Research (NIWA), Hamilton, New Zealand. The pre-combusted filter was weighed prior to filtering and following filtering it was heated at 100°C for 6 hours then weighed again, where the difference in weight gives the approximate amount of suspended solids (SS) or organic and inorganic carbon for the amount of water filtered. The filter was then combusted again (480°C for 6 hours) and the difference in weight (from the SS weight) gives the amount of particulate organic matter (POM) in the volume of filtered pool water.

2.1.7.3 Most probable numbers (MPN) testing

Pool water and/or sediment samples were collected in sterile tubes with any air-space minimised and transported back to the laboratory at room temperature. The samples were inoculated into triplicate dilution series of appropriate media ranging from 10^{-1} to 10^{-5}. After incubation at the designated temperature for at least three days growth was assessed by turbidity and confirmed by microscopic observation. Most probable numbers were derived using MPN tables (http://www.jlindquist.net/generalmicro/102dil3.html). MPN were also undertaken on biofilm removed from glass slide surfaces. A slide was rinsed in 0.9% saline then the biofilm was removed from the slide using a sterile razor blade into a weighing dish containing 1 ml of 0.9% saline and used to produce serial dilutions in 0.9% saline for the inoculation of appropriate media.

2.2 DNA analysis

2.2.1 DNA extraction

The sampling and DNA extraction methods used are briefly described below, with detailed descriptions of each method provided in the respective section below.

1. Biofilm: two chemical/heat-based methods, namely guanidine isothiocyanate (GIT), see Section 2.2.1.1 and CTAB (hexadecyltrimethylammonium bromide), see

Section 2.2.1.2 were tested for DNA extraction from biofilm removed from colonised glass slide samples. Both methods gave similar DNA extraction efficiencies from biofilm; however, the CTAB method was preferred as it was a less costly procedure. Bio-film that had developed on microscope slides incubated in hot pools (*in situ*) was aseptically scraped from the slide using a sterile scalpel blade into CTAB extraction buffer.

2. Pool water: pool water was filtered through a 0.22 or 0.45 μm membrane filter. The filter was then placed in CTAB extraction buffer for DNA extraction, see Section 2.2.1.2.

3. Pool sediment: a CTAB method was used initially (see Section 2.2.1.3), however, this gave poor quality DNA (dark material within DNA pellets) and DNA was not amplifiable by PCR. A bead beating method (Section 2.2.1.4) was then employed that gave clean DNA that was amplifiable by PCR.

4. Cultures of microorganisms:

4.1. Cultures grown in liquid medium: cells were concentrated by centrifugation at ~ 1,500 *g* for 15 minutes and the supernatant discarded and the cell pellet re-suspended in CTAB extraction buffer for DNA extraction as outlined in Section 2.2.1.2.

4.2. Cultures grown on solid medium: approximately 2 ml of 1X 'Solution One' (4X concentration in 4 litre aliquots containing: 200 ml of 1 M Tris HCl (pH 8.0), 23.38 g of NaCl, 80 ml of 0.5 M EDTA (pH 8.0) and 3720 ml of RO water) was dispensed onto the agar surface and cells on the surface re-suspended using a sterile spreader. Approximately 1.5 ml of the suspended cell solution was transferred into a sterile eppendorf tube. The tubes were then spun at ~ 1,500 *g* for 15 minutes to pellet the cells. The supernatant was decanted to waste and the cell pellet was re-suspended in CTAB extraction buffer for DNA extraction (Section 2.2.1.2).

2.2.1.1 GIT-based DNA extraction from biofilm

Slides were removed from the hot pool and transported to the laboratory in pool water. The surfaces of the slides were rinsed in sterile Milli-Q water or sterile 0.9%

saline and the biofilm on the slides was aseptically scraped into 100 µl of 5.0 M GIT. The samples were incubated at 65°C for 20 minutes and DNA extracted using the IsoQuick kit as per manufacturer's protocol (ORCA Research Inc., WA, USA).

2.2.1.2 CTAB-based DNA extraction from biofilm

This method is modified from Dempster *et al.*, (1999). Slides were transported back to the laboratory immersed in pool water. The surfaces of the slides were rinsed in sterile Milli-Q water or sterile 0.9% saline and the biofilm on both sides of the slides aseptically scraped into 0.5 ml of CTAB buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB, 1% (w/v), PVP (molecular weight 360,000), pH 8.0 and 0.4% (w/v) 2-mercaptoethanol (added before each use) using a sterile scalpel blade. The CTAB buffer was also used to wash biofilms from the slides and blades by pipette (the 2-mercaptoethanol was added to the CTAB buffer at this step). The resuspended biofilm was centrifuged quickly (1 minute at 16,100 *g*) to bring contents to the bottom of the tube and then incubated at 100°C for 20 minutes. Following heating, the samples were allowed to cool and an equal volume chloroform/isoamyl alcohol (24:1) added and mixed using a rocker/rotator for 20 minutes. Tubes were then spun for 15 minutes at 16,100 *g*. The aqueous phase was transferred to a new tube and 0.5 volume 5.0 M NaCl and 1 volume isopropanol (2-proponol or isopropyl-alcohol) added. The suspension was inverted to mix and incubated at -70 °C for at least 1 hour then centrifuged for 30 minutes at 16,100 *g*. The supernatant was decanted to waste and the sides of the tube and DNA pellet washed in 80% ethanol, using a 20 second centrifuge step to ensure that the DNA pellet remained at the bottom of the tube. The supernatant was decanted to waste and the tube incubated at room temperature in an inverted position. When all traces of ethanol had evaporated, the DNA pellet was suspended in 20 µl PCR grade water or Milli-Q water and stored at -20°C.

2.2.1.3 CTAB-based DNA extraction from sediment

Sediment was collected into a sterile 50 ml polypropylene tube using a sterile spatula and transported back to the laboratory under ambient conditions. Approximately, 2 grams of sediment was weighed into a 50 ml polypropylene tube containing 3 ml of CTAB buffer (see Section 2.2.1.2 for composition) and mixed by a vortex mixer and incubated at 100°C for 20 minutes. Approximately, 3 ml of the liquid phase was transferred into a 15 ml polypropylene tube and an equal volume chloroform:iso-amyl alcohol (24:1) added and the sample incubated for 20 minutes on a rotating mixer. The tubes were then spun at 800 *g* for 15 minutes and 0.6 ml aliquots of the aqueous phase transferred to as many 1.5 ml eppendorf tubes as required. Following which, the DNA was precipitated and stored as described for the standard CTAB-based DNA extraction method in Section 2.2.1.2. Some of the DNA pellets from the sediment samples contained a black/brown coloured precipitate; therefore, a phenol extraction was undertaken. Milli-Q water was added to the DNA to bring the total volume up to 100 µl, and 1 volume of phenol:chloroform:iso-amyl alcohol (25:24:1) added and mixed by vortexing, and the tubes were then spun at 16,100 *g* for 10 minutes. The aqueous phase was removed and 1 volume of chloroform:iso-amyl alcohol (24:1) added. Tubes were mixed by vortexing and spun at 16,100 *g* for 10 minutes. The aqueous phase was removed and the DNA precipitated and prepared as described above. Following this extended protocol, the DNA commonly contained dark coloured material and most samples could not be amplified by 16S rDNA PCR. Therefore, a bead-beating based method of DNA extraction was trialled, as explained in Section 2.2.1.4.

2.2.1.4 Bead-beating DNA extraction from sediment

Sediment was collected as described in Section 2.2.1.3. Approximately, 0.5 grams of sediment was weighed out into a 2 ml polypropylene tube containing approximately 0.5 grams of 0.1 mm and 3.0 mm oven baked (250°C for 2 hours) silica-zirconia beads (Biospec Products Inc., OK, USA). A 300 µl aliquot of phosphate buffer was added (100 mM $Na_2H_2PO_4$, pH 7.4) followed by 300 µl SDS lysis buffer (100 mM

NaCl, 500 mM Tris, pH 8.0, and 10% SDS). Tubes were shaken in a Fast Prep© FR120 Cell Disrupter (Bio101 Instruments, Irvine, CA, USA) at 4.0 m/s for 30 seconds. Tubes were removed and centrifuged for 1 minute at 16,100 *g* and 500 µl of chloroform:iso-amyl alcohol (24:1) added. Tubes were mixed by vortexing for 15 seconds and then centrifuged for 3 minutes at 9,300 *g*. The aqueous phase was dispensed into a new tube and 300 µl of chloroform:iso-amyl alcohol (24:1) added, vortex-mixed for 15 seconds and incubated on a rocker/rotator for 20 minutes. Tubes were then spun at 16,100 *g* for 5 minutes. The aqueous phase was dispensed into a new tube and 7.0 M ammonium acetate added to a final concentration of 2.5 M. Samples were mixed by inversion and centrifuged at 16,100 *g* for 5 minutes. The aqueous phase was dispensed into a new tube and 0.54 volume iso-propyl alcohol added and the tubes mixed by inversion and incubated at -70°C for at least an hour. Tubes were then removed and thawed and then centrifuged at 16,100 *g* for 15 minutes, supernatants discarded to waste and the DNA pellets and tubes washed in ~ 1 ml of 70% ethanol. Tubes were incubated in an inverted position and allowed to air dry. DNA was suspended in 20 µl Milli-Q water and stored at -20°C.

2.2.2 DNA quantification

DNA extracted from samples was quantified by dot titration (Section 2.2.2.1), or by absorbance at 260 nm using a spectrophotometer (Section 2.2.2.2).

2.2.2.1 DNA concentration estimation by dot titration

A gradient of standard DNA concentrations typically ranging from 10 to 150 ng were dispensed as 20 µl drops onto a clean Perspex surface. Then 1-2 µl of the sample DNA was diluted in Milli-Q water to a total volume of 20 µl and dispensed adjacent to the standard DNA drops. Finally, 2 µl of ethidium bromide (10 mg/ml) diluted 1:1000 in Milli-Q water was added to each drop and mixed by pipette. The concentration of the sample DNA was estimated by comparison to the intensity of the standard DNA concentrations viewed by UV light (260 nm) with the Eagle Eye System (Stratagene, USA).

2.2.2.2 DNA concentration and purity determination by spectrophotometry

The concentration of nucleic acids was also determined by the absorbance (optical density, OD) of UV light at 260 nm wavelength using a spectrophotometer (UltroSpec 3000, Pharmacia Biotech, USA) or the ND 1000 Nanodrop (Nanodrop, DE, USA) instrument, where one OD unit is assumed to be 50 µg/ml for DNA at 260 nm. The purity of nucleic acids was estimated by the ratio of readings taken at 260 nm and 280 nm wavelengths (both in the UV range) as proteins absorb UV light at 280 nm. A 260/280 nm ratio of approximately 1.8 and 2.0 was considered to represent DNA and RNA, respectively.

2.2.3 Computer-based DNA sequence analysis

2.2.3.1 16S rDNA sequences and similarity

All comparative 16S rDNA sequences were obtained from the National Centre of Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) and similarity between sequences were tested using the nucleotide to nucleotide, Basic Local Alignment Search Tool (BLASTn) (Altschul *et al.*, 1990). However, BLASTn only uses a basic algorithm for multiple sequence comparisons. Therefore, further phylogenetic analysis was undertaken (i.e. powerful analytical methods accounting for mutations and ignoring spurious regions between multiple sequence alignments) as discussed below to provide a more robust and confident indication of phylogenetic affiliations of 16S rDNA sequences.

2.2.3.2 16S rDNA sequence alignments and phylogenetic trees

DNA sequence alignment was undertaken using version 8 of ClustalW (www.ebi.ac.uk/clustalw/), ClustalX (version 1.81) or the ARB software package (Ludwig *et al.*, 2004) with phylogenetic analysis of 16S rRNA gene sequences being performed using the ARB software package. The phylogenetic positions of the sequences were determined using the PHYLIP package with analysis of sequences undertaken using DNADIST, DNAML, DNAPARS, FITCH, NEIGHBOR and SEQBOOT programs (Felsenstein, 1993) and phylogenetic trees viewed in TREEVIEW (Page, 1996).

2.2.3.3 Restriction endonuclease digestion

Cleavage sites of restriction endonuclease digestion and the corresponding restriction fragments were determined using: http://www.restrictionmapper.org/.

2.2.4 Polymerase Chain Reaction (PCR)

All PCRs were undertaken using an Eppendorf Mastercycler gradient thermocycler (Eppendorf AG, Hamburg, Germany). Unless otherwise stated, all PCR reagents (*Taq* polymerase, 10X PCR buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3) and 25 mM $MgCl_2$) were obtained from Roche Diagnostics, GmbH, Germany. All PCR runs contained a negative control, consisting of filter sterilised Milli-Q water that was UV treated for 30 minutes or PCR grade water (Eppendorf AG, Hamburg, Germany) replacing the template DNA. Particular PCR runs also contained positive controls whereby standard DNA was used to ensure the PCR was functioning correctly. All primers utilised in the study are listed in Table 2.1. Stock solutions of PCR primers were stored at 60 μM concentration in 1X TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) at -20°C. Commonly, a master mix of all PCR components was prepared and dispensed into 0.5 ml PCR tubes prior to the addition of template DNA. The master mix components were added in the respective order as presented in the Tables in the appropriate sections below. When PCR reactions on environmental samples failed, 5% dimethyl sulfoxide (DMSO) was added to the PCR reactions, which commonly overcame any PCR inhibition.

Table 2.1 PCR primers used in the thesis.

Primer #	Purpose	*E.coli* positioning and direction	Sequence (5'-3')
OPR13	RAPD	N.A.	GGACGACAAG
OPR12	RAPD	N.A.	ACAGGTGCGT
RR164[A]	Bacterial 16S rDNA DGGE	338F(GC)	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCC-CTCCTACGGGAGGCAGCAG
RR148	Bacterial 16S rDNA DGGE	519R	ATTACCGCGGCTGCTGG
RR71	Bacterial 16S rDNA DGGE (non-GC)	338F	CTCCTACGGGAGGCAGCAG
RR69	Bacterial 16S rDNA	27F	AGATTTGATCCTGGCTCAG
RR70	Universal 16S rDNA	1392R	ACGGGCGGTGTGTRC
RR77	Universal 16S rDNA	1522R	AAGGAGGTGATCCARCCGCA
RR79	Universal 16S rDNA	907R	CCGTCAATTCCTTTGAGTTT
RR149	Archaeal 16S rDNA	~2F	CCGGTTGATCCTGCCGG
RR150	Archaeal 16S rDNA	347F	CCAGGCCCTACGGGGCGCA
RR151	Archaeal 16S rDNA	1335R	GTGTGCAAGGAGCAGGGAC
RR154[B]	Archaeal 16S rDNA DGGE	1335R(GC)	CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC-TGTGCAAGGAGCAGGGACG
RR155	Archaeal 16S rDNA DGGE	915F	AGGAATTGGCGGGGGAGCAC
RR160[C]	Nanoarchaeal 16S rDNA	7F	CTGCCGTTGATCCTGCG
RR161[C]	Nanoarchaeal 16S rDNA	1511R	CGGCTACCTTGTGTCGACTTAG
9bF[D]	Nanoarchaeal 16S rDNA	9F	GRGTTTGATCCTGGCTCAG
511mcR[D]	Nanoarchaeal 16S rDNA	511R	CTTGCCCACCGCTT

[A] Contains GC-clamp at 5' end. [B] Based on primer RR151, contains GC-clamp at 5' end. GC-clamp from Ferris *et al.*, (1996b). [C] Hohn *et al.*, (2002). [D] Huber *et al.*, (2002).

2.2.4.1 Randomly amplified polymorphic DNA (RAPD) PCR

A modified version of the RAPD assay developed by Ronimus *et al.*, (1997) was used in this thesis. RAPD assays were undertaken in 25 µl volume reactions containing the items listed in Table 2.2.

Table 2.2 RAPD PCR.

PCR component	Volume (µl)
Water	To 25
$MgCl_2$ (25 mM)	2.5
10x PCR buffer (No $MgCl_2$)	2.5
dNTP (2 mM)	2.5
Primer (10 µM)	5.0
Taq Polymerase (1U/µl)	1.25
Template DNA	~ 20 ng

Template DNA was amplified by a RAPD PCR program involving an initial denaturation temperature of 94°C for 3 minutes and 45 seconds, then 35 cycles of; 94°C for 15 seconds, primer annealing at 36°C for 15 seconds and primer extension at 72°C for 2 minutes followed by an additional final extension at 72°C for 4 minutes.

2.2.4.2 Bacterial 16S rDNA PCR

Near-full length bacterial 16S rDNA (approximately 1522 bp) was amplified using primers RR69 (27F) and RR77 (1522R). A master mix of PCR components was made (excluding *Taq* polymerase and template DNA) and dispensed into 0.5 ml PCR tubes (See Table 2.3 for the components). The aliquots were then exposed to UV light for 10 minutes to reduce the PCR amplification of any contaminating background DNA. Control reactions were run to ensure primers were not affected by this treatment. Template DNA and *Taq* polymerase were then added and amplification initiated.

Table 2.3 Near-full length bacterial 16S rDNA PCR.

PCR component	Volume (µl)
Water	To 25
$MgCl_2$ (25 mM)	1.5
10x PCR buffer (No $MgCl_2$)	2.5
dNTP (2 mM)	2.5
Forward primer RR 69 (10 µM)	2.5
Reverse primer RR 77 (10 µM)	2.5
Taq Polymerase (1U/µl)	1.25
Template DNA	~ 10-20 ng

The thermocycling conditions involved an initial denaturation at 94°C for 2 minutes and 30 seconds, then 5 cycles of: 94°C for 30 seconds, primer annealing at 62°C for 15 seconds and primer extension at 68°C for 20 seconds. This was followed by 30 cycles involving denaturation at 92°C for 30 seconds, annealing at 58°C for 15 seconds and extension at 68°C for 20 seconds followed by a final extension at 72°C for 7 minutes.

2.2.4.3 Bacterial DGGE PCR

The DGGE PCR protocol was undertaken as suggested by Cary, C., (2001, personal communication) and involved 50 µl reaction volumes. PCR primers, RR164 (338F-GC) and RR148 (519R) were used to amplify an approximate 220 bp region of the 16S rRNA gene. A master mix of the PCR reagents (excluding *Taq* polymerase and DNA template) was prepared and dispensed into 0.5 ml PCR tubes. PCR tubes containing master mix were exposed to UV light for 10 minutes to decrease background amplification of any contaminating DNA, following which template DNA was added. Each reaction tube was then loaded into the thermocycler and the *Taq* polymerase dispensed into each reaction tube when the initial PCR step reached 94°C (see below). This 'hot start' was undertaken to improve the fidelity of the PCR amplification, i.e. the *Taq* polymerase had less chance to replicate the DNA in a non-specific manner due to primer binding at low temperatures. Also, a touchdown PCR program was undertaken (see below), i.e. a high primer annealing temperature was used for the initial cycle which was lowered for each of the next 22 successive amplification cycles. Theoretically, this ensured specific binding of the primer to template DNA, again facilitating high fidelity during the PCR amplification. Table 2.4 contains the PCR components for the bacterial DGGE PCR. Where bands in the DGGE profile were required for sequencing (outlined in Section 2.2.5.3) the forward primer (RR164; 338F-GC) was replaced with the primer without the GC-clamp (RR71).

Table 2.4 Bacterial DGGE PCR.

PCR component	Volume (μl)
Water	To 50
$MgCl_2$ (25 mM)	2.5
10X PCR buffer (No $MgCl_2$)	5.0
dNTP (2 mM)	5.0
Forward primer RR164 (10 μM)	1.0
Reverse primer RR148 (10 μM)	1.0
Taq Polymerase* (1U/μl)	1.25
Template DNA	~ 20 ng

*** *Taq* polymerase is added as a hot start**

Template DNA was amplified by a PCR program involving an initial denaturation temperature of 94°C for 5 minutes (*Taq* polymerase added during this step), then 22 cycles of: 94°C for 30 seconds, primer annealing at 65°C for 1 minute (-0.5°C at each subsequent cycle) and primer extension at 72°C for 1 minute. Then 14 cycles of an initial denaturation of 94°C for 1 minute followed by primer annealing at 55°C for 1 minute and extension at 72°C for 1 minute followed by a final extension at 72°C for 5 minutes. A 30 minute final extension at 72°C was also trialled; however, this did not affect DGGE results (Janse *et al.*, 2004).

2.2.4.4 Near-full length archaeal 16S rDNA

The near-full length archaeal 16S rRNA gene (approximately 1500 bp) was amplified using primers RR149 (~ 2F) and RR77 (1522R). The components of these reactions are listed in Table 2.5.

Table 2.5 Near-full length archaeal 16S rDNA PCR.

PCR component	Volume (µl)
Water	To 25
$MgCl_2$ (25 mM)	1.5
10X PCR buffer (No $MgCl_2$)	2.5
dNTP (2 mM)	2.5
Forward primer RR149 (10 µM)	1.5
Reverse primer RR77 (10 µM)	1.5
Taq Polymerase (1U/µl)	1.25
DMSO	1.25
DNA	~ 20 ng

The thermocycling conditions involved an initial denaturation step of 94°C for 3 minutes and 30 seconds, followed by 33 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 2 minutes with a final extension at 72°C for 6 minutes.

2.2.4.5 Archaeal DGGE PCR

Archaeal DGGE PCR was undertaken using a semi-nested PCR approach, due to the direct PCR not functioning when the GC-clamp primer was used on DNA extracted from pure archaeal cultures. Initially, an approximate 1000 bp region of 16S rDNA was amplified using primers RR150 (347F) and RR151 (1335R) and the components outlined in Table 2.6.

Table 2.6 Initial DGGE PCR of archaeal 16S rDNA.

PCR component	Volume (µl)
Water	To 25
$MgCl_2$ (25 mM)	1.5
10X PCR buffer (No $MgCl_2$)	2.5
dNTP (2 mM)	2.5
Forward primer RR150 (10 µM)	1.0
Reverse primer RR151 (10 µM)	1.0
DMSO	1.25
Taq Polymerase (1U/µl)	1.25
Template DNA	~ 20 ng

The thermocycling conditions consisted of an initial denaturation step of 94°C for 3.5 minutes followed by 32 cycles of denaturation at 94°C for 30 seconds then primer annealing at 60°C for 30 seconds then extension at 72°C for 2 minutes with a final extension of 6 minutes.

The success of this PCR was determined by running an agarose gel to confirm amplification of the expected amplicon size, and when confirmed, typically 0.2 to 1.0 µl of this PCR product was used for a second PCR using a GC-clamped reverse DGGE primer and the protocol outlined in Table 2.7. When bands in the DGGE profile were required for sequencing (outlined in Section 2.2.5.3) the reverse primer, RR154 (1335R-GC) was replaced with the primer without the GC-clamp (RR151).

Table 2.7 Second DGGE PCR of archaeal 16S rDNA.

PCR component	Volume (µl)
Water	To 50
$MgCl_2$ (25 mM)	2.5
10X PCR buffer (No $MgCl_2$)	5.0
dNTP (2 mM)	5.0
Forward primer RR155 (10 µM)	1.0
Reverse primer RR154 (10 µM)	1.0
Taq Polymerase (1U/µl)	1.25
Template DNA	0.1-1.0 (PCR product)

The thermocycling conditions consisted of an initial denaturation step of 94°C for 5 minutes followed by 22 cycles of 94°C for 30 seconds, 60°C at 1 minute, 72°C for 1 minute and 14 cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, with a final extension of 5 minutes at 72°C.

2.2.4.6 Nanoarchaeal 16S rDNA

Initially, attempts to detect near-full length nanoarchaeal 16S rDNA by PCR were undertaken using primers RR160 (7F) and RR161 (1511R) designed by Hohn *et al.*, (2002), with the appropriate PCR protocol (Eder *et al.*, 1999) as described below and in Table 2.8. However, another PCR protocol was adopted to detect nanoarchaeal 16S rDNA that proved to be more reliable (as developed by McCliment *et al.*, in press) and is described below and in Table 2.9.

Table 2.8 Nanoarchaeal 16S rDNA PCR (based on Eder *et al.*, 1999).

PCR component	Volume (μl)
Water	To 25
$MgCl_2$ (25 mM)	2.0
10X PCR buffer (No $MgCl_2$)	2.5
dNTP (2 mM)	2.5
Forward primer RR160 (10 μM)	0.5
Reverse primer RR161 (10 μM)	0.5
DMSO	1.25
Taq Polymerase (1U/μl)	1.25
Template DNA	~ 20 ng

The thermocycling conditions based on Eder *et al.*, (1999) involved initial denaturation at 96°C for 1.5 minutes, 10 cycles of 96°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute. Then 25 cycles of 94°C for 20 seconds, 60°C for 30 seconds and 72°C for 1 minute with 2 seconds added on to the extension time at every successive cycle, with a final extension at 72°C of 10 minutes.

Table 2.9 Nanoarchaeal 16S rDNA PCR (McCliment *et al.*, in press).

PCR component	Volume (µl)
Water	To 50
$MgCl_2$ (25 mM)	4.0
10X PCR buffer (No $MgCl_2$)	5.0
dNTP (2 mM)	5.0
Forward primer 9bF (10 µM)	1.4
Reverse primer 511mcR (10 µM)	1.4
DMSO	4.0
Taq Polymerase (1U/µl)	1.25
Template DNA	~ 50 ng

The thermocycling conditions consisted of 40 cycles of 94°C for 1 minute, then 1 minute of annealing beginning at 65°C and decreasing 0.5°C every cycle until 55°C and extension at 72°C for 1 minute (McCliment *et al.*, in press).

2.2.4.7 Preparative PCR for DNA sequencing

If a DNA sample was to be sequenced, then a preparative PCR was commonly run whereby 10-15 replicates of 25 µl PCR reaction were performed for the DNA region of interest. All replicate tubes were then combined for electrophoresis (Section 2.2.5.2).

2.2.5 Electrophoresis

2.2.5.1 Agarose gel electrophoresis

The electrophoresis of DNA fragments through 1-3% agarose gels was used to separate PCR products and restriction endonuclease DNA digests. Appropriate amounts of agarose was added to either: 1X TBE buffer (1 L of 5X TBE buffer stock solution contained; 54 g Tris, 27.5 g boric acid and 20 ml of 0.5 M EDTA, pH 8.0); or 1X TAE buffer (1 L of 50X TAE buffer stock solution contained; 242 g Tris, 57.1

ml glacial acetic acid, 100 ml 0.5 M EDTA, pH 8.0) or 1X SB buffer (1 L of 20X SB buffer consisted of 38 g $Na_2B_4O_7$ and 36 g H_3BO_3 dissolved in one litre of water) and boiled until the agarose was dissolved. TBE buffer was commonly used; however, TAE buffer was preferred when DNA was to be extracted from the gel matrix and used for further analysis. SB buffer was adopted in the final year of the Ph.D. project to check DGGE PCR products (Brody and Kern, 2004). The advantages of using SB buffer are that it does not contain Tris, making it less costly; moreover, it can be run above 300 V providing shorter run times. Once the agarose solution had cooled to approximately 55°C it was poured onto the gel electrophoresis platform and allowed to set. The appropriate buffer was added to ensure adequate buffer recirculation between the anode and cathode reservoirs. A 6X gel-loading buffer (0.04% bromophenol blue, 30% glycerol) was added to samples prior to the loading of samples into the agarose gel. A 3X gel-loading buffer (GLB) containing SDS (see Section 2.2.7) replaced the 6X GLB for restriction digest samples.

All agarose electrophoresis runs included a 1 kb size standard containing approximately 1 µg of DNA (Gibco, BRL, Life technologies, Gaithersburg, USA). The profile of this ladder is presented in Appendix C. Electrophoresis runs were typically undertaken at 10 V/cm and 30 V/cm for TBE/TAE and SB buffers, respectively. Following electrophoresis, gels were stained with 0.5 mg/L of ethidium bromide for between 10 and 40 minutes and de-stained for up to 30 minutes in RO water, and the DNA visualised and photographed under UV (260 nm) light with an Eagle Eye System (Stratagene, USA) or an AlphaImager System (Alpha Innotech, CA, USA).

2.2.5.2 Preparative agarose gels and preparation of PCR product for DNA sequencing

Commonly, 10 to 15 PCR reactions (25 µl) were undertaken and run in a broad single lane of an agarose gel (1X TAE buffered) as outlined in Section 2.2.5.1. The band of interest was cut out of the agarose under a UV light using a cover slip with attempts to minimise the exposure of the DNA to UV light. The extracted band was wrapped in parafilm and stored overnight at -20°C. The frozen band was squeezed and the liquid collected into as many eppendorf tubes as required. The tubes were then centrifuged for approximately 30 seconds to pellet any remnants of agarose and the supernatant transferred to clean eppendorf tubes. One volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, mixed by vortexing and centrifuged at 16,100 *g* for 10 minutes. The top layer was transferred into a clean tube and one volume chloroform:isoamyl alcohol (24:1) added, mixed well and the centrifugation step repeated. The supernatant was then transferred to a new tube and both ammonium acetate (to a final concentration of 2.5 M) and an equal volume isopropyl-alcohol added. Samples were incubated at -70°C for at least 1 hour, following which they were centrifuged at 16,100 *g* for 30 minutes. The supernatant was removed and the tube washed with ~ 1 ml of 70% ethanol. Ethanol was removed by pipette or decanting to waste (a 5 second centrifugation step was included to ensure the DNA pellet remained at the bottom of the tube) and the DNA pellet dried by positioning the tube in an inverted position at room temperature on tissues. Finally, the DNA was re-suspended in PCR grade water (Eppendorf AG, Hamburg, Germany) or Milli Q-water by vortexing and if needed, appropriate samples combined. Samples were sent to the Waikato DNA Sequencing Facility and sequenced as detailed in Section 2.2.8.

2.2.5.3 Denaturing gradient gel electrophoresis (DGGE)

The bacterial and archaeal DGGE PCR methods used are described in Sections 2.2.4.3 and 2.2.4.5, respectively. All DGGE gels described in the thesis were replicated at least twice. The success of DGGE PCR was initially checked by electrophoresis of 5 μl of DGGE PCR product (with appropriate amount of 6X GLB added, see Section 2.2.5.1) on a 1X TBE or 1X SB agarose gel. A Gradient Delivery system (Model 475, Bio-Rad Laboratories, NY, USA) was used to cast parallel denaturant gradient gels as described in the denaturing gel electrophoresis system instruction manual and applications guide (Bio-Rad, New York, USA). Gels of 8%

acrylamide concentration (25 to 65% denaturant gradient concentration) were used for bacterial DGGE (for expected amplicons of 200-300 bp) and 6% acrylamide concentration (40 to 65% denaturant gradient concentration) for archaeal DGGE (for expected amplicons of 400-500 bp). Gels were then loaded into a *D GENE*™ Denaturing Gel Electrophoresis System, with 7 litres of 1X TAE buffer (see Section 2.2.5.1 for composition) added to the electrophoresis chamber. Prior to and during electrophoresis the 1X TAE buffer was held at 60°C. Gels were left for approximately one hour to allow the gel to set.

One volume of DGGE gel-loading buffer solution (0.05% BPB, 0.05% xylene cyanol and 70% glycerol diluted in 1X TAE buffer) was added to samples prior to loading onto the gels. A maximum of 40 µl can be loaded in a single track of a gel, therefore, 10-20 µl of PCR product was commonly run on a gel. Electrophoresis was undertaken at 140 V for 5 hours. Gels were stained in 500 ml of 1 mg/L ethidium bromide for 10 minutes and de-stained in 500 ml RO water for 10 minutes and visualised as described for agarose gel electrophoresis (Section 2.2.5.1).

In the case of low yield and faint bands on a DGGE profile, two 50 µl PCR reactions were combined and concentrated to a volume that allowed loading into a DGGE gel. To the two 50 µl PCR reactions, 0.1 volume 3 M sodium acetate (pH 5.2) and 2.5 volume 100% ethanol was added. The mixture was mixed well by inversion and centrifuged at 16,100 *g* for 20 minutes. The supernatant was discarded and the DNA pellet washed with 70% ethanol, and the DNA pellet dried and suspended in 15 to 20 µl of Milli-Q water.

Any DNA bands of interest were cut from the DGGE using a pipette tip and transferred into a 1.5 ml eppendorf tube, 20 µl of Milli-Q water added and the tubes stored at -20°C. Bands of interest that were to be ultimately sequenced were incubated overnight at 4°C prior to re-amplification. The following morning the gel was disrupted by vortex mixing and 10 µl used as template DNA for a repeat DGGE PCR (Ibekwe *et al.*, 2001) as described in the appropriate section. This DGGE PCR product was then run on a DGGE gel against the original PCR to check its purity and position. If a single band at the correct position was obtained the PCR product (from the band stab) was used for a preparative PCR (using 1 µl of PCR product per reaction) and the non-GC-clamped primer (see Section 2.2.4.3 for bacteria and Section 2.2.4.5 from archaea) was used and then run on a preparative gel, extracted and sequenced (see Section 2.2.5.2).

2.2.6 Cloning

Cloning of 16S rDNA amplicons obtained from thermophilic microorganisms by insertion into vectors and introduction into *E. coli* was undertaken under the approval code GMD 002630 (GMO031/UOW001) obtained from ERMA (Environmental Risk Management Authority, Wellington, NZ).

2.2.6.1 Preparation of electro-competent cells

The following method was performed essentially as described by Sambrook and Russell, (2001). A single colony of *E. coli* (strain JM109) was transferred from a fresh LB plate (see Appendix E for composition) into 50 ml of LB medium and grown overnight at 37°C with shaking at approximately 200 rpm. Two 2 litre flasks containing 500 ml of LB medium were pre-warmed at 37°C and 25 ml of the overnight culture transferred into each flask. The flasks were incubated at 37°C shaking at approximately 200 rpm and the OD_{600} taken at regular intervals. When the OD_{600} reached approximately 0.4, the flasks were cooled by putting on ice for approximately 15-30 minutes, swirling occasionally. It was essential that the rest of the procedure was always kept at or below 4°C, i.e. centrifuge rotors were cooled to 4°C prior to use and all tubes and buffers were kept on ice and re-suspension of cells was gentle. The cooled suspension was transferred to ice-cold centrifuge tubes and the cells harvested by centrifugation at approximately 1,000 *g* for 15 minutes. The supernatant was decanted to waste and cells were re-suspended gently in 500 ml ice-cold Milli-Q water. Cells were harvested again at 1,000 *g* for 20 minutes; this step was repeated using 250 ml and then 10 ml of ice-cold 10% glycerol. At the final harvest step the 10% glycerol supernatant was removed with a sterile Pasteur pipette and cells re-suspended in 1 ml of ice-cold GYT (10% glycerol, 0.125% yeast extract and 0.25% trypticase peptone) medium. The cell suspension was diluted 1:100 in GYT and the OD_{600} measured, the cells were diluted to approximately 2 X 10^{10} to 3 X 10^{10} cells/ml (1.0 OD_{600} = ~ 2.5 X 10^{8}) in ice-cold GYT medium. Aliquots of 80 µl were snap frozen in liquid nitrogen and stored at -70°C.

2.2.6.2 Ligation

The pGEM-T Easy vector system (Promega, Madison, WI, USA) utilises 3'-T overhangs at the insertion site which prevents re-circulisation of the vector and provides compatible overhangs for PCR products generated by certain DNA polymerases that add a single deoxyadenosine nucleotide to the 3' termini. The vector also contains multiple restriction sites, facilitating single restriction endonuclease digests that can release the inserted DNA fragment. The vector contains ampicillin resistance and blue/white screening, allowing easy selection of clones containing inserts by use of both IPTG (isopropyl β-D-thiogalactoside), which is an artificial inducer of the Lac operon and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) which is a substrate that detects galactosidase activity, that subsequently produces a blue colour.

The components of a single ligation reaction are presented in Table 2.10. The reaction was incubated at 4°C overnight.

Table 2.10 Cloning ligation reaction.

Component	Supplier	Volume (µl)
5 X Ligation buffer	Invitrogen, CA, USA	2.0
pGEM-T Easy Vector (50 ng/µl)	Promega, Madison, WI, USA	1.0
PCR product*		Dependent on required ratio of vector/insert
T4 DNA ligase (1U/µl)	Invitrogen, CA, USA	1.0

Milli-Q water	Up to 10.0

***PCR product was purified as detailed in Section 2.2.5.2.**

2.2.6.3 Electroporation

The ligation reactants, an 80 µl aliquot of competent cells, and a Gene Pulser 0.1 cm cuvette (Bio-Rad Laboratories, Hercules, CA, USA) were all kept on ice. A 1.25 µl aliquot of the ligation reaction was added to the competent cells, and mixed gently. This was then transferred to the cuvette and the cells subjected to electroporation using a Genepulser (Bio-Rad, NY, USA) set at 200 Ohms resistance, 25 µFD capacitance and 1.8 kV (due to the use of a 0.1 cm cuvette). Immediately following electroporation the cells were transferred to 920 µl of SOC (see Appendix E for composition) media and incubated at 37°C shaking at 200 rpm for 1.5 hours. During the 1.5 hours incubation, 100 µl of 100 mM IPTG and 20 µl X-Gal were spread onto LB agar plates (see Appendix E) containing 100 µg/ml ampicillin and dried by incubation at 37°C for up to an hour. Aliquots of the transformed cells were then spread out onto the X-Gal/IPTG/ampicillin LB agar plates (if required transformed cells were concentrated by centrifugation at 800 *g* for 5 minutes and an aliquot of the supernatant removed and the cells re-suspended). Plates were inverted and incubated at 37°C overnight.

2.2.6.4 Plasmid isolation by alkaline lysis

This method was obtained from Ronimus, (1993), a derivation of the alkaline lysis method of Birnboim and Doly, (1979). White colonies from the LB plates in Section 2.2.6.3 were transferred into 10 ml of LB medium containing 100 µg/ml ampicillin and incubated overnight at 37°C (shaking at 200 rpm). Cells were harvested by centrifugation at 1,000 *g* for 5 minutes at room temperature. The supernatant was decanted and cells re-suspended in 0.2 ml of 'Solution One' (50 mM glucose, 25 mM Tris, pH 8.0 and 10 mM EDTA). Suspensions were vortexed to mix then 0.4 ml of fresh 'Solution Two' (1% SDS and 0.2 M NaOH) was added to the cell suspension and tubes inverted to mix and incubated on ice for 2 to 3 minutes (timing was essential so the plasmid was not excessively denatured). A 0.3 ml aliquot of 'Solution Three' (3.0 M potassium acetate, 5.0 M glacial acetic acid, pH 4.8) was added and the sample vortex mixed for 10 seconds, then incubated on ice for at least 5 minutes. The solution was then centrifuged for 5 minutes at 16,100 *g* at room temperature.

A 0.75 ml aliquot of supernatant was transferred to a fresh tube to which 0.75 ml of iso-propyl alcohol had already been added. Tubes were mixed by repeated inversion and incubated for at least 5 minutes at room temperature. Samples were then centrifuged at 16,100 *g* for 7 minutes. The resulting DNA pellet was washed twice with 80% ethanol, and centrifuged for 30 seconds between washes to keep the pellet at the bottom of tube. The DNA pellets were dried by incubating tubes in an inverted position at room temperature for at least 10 minutes. DNA was then re-suspended in 100 µl TE (10 mM Tris, 1 mM EDTA, pH 8.0) containing 1 µl of 10 mg/ml RNAase in each 100 µl aliquot and incubated at 37°C for 30 minutes or 4°C overnight. The plasmid preparations were stored at -20°C.

Plasmids were checked for inserts by electrophoretic separation through a 1% 1X TBE agarose gel. Aliquots of the plasmid preparation (10 µl) was mixed with 10 µl Milli-Q water and 10 µl SDS GLB (30% glycerol, 3% SDS, 0.025% BPB and 1 mM EDTA, pH 8.0) incubated at 65°C for 15 minutes and then loaded onto the gel. Plasmids were screened using restriction endonuclease digestion (Section 2.2.7) and selected clones sequenced (Section 2.2.8).

2.2.7 Restriction endonuclease digestion

Typically a master-mix of components was made and dispensed into 0.5 ml tubes and then plasmid or PCR product added (Table 2.11).

Table 2.11 Restriction endonuclease digestion reaction.

Component	Volume (µl)
Restriction endonuclease buffer (10 X)	2.0
Restriction endonuclease (10 U/µl)	0.4
DNA – Plasmid	10.0
PCR product	2.0 to 10.0
Milli-Q water	Up to 20.0

Reactions were undertaken at the appropriate temperature for 3 hours, then 10 µl of 3X SDS GLB (30% glycerol, 3% SDS, 0.025% BPB and 1 mM EDTA) was added to stop the reaction and the samples incubated at 65°C for 20 minutes prior to loading onto a 2.5% TBE agarose gel (see Section 2.2.5.1). The restriction endonucleases used in the thesis are listed in Table 2.12.

Table 2.12 Restriction endonucleases employed in thesis

Restriction endonuclease	Corresponding buffer	Cut site	Incubation temp. (°C)	Supplier
*Eco*R I	REact 3	G↓AATTC	37	Invitrogen, CA, USA
Rsa I	Surecut A	GT↓AC	37	Roche Applied Science, IN, USA
Hind III	REact 3	A↓AGCTT	37	Invitrogen, CA, USA
Hha I	Multicore	GCG↓C	37	Promega, WI, USA

2.2.8 DNA sequencing

DNA sequencing was undertaken by the Waikato DNA Sequencing Facility based at the University of Waikato, Hamilton New Zealand using the MegaBace capillary analysis system (Amersham Biosciences Corp., NJ, USA). All sequences obtained and used for analysis during the study are listed in Appendix D in FASTA format.

2.3 Cultivation of microorganisms

All microorganisms were cultured using standard aseptic laboratory procedures. Samples taken from hot springs for culturing were transported to the laboratory under ambient conditions in sterile tubes with the air space minimised to prevent oxygenation. Chapter 5 describes the characterisation of some novel archaea and contains a Materials and Methods section specific for this study.

2.3.1 Observation of cultures

2.3.1.1 Phase-contrast microscopy

Microorganisms were viewed using a 100X phase-contrast objective lens (Olympus, BH-2, Japan) and images recorded taken using an Olympus SC35, type 12 camera (Olympus, BH-2, Japan) or a Nikon CoolPix 4500 digital camera (Nikon, Tokyo, Japan).

2.3.1.2 Scanning electron microscopy (SEM)

Samples were filtered through a 0.22 µm filter followed by 2.5% glutaraldehyde to fix any microbes onto the filter membrane. The filter was given to the Waikato University Scanning Electron Microscopy (SEM) laboratory where the filter was exposed to four changes of 0.1 M sodium cacodylate buffer (pH 7.5), rinsed in water and dehydrated in increasing concentrations of ethanol (50, 75 and 90%) and finally four changes of absolute ethanol. The filter was critically point dried sputtered with platinum and viewed using a Hitachi S-4100 field emission SEM.

2.3.2 Media preparation

All media utilised in the thesis are listed in alphabetical order in Appendix E. Reagents were obtained from Fort Richard (Auckland, NZ). Media were sterilised by autoclaving (121°C, 1 bar pressure) for 20 min. However, some media containing elemental sulfur were sterilised by exposure to steam at 98°C for 15 minutes on 3 consecutive days.

2.3.3 Roll tubes

This method was modified from Götz, (1998). Gelrite (Merck & Co., Inc., NJ, USA) and/or Phytagel (Sigma Aldrich, USA) was added at 8 g/L final concentration to growth medium, boiled and 3 ml dispensed into Hungate tubes and autoclaved. Following autoclaving the tubes were held at the appropriate incubation temperature for the sample and approximately 100 µl of inoculum and 50 µl of sterile 1 M $MgSO_4 \cdot 7H_2O$ added. Tubes were then reduced with sterile anaerobic 10% $Na_2S \cdot 9H_2O$ and rolled on ice until set. The roll tubes were incubated on an incline due to the development of liquid at the bottom of the tubes.

2.3.4 Storage of microorganisms

2.3.4.1 Cryo-preservation in glycerol-based solution

Broth cultures were cooled to room temperature and then centrifuged (approximately 800 to 1500 *g* for 15 minutes) to pellet cells. Cells were then gently re-suspended in approximately 0.5 ml anaerobic cryo-protectant (see Appendix E for composition). The cell suspension was transferred to a Nunc-cryo tube (NY, USA) and snap-frozen in liquid N_2 and stored at -70°C. The microorganisms and consortia stored in cryoprotectant at -70°C are listed in Table F.1 in Appendix F.

2.3.4.2 Freeze–drying

For long-term storage, pure cultures were freeze-dried by Lynne Parker and deposited in the Thermophile Research Unit Collection Culture (TRUCC) at the University of Waikato, Hamilton, New Zealand. The organisms deposited into the TRUCC are listed in Table F.1 in Appendix F.

2.3.4.3 Reviving freeze-dried microorganisms

The glass freeze-dried vial was wiped in 70% ethanol and scratched with a diamond pen above the wool bung. The vial was then wrapped in a tissue and snapped across the scratch. A 1 ml aliquot of medium was dispensed into the vial and left for 2 to 3 minutes to allow suspension of cells into the medium and then used to inoculate a fresh tube of medium and an additional 10^{-1} dilution into a further fresh tube of medium. This was necessary so that the $FeSO_4$ contained in the freeze dry vial was sufficiently diluted not to cause any inhibition of growth. The inoculated tubes were then incubated at 5°C less than the optimum growth temperature of the microorganism.

Chapter 3 : The colonisation of surfaces and the microbial ecology of three New Zealand hot pools

3.1 Introduction

As outlined in the Introduction (Chapter 1), the aim of the study was to identify the 'active' microbial members of New Zealand's geothermal habitats using DNA-based molecular approaches. In an attempt to target the 'metabolising/active' microbial members of the community it was decided to incubate surfaces within these environments and document the microorganisms colonising the surfaces. Consequently, microbial colonisers could be compared to the microbes that reside within the water column of the pool and the microbes enriched for using culture-based approaches.

Two hot pools (designated KP1 and AQ1, both are described in detail below) located in Kuirau Park, Rotorua were used for the microbial colonisation and ecology study. Both pools were chosen because they had similar characteristics, i.e. clear water, silica-depositing, similar geochemistry profiles, near-neutral pH, but differed in temperature. The expectation was that the higher temperature AQ1 pool (~ 95°C) would consist mainly of an archaeal community whereas the lower temperature KP1 pool (~ 75°C) would be comprised of mostly bacteria. Champagne Pool located in Waiotapu, Rotorua, was also included in the initial stages of the study so comparisons could be made to a pool with differing water chemistry profile and pH. The in-depth study and comparison of the microbial ecology of these hot pools also gave the opportunity to develop molecular-, microscopy- and culture-based techniques which could then be applied to a survey of the microbial colonisation of surfaces incubated in hot pools situated in New Zealand and Yellowstone National Park, WY, USA as discussed in Chapter 4.

The physical and chemical parameters of the three pools (KP1, AQ1 and Champagne Pool) are initially described in Section 3.2, below, followed by a study involving the documentation of colonisation in unison for both KP1 and AQ1 (Section 3.3). Further in-depth colonisation studies, culture attempts, and microbial ecological analysis are presented in an individual section for each hot pool; i.e. Sections 3.4, 3.5 and 3.6 for KP1, AQ1 and Champagne Pool, respectively. The corresponding section for each pool has its own individual discussion and conclusion with comparisons between the studied pools and other international studies being included in a final conclusion and discussion in Section 3.7.

3.2 Description of the hot pools

3.2.1 Physical parameters

The global position, temperature and pH of the three pools are presented in Table 3.1. A photograph of each pool is contained in Appendix A. Access to KP1 was via Tarewa Road, Rotorua, and via the Rotorua, Aquatic centre car park for AQ1 (located in Kuirau Park).

Table 3.1 Physical parameters of KP1, AQ1 and Champagne Pool.

Pool	Location	GPS position	Temp. (°C)	pH
KP1	On Tarewa road, Kuirau Park, Rotorua. On a previous house site.	38° 07' 40" S 176° 14' 30" E	~ 75*	~ 7.5*
AQ1	Kuirau Park, Rotorua. Access via car park of the Rotorua Aquatic centre.	38° 07' 54" S 176° 14' 31" E	~ 95*	~ 7.5*
Champagne Pool	Waiotapu thermal park	N.D.	~ 75*	~ 5.5*

***This is only an indicative figure, the temperature and pH of the pools fluctuated during the sampling period and is discussed below in Sections 3.4, 3.5 and 3.6 for KP1, AQ1 and Champagne Pool, respectively.**

3.2.2 Chemical analyses of pool water

Pool water was collected from AQ1 and KP1 on the 2nd July 2002 and the chemical composition determined at the Institute of Geological and Nuclear Sciences (IGNS), Taupo, New Zealand, as presented in Table 3.2. The chemical composition of Champagne Pool water was obtained from Dr. Bruce Mountain (IGNS).

Table 3.2 Chemical composition of KP1, AQ1 and Champagne Pool water.

	KP1	AQ1	Champagne Pool
Bicarbonate (total) mg/L	273	325	195
pH	8.11	8.1	5.18
Analysis temp. (°C)	16	17	22
HCO_3/Date analysed	06/08/2002	06/08/2002	07/08/2001
Aluminium mg/L	<0.1	0.16	0.19*
Ammonia (total as NH_3) mg/L	0.4	0.46	35
Arsenic mg/L	<0.1	<0.1	4.7*
Boron mg/L	6.1	6.4	24
Calcium mg/L	1.3	0.85	34
Caesium mg/L	0.31	0.36	1.3
Chloride mg/L	325	332	1908
Iron mg/L	<0.02	<0.02	0.04*
Lithium mg/L	2.0	2.9	7.7
Magnesium mg/L	0.033	0.013	0.02
Manganese mg/L	0.012	0.007	0.08*
Potassium mg/L	32	33	167
Rubidium mg/L	0.33	0.35	1.6
Silica (as SiO_2) mg/L	255	331	456
Sodium mg/L	333	338	1154
Sulphate mg/L	77	43	216
Sulphide (total as H_2S) mg/L	0.49	3.0	6.8
H_2S date analysed	06/08/2002	06/08/2002	07/08/2001
Ion Balance	1.2	1.0	0.7

***Sample taken on 03/04/2001 and measurements made on 04/04/2001.**

From Table 3.2 the water chemistry results between KP1 and AQ1 were generally similar, with the exception of sulphide being higher for AQ1 (explained by the fact that the solubility of sulphide increase with temperature), indicating that water for both pools derives from the same aquifer system. However, Champagne Pool typically had considerably higher concentrations of most elements (with the exception of bicarbonate) as compared to AQ1 and KP1.

3.2.3 Carbon content of pool water

The particulate organic matter (POM), suspended solids (SS) and dissolved organic carbon (DOC) levels of pool water from KP1 and AQ1 was determined and the results presented in Table 3.3, below (POM and SS was not determined for Champagne Pool).

Table 3.3 Carbon parameters of KP1, AQ1 and Champagne Pool water.

	POM (mg/L)	SS (mg/L)	DOC (mg/m^3)
KP1	6.25	17.19	1270
AQ1	0.61	4.55	590
Champagne pool	N.D.	N.D.	700

3.3 Colonisation in pools KP1 and AQ1

The microbial colonisation of glass slides incubated *in situ* was followed in unison for KP1 and AQ1 and documented by:

1. ATP assay – amount of ATP on slides surfaces used as an estimate of biomass.
2. Microscopy – phase-contrast and fluorescent (DNA staining using DAPI) microscopy on colonised slides.
3. Denaturing Gradient Gel Electrophoresis (DGGE) – DNA extracted from colonised slide surfaces and used for DGGE analysis.
4. Most Probable Number (MPN) – biofilm was removed from the slide and used to inoculate growth media for MPN enumeration.

During the sampling period the temperature and pH ranges for KP1 and AQ1 were 65-72°C, pH 7-7.5 and 94-95°C, pH 7.5-7.8, respectively. The times when slides were removed for testing and the tests undertaken are summarised in Table 3.4.

Table 3.4 Time and test schedule for slide incubations in pools KP1 and AQ1.

	Time of incubation (hours)									
Test	**0**	**15**	**19**	**24**	**38**	**50**	**62**	**74**	**88**	**137**
Phase-contrast	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
ATP assay		✓	✓	✓	✓	✓	✓	✓	✓	✓
DAPI staining		One slide used for 15-24 hr			✓		✓		✓	✓
DGGE				✓		✓		✓	✓	✓
MPN enumeration			✓					✓	✓	✓

3.3.1 ATP results

The ATP levels measured on the surfaces of incubated slides are presented in Table 3.5 as relative light units (RLU). The background levels of ATP in the pool were determined by measuring ATP levels in 25 µl of pool water.

Table 3.5 Total ATP (RLU) from colonised slides for KP1 and AQ1.

	25 µl water	15 hr	19 hr	24 hr	38 hr	50 hr	62 hr	74 hr	88 hr	137 hr
KP1	386	10031	37829	5327	6090	6325	57826	17023	25869	8746
AQ1	42	153770	Overload	72456	391460	139544	Overload	385652	Overload	Overload

A quarter of the total area of a slide was swabbed for AQ1 samples from 24 to 137 hr which have been converted to total slide area values. Overload is a measurement above ~ 180 000 RLU.

The rapid accumulation of ATP on slides over a short period of time, as compared to background levels (pool water) of ATP was indicative of colonisation and outgrowth (i.e. exponential increase) of microbes on the slide, rather than the random linear accumulation of microorganisms on the slide surface. Furthermore, there was not a continuous increase in the ATP level on the slides, which suggested either uneven colonisation occurred between slides, which was also supported by microscopic observation (Section 3.3.2) or variability between ATP assays. Microscopic observation also showed uneven colonisation between different parts of the same slide. Interestingly, the accumulation of ATP occurred more rapidly on slides in the higher temperature pool, AQ1. Moreover, it was necessary to swab a smaller area of the slides from pool AQ1 to prevent overloading the ATP meter, although this still occurred for some sample times.

3.3.2 Microscopic observations

All micrographs of colonised slides for KP1 and AQ1 are contained on the supplementary compact disc, located on the inside of the back cover of the thesis. For the slides incubated in KP1, short rods were observed to be the pioneer colonisers (example micrograph; 24 hours incubation, Figure 3.1(a)) and after 88 hours incubation there was obvious biofilm development in the form of micro-colonies of longer rods (example micrograph; 137 hours incubation, Figure 3.1(b)).

In contrast to KP1 colonisation, dense micro-colonies of short rods were observed on AQ1 slides after only 24 hours incubation (example micrograph; Figure 3.2(a)), with evidence of heavy colonisation by rods and dense silicification occurring after 137 hours incubation (example micrograph; Figure 3.2(b)).

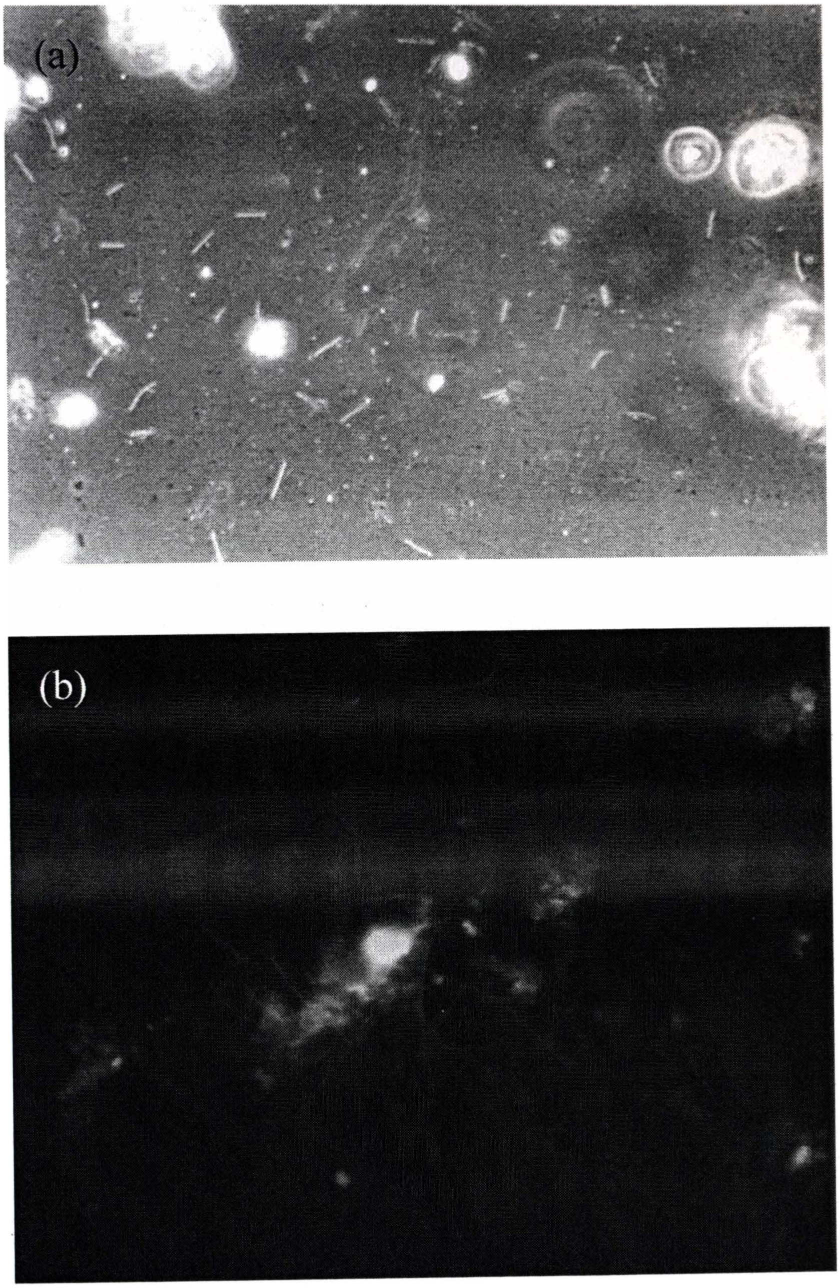


Figure 3.1 Colonisation micrographs (1000X) for KP1: (a) 24 hours incubation (phase-contrast); (b) 137 hours incubation (DAPI).

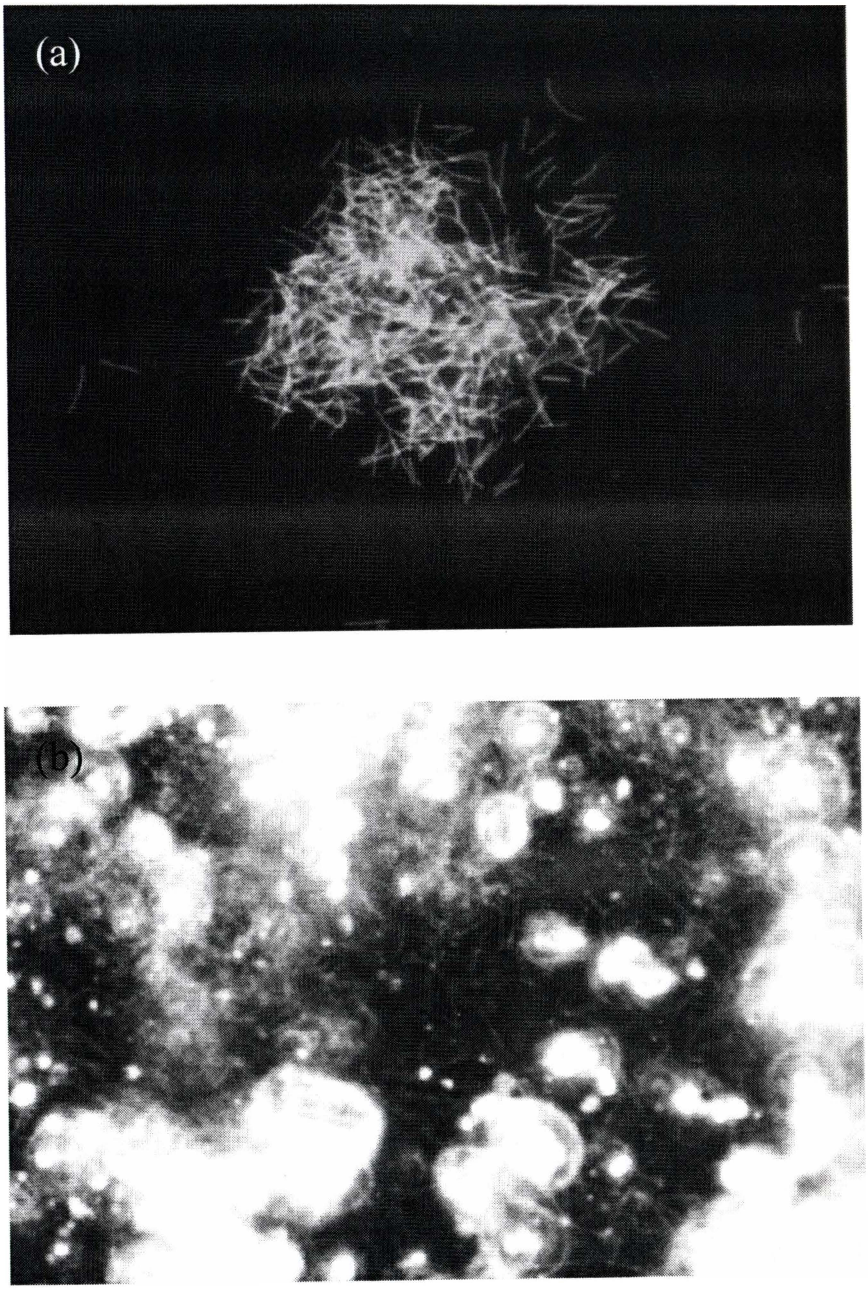


Figure 3.2 Colonisation micrographs (1000X) for AQ1: (a) 24 hours incubation (DAPI); (b) 137 hours incubation (phase-contrast).

Colonisation of slides for both pools were dominated by rods, although small numbers of cocci and *Fervidobacterial*-like cells (shorter rods with terminal spheroids) were also observed on KP1 incubated slides. The concentration of cells reflected the ATP results of Section 3.3.1, whereby, AQ1 slides which had the highest ATP levels also had the highest concentrations of cells as observed by microscopy.

3.3.3 MPN results

The biofilm from the incubated slides was used to inoculate MPN tubes over a 10^{-1} to 10^{-4} dilution series in triplicate. MPN tubes were incubated at 70°C for KP1 and 90°C for AQ1. The compositions of all tested media are included in Appendix E and included anaerobic heterotrophic media, 2/1+C, Db+S and *Thermococcus zilligii* medium. Aerobic CMD agar spread plates (duplicate at 70°C) were also used with the 137 hour slides for KP1. The MPN results, including dominant cell morphologies are listed in Table 3.6.

Table 3.6 MPN results from colonised slides for KP1 and AQ1.

Hot Pool	Medium	19 hours	74 hours	88 hours	137 hours
KP1	2/1+C	2.1×10^1	7.3×10^0	9.3×10^2 fervido/cocci	2.4×10^2 cocci
	AN1	9.3×10^2	-	-	2.4×10^3 long rods
	Db+S	2.1×10^1	-	4.3×10^2 fervido	2.4×10^4 fervido
	CMD agar	Not determined	Not determined	Not determined	21.5×10^{4}*
AQ1	2/1+C	-	-	1.5×10^2 cocci	-
	AN1	-	-	-	-
	Db+S	2.4×10^2 few rods	2.4×10^2 few rods	2.1×10^1 many rods	2.1×10^1 many rods

- = No growth. fervido = '*Fervidobacterial*' morphology, motile cells with terminal spheroids. *CMD agar plates were dominated by orange colonies of likely '*Thermus*' and '*Thermonema*' cells.

3.3.4 DGGE results

DNA was isolated from colonised glass slides (24, 50, 74, 88, 137 hour samples) for both KP1 and AQ1, and bacterial and archaeal 16S rDNA (near full-length and DGGE) PCR amplifications undertaken. No amplification was obtained using archaeal primers on DNA from KP1 slides, or with bacterial primers on DNA from AQ1 slides. The amplicons obtained using bacterial DGGE primers and DNA of slides from pool KP1 were separated by DGGE and the results presented in Figure

3.3. Included in the KP1 DGGE gel are DGGE amplicons obtained from pure cultures isolated from pool KP1 (see Section 3.4.2), DNA extracted from a 0.22 μm filter used to filter KP1 pool water and DNA from a slide that had been incubated in the pool for 33 days.

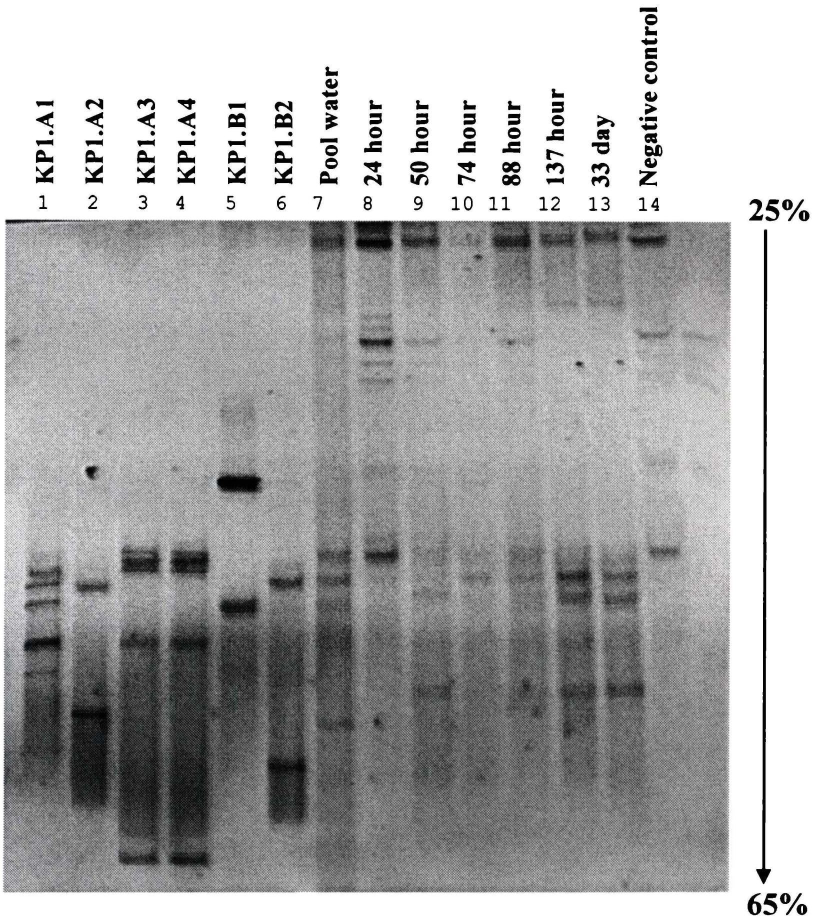


Figure 3.3 Bacterial DGGE of DNA from pure cultures and colonised slides from pool KP1. The track numbers and corresponding samples are: (1) KP1.A1; (2) KP1.A2; (3) KP1.A3; (4) KP1.A4; (5) KP1.B1; (6) KP1.B2; (7) pool water; (8) 24 hr glass slide; (9) 50 hr glass slide; (10) 74 hr glass slide; (11) 88 hr glass slide; (12) 137 hr glass slide; (13) 33 day glass slide; (14) Negative control.

From the KP1 DGGE (Figure 3.3) the DGGE profiles of the colonised slides were complex with many faint bands. The 24 hour sample (lane 8) shared the same profile as the negative control (lane 14) and thus, may be indicative of low DNA yields from the slide. However, the DNA from slides colonised for longer periods (lanes 9 to 12) shared similar profiles including the slide incubated for 33 days (lane 13) and the pool water sample (lane 7). As discussed in Section 3.4.1.3, all of the organisms isolated from pool KP1 contained more than one band in their DGGE profiles (lanes 1 to 6).

DGGE analysis using archaeal primers on DNA from slides colonised in pool AQ1 is presented in Figure 3.4. The 24, 50 and 74 hour samples did not provide PCR amplification, due to PCR inhibition, which was later overcome with the addition of DMSO to the PCR. Therefore, only the 88 hour and 137 hour samples are included in this DGGE profile. The DGGE profiles of two cultures isolated from AQ1 (a coccal isolate, AQ1.S1^T and a rod isolate, AQ1.S2, discussed in Section 3.5.4) are included in the DGGE analysis, as well as DNA extracted from a 0.22 µm filter used to filter AQ1 pool water.

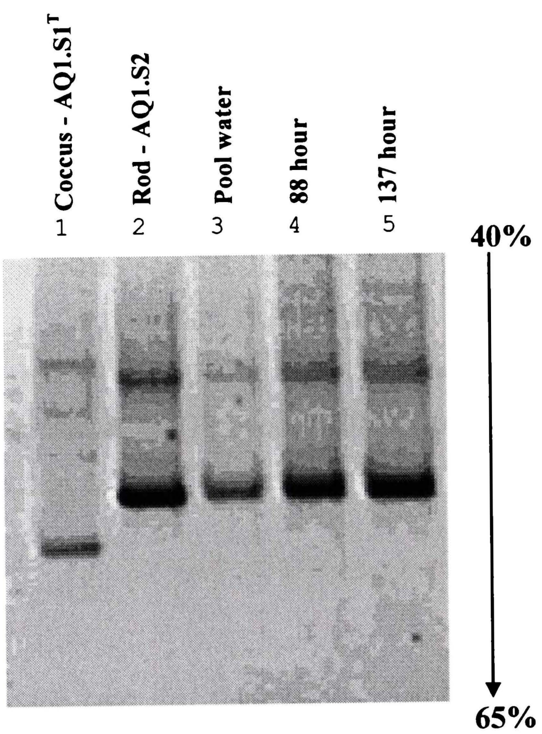


Figure 3.4 Archaeal DGGE of DNA from colonised slides and pure cultures from pool AQ1. Samples and the corresponding track numbers are: (1) coccal isolate, AQ1.S1^T; (2) rod isolate, AQ1.S2; (3) pool water; (4) 88 hr glass slide; (5) 137 hr glass slide.

3.3.5 Discussion and Conclusion

Microbial colonisation was observed on all slides for both pools and ATP levels were indicative of the concentration of cells on slides as observed by microscopy. At least three distinct cell morphologies were observed on colonised slides incubated in pool KP1 and in the MPN media used. This diversity of microorganisms was reflected by complex colonisation DGGE profiles. As was expected, slides in KP1 were completely dominated by bacteria and archaea were not detected. A bacterial dominance in KP1 is reflected in further isolation attempts, with seven pure cultures of bacteria being isolated from KP1 pool water as discussed in Section 3.4.2.

Slides incubated in AQ1 were colonised by rods only, similarly, MPN media contained a low diversity of cell morphologies consisting of rods and cocci. As was expected, slides in AQ1 were completely dominated by archaea and bacteria were not detected. This was also reflected in further extensive culture-based results, in which two archaea were isolated from AQ1 pool water, namely, a coccus designated AQ1.S1^T (*Ignisphaera aggregans*) and rod designated AQ1.S2 (*Pyrobaculum* sp.) as discussed in Section 3.5.4. The dominant DGGE band of the DNA amplified from colonised slides for AQ1 (lanes 4 and 5 of Figure 3.4) and pool water (lane 3) were in the same position as the dominant DGGE band of DNA from the pure culture of the rod, AQ1.S2. As discussed in Section 3.5 these two DGGE bands have an identical DNA sequence and are *Pyrobaculum* in identity, which accounts for the rod-shaped dominance of the colonised slides.

Although, it is generally the case that thermophilic bacteria have faster optimum growth rates than hyperthermophilic archaea, in this instance the rate of colonisation by the hyperthermophiles of pool AQ1 greatly exceeded that of the KP1 bacterial colonisers. Further analysis into the colonisation of surfaces and microbial ecology for KP1 and AQ1 are presented in Sections 3.4 and 3.5, respectively.

3.4 Hot pool: KP1

The water level of KP1 varied during the sampling period, changing from a stagnant pool to one which overflowed into a drain located on Tarewa Road. The pool was sampled periodically from the 15th July 2002 till the 27th April 2003, during this time the temperature of the pool fluctuated between 66 and 77°C and the pH between 7.0 and 8.5.

By the 7th of July, 2003, the temperature of the pool increased to 82°C. Glass slides were then incubated in the pool to observe any differences in colonisation due to the increase in temperature. Slides were collected after 11 days incubation and the temperature of the pool was 85°C and the pH 7.5. No colonisation of the glass slides was apparent by phase-contrast microscopy and the DNA yields from the slide surfaces were below the level required for amplification using either bacterial or archaeal primers. On the 22nd November 2003 the temperature of the pool had returned to 70°C and pH 7.5. Subsequently, the pool continued to cool to temperatures barely above 50°C and water flow declined so that no or minimal run-off occurred. Further studies on this pool were abandoned.

3.4.1 Colonisation results

Surfaces (glass, steel and Teflon) were initially incubated in the water run-off channel; however, they had to be moved to the edge of the pool when the water level of the pool dropped. Colonisation of surfaces (glass, Teflon and stainless steel) was examined using DGGE, ATP, protein and microscopical methods (phase-contrast and fluorescent, DNA staining using DAPI). Typically, after approximately 1 month incubation the slides were heavily silicified, bacteria were no longer observable by phase-contrast microscopy and DNA could not be isolated. The Teflon slides could not be observed by phase-contrast or fluorescent illumination, since insufficient light was transmitted through the Teflon coating for phase-contrast, and the Teflon coating auto-fluoresced. Therefore, only the glass-Teflon interfaces on the Teflon slides were documented by microscopy.

3.4.1.1 ATP and protein analyses

The concentration of ATP on slides and protein on glass coverslips reflecting colonisation were measured and the results presented in Table 3.7.

Table 3.7 ATP and protein measured on colonised surfaces incubated in pool KP1.

Incubation period	ATP (RLU)	ATP (RLU) adjusting for area swabbed	μg of protein on glass coverslip
200 μl pool water	226	-	-
4 days – Glass slide	4777	4777	Below detection limits
8 days – Glass slide	6084*	12168	1.02
8 days – Stainless steel slide	7924*	15848	-
10 days – Glass slide	12549	12549	-
17 days – Glass slide	6488*	12976	4.08
17 days – Stainless steel slide	2923*	5846	-
17 days – Teflon slide	9576*	19152	-
28 days	Not determined	-	4.4

***half of an entire surface of a slide swabbed, i.e. one side of a slide.**

3.4.1.2 Microscopic observation

All phase-contrast and DAPI-stained micrographs of slides colonised in pool KP1 are contained on the supplementary compact disc, located on the inside of the back cover of the thesis. Colonisation of surfaces incubated in KP1 was initially by isolated rods typically at locations where crystalline deposits formed on the surface, which over time developed into small micro-colonies (example micrograph, Figure 3.5(a)). After approximately 1 week incubation, a well developed biofilm was present (Figure 3.5(b)) consisting of dominant rods, shorter more refractile rods and a few cocci. There was no noticeable difference in colonisation between the different surfaces (steel, glass and Teflon-coated glass). After approximately 17 days incubation, microscopy was difficult due to heavy deposition of particulate and silicate deposits.

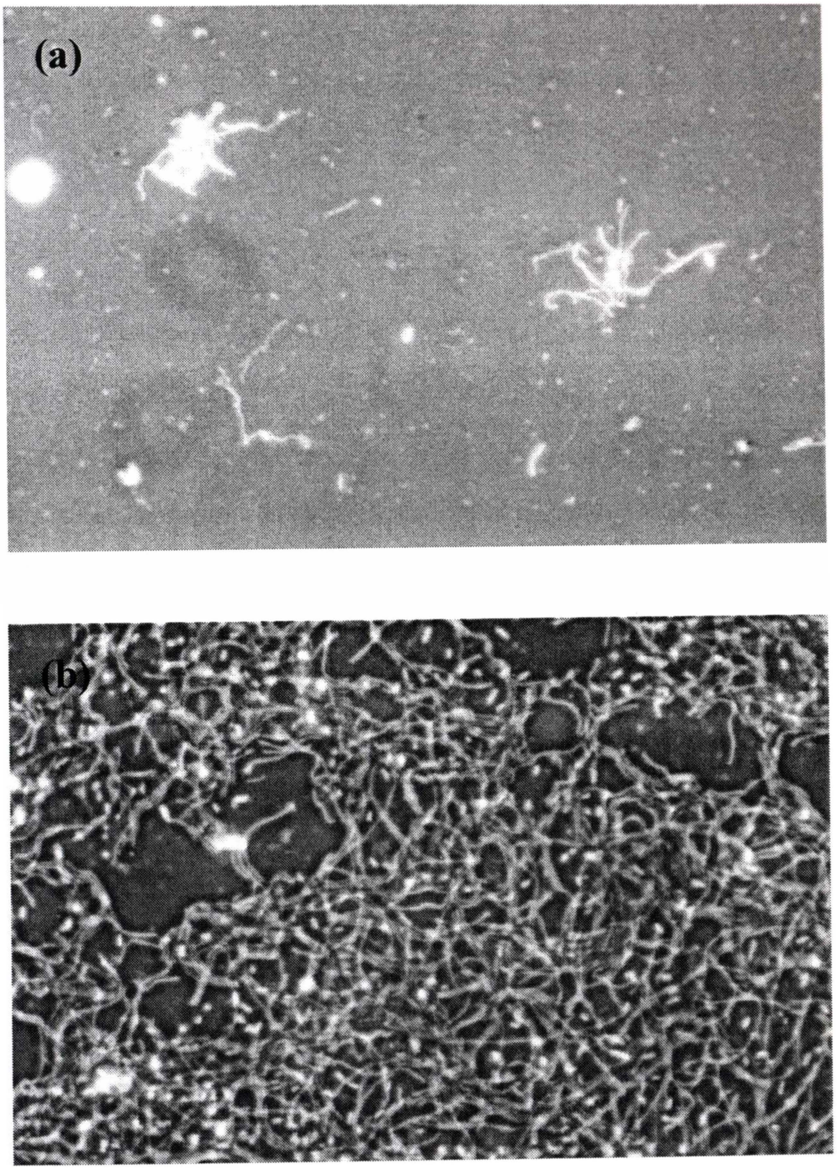


Figure 3.5 Colonisation micrographs (phase-contrast, 1000X) of glass slides incubated in pool KP1: (a) 4 days incubation; (b) 9 days incubation.

3.4.1.3 Molecular analyses

Biofilm was removed from colonised surfaces for DNA extraction and DGGE analysis. Amplification was not obtained using archaeal primers (either those for the full-length 16S rDNA gene or using DGGE primers). Positive amplification was obtained using primers designed for bacterial DGGE. Commonly, the shorter *in situ* incubation times for slides gave faint profiles on DGGE. Therefore, two DGGE PCR reactions were undertaken on a single DNA sample, the PCR products precipitated, combined and loaded together in a single lane of a gel. The DGGE results are presented in Figures 3.6 and 3.7, below; including the DGGE profiles of pure cultures of isolates from KP1 (see Section 3.4.2) as reference. Figure 3.6 contains the DGGE for DNA obtained from colonised glass slides, Figure 3.7 contains profiles from glass and steel surfaces as well as those from pure cultures isolated from pool KP1. KP1 pool water (2 litres) was also filtered through a 0.44 μm filter and DNA extracted from the filter surface was used for DGGE. The filtrate was further ultra-filtered through an YM10 cut-off membrane and DNA extracted from the liquid concentrate. Bacteria, archaea and nanoarchaea (PCR protocol by Eder *et al.*, 1999) were not detected by 16S rDNA PCR (near full-length and DGGE) in the ultra-filtrate concentrate.

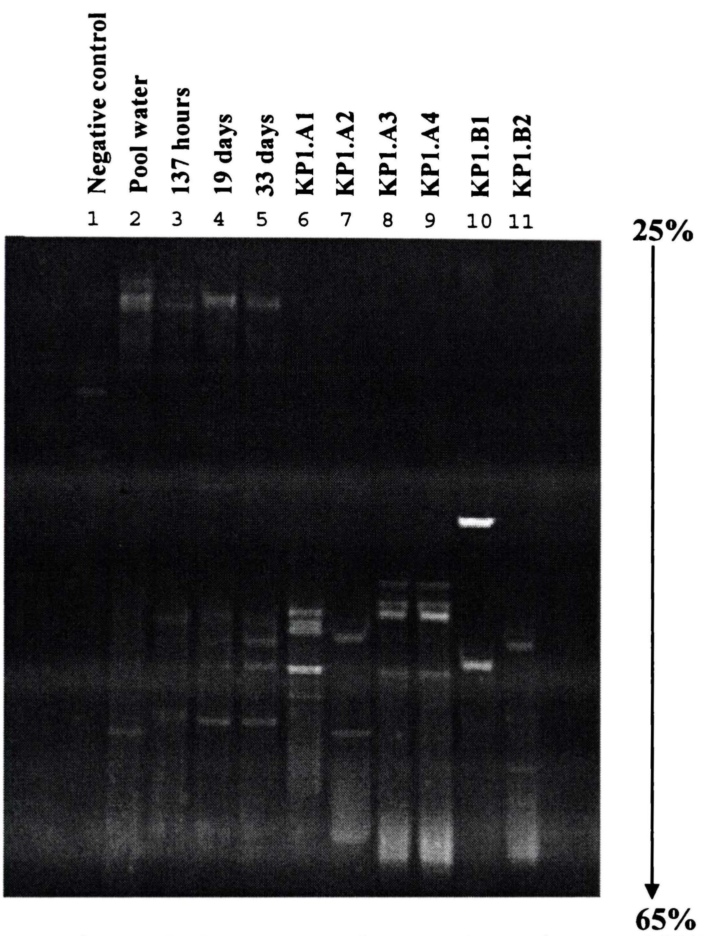


Figure 3.6 DGGE of DNA from colonised slides and pure cultures from pool KP1. The track numbers and corresponding samples are: (1) negative control; (2) pool water; (3) 137 hours; (4) 19 days; (5) 33 days; (6) KP1.A1; (7) KP1.A2; (8) KP1.A3; (9) KP1.A4; (10) KP1.B1; (11) KP1.B2.

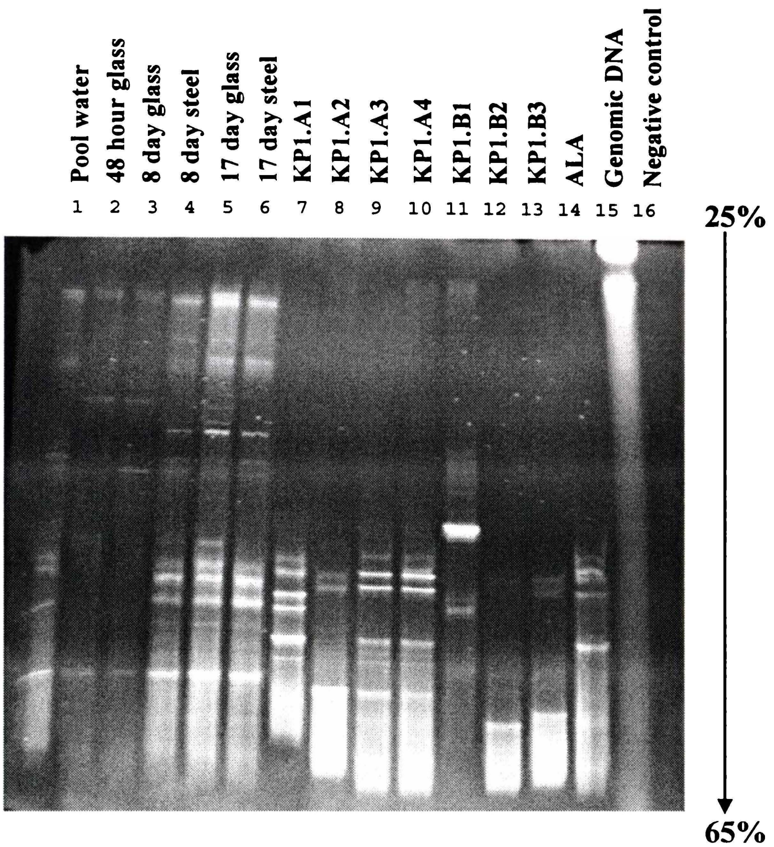


Figure 3.7 DGGE of DNA from colonised glass and steel slides and pure cultures from pool KP1. The track numbers and corresponding samples are: (1) pool water; (2)) 48 hour glass; (3) 8 day glass; (4) 8 day steel; (5) 17 days glass; (6) 17 day steel; (7) KP1.A1; (8) KP1.A2; (9) KP1.A3; (10) KP1.A4; (11) KP1.B1; (12) KP1.B2; (13) KP1.B3; (14) ALA; (15) genomic DNA (KP1.B1); (16) negative control.

From the DGGE results, complex profiles resulted with many faint bands from the DNA from all the colonised slides. It was also difficult to correlate profiles for the pure cultures isolated from the pool with profiles, of presumably mixed cultures, from colonised slides. In part, this reflects the rather complex profile of patterns obtained even with pure cultures and presumably reflects the presence of multiple 16S rDNA gene copies with their genomes (Klappenbach *et al.*, 2001). This means that the identification of signature bands within the profile obtained from DNA of colonised slides by reference to similar bands in the profiles of pure cultures cannot be made with absolute certainty. The isolates, KP1.A3 and KP1.A4 (lanes 9 and 10, Figure 3.7) have identical DGGE profiles and thus they may be closely related. As demonstrated below in Section 3.4.2.1, randomly amplified polymorphic DNA (RAPD) profiles for KP1.A3 and KP1.A4 also support a close relationship.

3.4.2 Isolation of pure cultures

Pool water and sediment from KP1 was used to inoculate various enrichment media. The compositions of all media used are listed in Appendix E. The organisms isolated into pure culture from KP1 pool water and sediment are presented in Table 3.8. The isolates that were freeze-dried and submitted to the Thermophile Research Unit Culture Collection (TRUCC) are also listed in Table F.1 in Appendix F. CMD agar spread plates incubated aerobically at 65°C were dominated by suspected '*Thermus*' species (orange colonies of cells with short rod morphology) and isolated into pure culture: designated KP1.A2. The CMD plates also contained a diversity of other colonies, from which pure cultures were obtained. These included orange colonies of long filamentous '*Thermonema*'-like cells which were designated KP1.A1; a spore-forming *Bacillus* species designated KP1.A3 and a non-spore-forming '*Bacillus*'-like species designated KP1.A4. Db basal^1 agar plates supplemented with various substrates (see Table 3.8) and incubated under aerobic conditions at 70°C were dominated by colonies of rod-shaped organisms, from which eight pure cultures were obtained (Table 3.8). From randomly amplified polymorphic DNA (RAPD) tests (see Section 3.4.2.1) it was determined that these eight rod-shaped isolates were all closely related.

Inocula of KP1 water into most of the anaerobic media used gave rise to the growth of microorganisms with diverse morphologies. However, short motile rods with terminal vesicles ('*Fervidobacterial*'-like morphology) were most common and out-competed other species on transfer. This occurred in 2/1+C medium, *Dictyoglomus* medium (from which an organism with '*Fervidobacterial*'-like morphology was isolated into pure culture, designated KP1.B1), Db+S and Db basal^2 (Db medium with low levels of carbon sources, i.e. 0.1 g/L trypticase peptone and yeast extract) without supplements or supplemented with either 1% sodium pyruvate, sodium malate or sodium-β-glycerophosphate. Growth was not observed when Db basal^2 was supplemented with 1% putrescine, β-alanine, glutamic acid, glycine or di-ammonium succinate (incubation at 70°C). Again no enrichments were obtained on Db basal^2 medium prepared as a solid Gelrite medium supplemented with 1% β-alanine, putrescine, sodium-β-glycerophosphate, glutamic acid and glycine, and a 200 µl aliquot of KP1 pool water spread on the surface of the media and incubated at 85°C under anaerobic conditions (CO_2 Systems Envelope, BBL GasPak, Becton-Dickson, USA).

Many morphologically distinct cells were observed growing in AN1 medium (at 70°C); however, a rod morphology dominated and was isolated into pure culture (designated KP1.B2). This isolate did not grow in 2/1+C medium at 70°C. Another rod-shaped bacterium (KP1.B3) was isolated using an anaerobic arsenic-based (Huber *et al.*, 2000b) medium incubated at both 65 and 70°C. This organism did not grow without the arsenic present, and therefore, arsenic may be a required growth factor. Interestingly, this was cultured from a co-culture of a tumbling, highly motile, short curved rod, which did not grow without the presence of KP1.B3. Attempts were made to isolate the motile rod, including serial dilutions, differing incubation temperatures (55, 65, 80 and 85°C) and growth in another arsenic-based medium (Macy *et al.*, 2000). However, these attempts failed and the motile rod did not grow at either 80 or 85°C. KP1 water (100 µl) was also spread onto solid Gelrite medium prepared with and without arsenic and incubated at 70°C, but, growth was not observed. Likewise, growth was not observed in anaerobic Db basal^2 roll tubes supplement with 1% pyruvate, β-alanine or with the supplements omitted when incubated at 70°C.

Table 3.8 Microorganisms isolated into pure culture from KP1 pool water.

Isolate	Morphology	Medium[D]	Temp. (°C)	pH	Aerobe/ Anaerobe	Suspected Genus	Isolated by:
KP1.A1	Long rod, (orange colonies)	CMD agar	65	7.0	Aerobe	'*Thermonena*'	H.M.[A]
KP1.A2	Short rod, (orange colonies)	CMD agar	65	7.0	Aerobe	'*Thermus*'	H.M.[A]
KP1.A3	Short rod (spore former)	CMD agar	65	7.0	Aerobe	'*Bacillus*'	H.M.[A]
KP1.A4	Short rod (non-spore former)	CMD agar	65	7.0	Aerobe	'*Bacillus*'	H.M.[A]
KP1.B1	Motile rod (terminal spheroids)	2/1+C and *Dictyoglomus*	70	7.0	Anaerobe	'*Fervidobacteria*'	T.N.[B]
KP1.B2	Long rod	AN1	70	7.0	Anaerobe	Unknown	T.N.[B]
KP1.B3	Long rod	As broth	65	6.5	Anaerobe	Unknown	T.N.[B]
BGP	Rod	Db basal[1] + β-glycero-phosphate	70	7.0	Aerobe	'*Geobacillus*': RAPD profile as for ALA isolate	R.R.[C]
GLU	Rod	Db basal[1] + glutamic acid	70	7.0	Aerobe	'*Geobacillus*': RAPD profile as for ALA isolate	R.R.[C]
PYR1	Rod (rough colony)	Db basal[1] + pyruvate	70	7.0	Aerobe	'*Geobacillus*': RAPD profile as for ALA isolate	R.R.[C]
PYR2	Rod (smooth colony)	Db basal[1] + pyruvate	70	7.0	Aerobe	'*Geobacillus*': RAPD profile as for ALA isolate	R.R.[C]
ALA*	Rod	Db basal[1] + L-alanine	70	7.0	Aerobe	*Geobacillus*	R.R.[C]
ASN	Rod	Db basal[1] + L-asparigine	70	7.0	Aerobe	'*Geobacillus*': RAPD profile as for ALA isolate	R.R.[C]
GABA	Rod	Db basal[1] + γ-aminobuytric acid	70	7.0	Aerobe	'*Geobacillus*': RAPD profile as for ALA isolate	R.R.[C]
AABA	Rod	Db basal[1] + α-aminobuytric acid	70	7.0	Aerobe	'*Geobacillus*': RAPD profile as for ALA isolate	R.R.[C]

[A] Prof. Hugh Morgan. [B] Thomas Niederberger. [C] Dr. Ron Ronimus. [D] See Appendix E for media compositions. *16S rRNA gene sequenced as discussed in Section 3.4.2.1.

3.4.2.1 Molecular analyses of KP1 isolates

Randomly amplified polymorphic DNA (RAPD) testing was undertaken on DNA isolated from all KP1 isolates to determine the similarity between isolates using primers OPR13 and OPR12 (Figures 3.8 and 3.9, respectively).

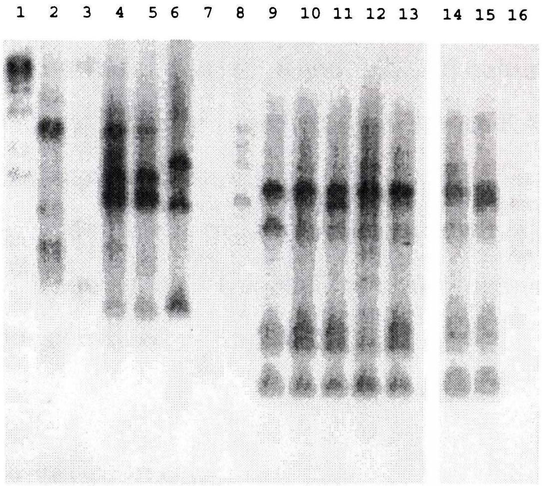


Figure 3.8 RAPD profiling of KP1 isolates using primer OPR13. The track numbers and corresponding samples are: (1) 1 kb ladder; (2) KP1.A1; (3) KP1.A2; (4) KP1.A3; (5) KP1.A4; (6) KP1.B1; (7) KP1.B2; (8) KP1.B3; (9) BGP; (10) GLU; (11) PYR1; (12) PYR2; (13) ALA; (14) ASN; (15) GABA; (16) AABA.

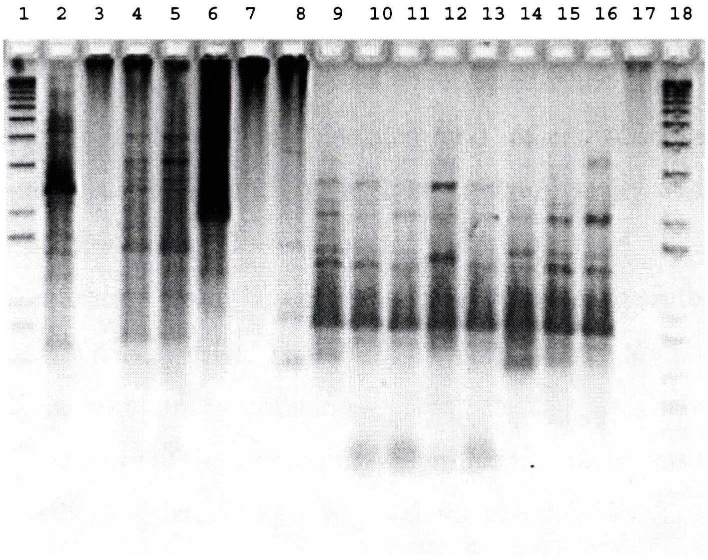


Figure 3.9 RAPD profiling of KP1 isolates using primer OPR12. The track numbers and corresponding samples are: (1) 1 kb ladder; (2) KP1.A1; (3) KP1.A2; (4) KP1.A3; (5) KP1.A4; (6) KP1.B1; (7) KP1.B2; (8) KP1.B3; (9) BGP; (10) GLU; (11) PYR1; (12) PYR2; (13) ALA; (14) ASN; (15) GABA; (16) AABA; (17) negative control; (18) 1 kb ladder.

Unfortunately, a RAPD profile was not obtained for isolates KP1.A2 and KP1.B2, possibly due to PCR inhibition. Isolate KP1.A2 was assumed to be a species of *Thermus* and it has previously been shown that obtaining a RAPD profile from *Thermus* is only possible with highly purified DNA (Shroll, 1997). The isolates KP1.A3 and KP1.A4 shared similar RAPD profiles as indicated in lanes 4 and 5 of Figures 3.8 and 3.9, suggesting they may be closely related which is supported by their respective DGGE profiles (Figures 3.6 and 3.7). All cultures isolated by aerobic incubation on supplemented Db basal[1] medium had similar RAPD profiles. The 16S rRNA gene of a representative culture (the ALA isolate, Table 3.8) was partially sequenced (248 bp) and found to be most closely related to *Geobacillus thermocatenulatus* (98% similar) and *Geobacillus stearothermophilus* (97% similar). The original sequence is contained in Appendix D.

3.4.3 Discussion and conclusion

As was observed for the initial study of KP1 in Section 3.3, colonised surfaces were dominated by diverse bacterial morphologies and archaea were not detected in any samples by PCR. However, the presence of archaea can not be fully discounted as the concentration of archaeal cells may be below the resolution of molecular-based detection. Bacterial dominance of KP1 was also reflected in the culturing results, with many different pure cultures being isolated and others observed which were recalcitrant to attempts at isolation. This high microbial diversity was also supported by the DGGE profiles using DNA extracted from colonised slides. The DGGE profiles of DNA extracted from the pure isolates were not represented in pool water or colonised slide DGGE profiles. Therefore, the pure isolates are possibly minority members within the community, growing well in selective enrichments but are not the major active members of the community. Significantly, there was no difference in DGGE profiles on the different surfaces used for colonisation, i.e. glass, steel or Teflon. The DGGE profiles of colonisation and the pure isolates from KP1 were complex containing many faint bands. Therefore, two PCR products derived from the same template DNA were combined and run in the same lane of a DGGE; however, this did not improve resolution, with bands being smeary.

Unfortunately, due to variable pool water level, temperature and poor DGGE resolution, the study of KP1 was suspended and the focus moved to higher temperature pools. The KP1 results are compared to AQ1, Champagne Pool and international studies in the final discussion and conclusion in Section 3.7.

3.5 Hot pool: AQ1

Over the major sampling period (15th July 2002 to 7th July 2003), the temperature of the pool fluctuated irregularly between 90 and 98°C and the pH between 7.4 and 8.2. The pool was well mixed with very active bubbling near the centre. By the 22nd November, 2003, the water level had dropped by approximately 1 meter, the bubbling had discontinued and the temperature and pH were 91°C and 7.91, respectively. Subsequently, the water level and temperature of AQ1 dropped further with the pool receding into three separate pools with temperatures below 80°C, sampling became dangerous and as a consequence, the study was suspended. Pool AQ1 was also included in a survey of microbial colonisation of glass slides in a number of high-temperature hot pools of New Zealand (Chapter 4).

3.5.1 Colonisation results

Microbial colonisation of slides (glass, Teflon and stainless steel) was documented by ATP, protein, DGGE, and microscopic observation (phase-contrast and DAPI staining). Initial studies of colonisation were undertaken on slides that were incubated in the shallow part of the pool near its periphery (5 cm below the water surface). Typically, slides were heavily silicified after 30 days incubation and DNA could not be isolated or amplified by PCR. Slides were also incubated directly within the sediment layer of the pool but colonisation was not observed by microscopy, DNA yields were below detectable levels and positive archaeal PCR amplifications were not obtained. Slides were also incubated deeper and closer to the centre of the pool by suspending them ~ 1 meter below the water surface.

3.5.1.1 ATP and protein analyses

The ATP and protein (on glass coverslips) levels measured on colonised surfaces are presented in Table 3.9, below.

Table 3.9 ATP and protein measured on colonised surfaces of AQ1.

Incubation period	ATP (RLU)	ATP (RLU) adjusting for area swabbed	µg of protein on glass cover slip
50 µl pool water	340:387[A]	-	-
200 µl pool water	327	327	-
3 hr	1618:1561[A]	1618:1561	-
5.5 hr	705	705	-
3 day glass	Not determined	-	Below detection limits
4 day glass	71006	71066	-
8 day glass	Overload[B]	Overload	Below detection limits
8 day steel (1 side)	135070:130172[A]	135070:130172	-
11 day glass	Overload	Overload	-
17 day glass	89033[B]	356132	9.0
17 day Teflon	163642[B]	654568	-
17 day steel	109546[B]	438184	-

[A]Duplicate measurements made. [B]Quarter of slide swabbed, i.e. half of one side. Overload relates to a measurement above ~ 180 000 RLU.

3.5.1.2 Microscopy of colonised slides

All micrographs of colonised slides for AQ1 are contained on the supplementary compact disc, located on the inside of the back cover of the thesis. All surfaces incubated in AQ1 were initially (up to 24 hour) colonised by short rods with an example of a typical micrograph presented in Figure 3.10(a). Subsequently, the slides incubated in the shallow part of the pool developed a thick biofilm consisting of mainly filamentous rods (observed for all surfaces, i.e. steel and glass) as presented in Figure 3.10(b). For slides incubated in the deeper part of the pool this transition to filamentous organisms was not observed even after 11 days incubation. Additionally, only on the slides incubated at the deeper region were cocci also observed on the surfaces (Figure 3.10(c)).

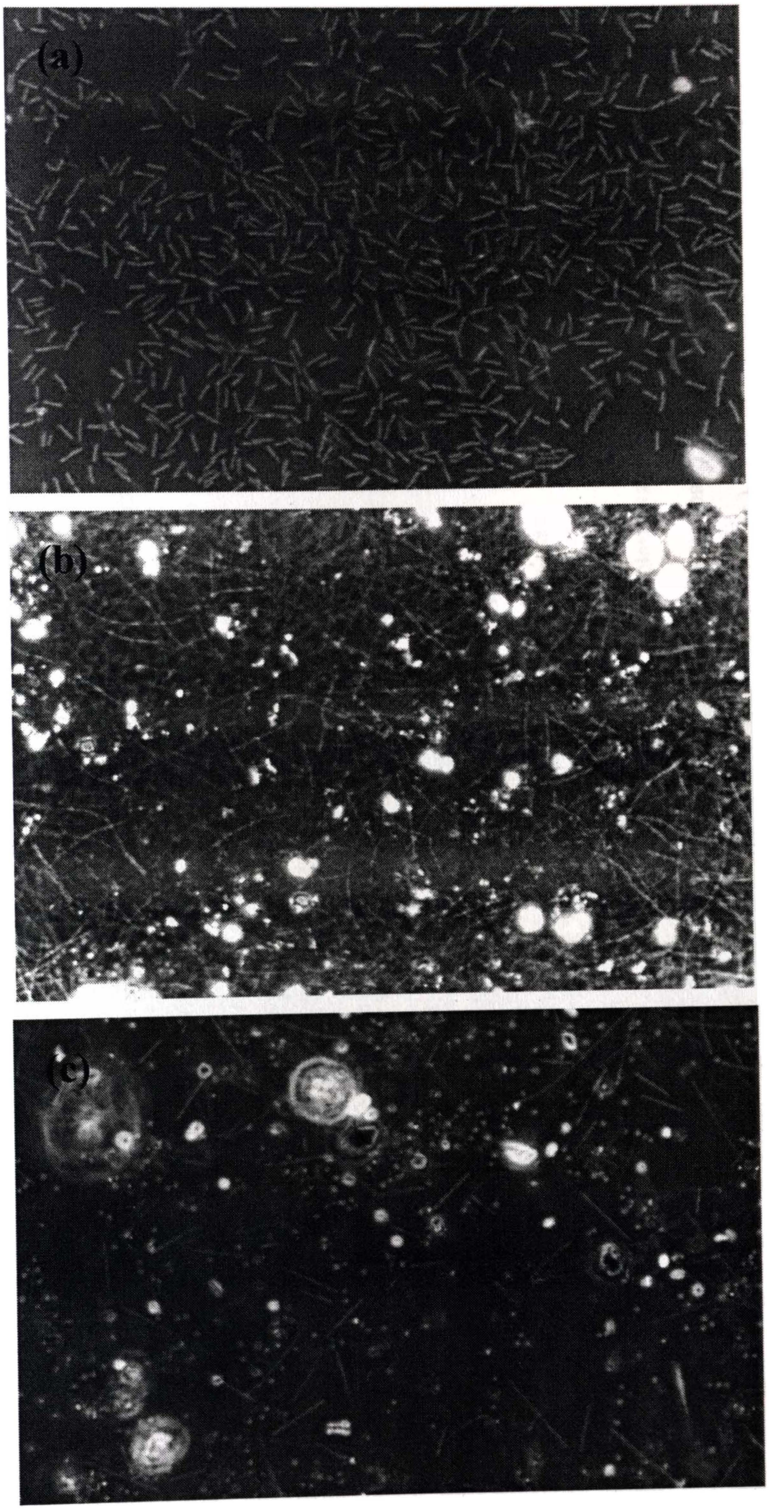


Figure 3.10 Phase-contrast micrographs of colonised glass slides (1000X) incubated in AQ1: (a) 24 hours incubation; (b) 19 days incubation; (c) deep incubation.

3.5.1.3 Molecular analyses of colonised surfaces

An archaeal DGGE was undertaken (bacteria were not detected by DGGE PCR) on DNA isolated from slides incubated in both the shallow and deep positions of the pool (Figure 3.11). Included in the DGGE were the DGGE profiles of DNA extracted from the pure cultures isolated from AQ1, i.e. coccal isolate, AQ1.S1^T and rod isolate, AQ1.S2 (see Section 3.5.4) and DNA extracted from a 0.22 μm filter used to filter AQ1 pool water.

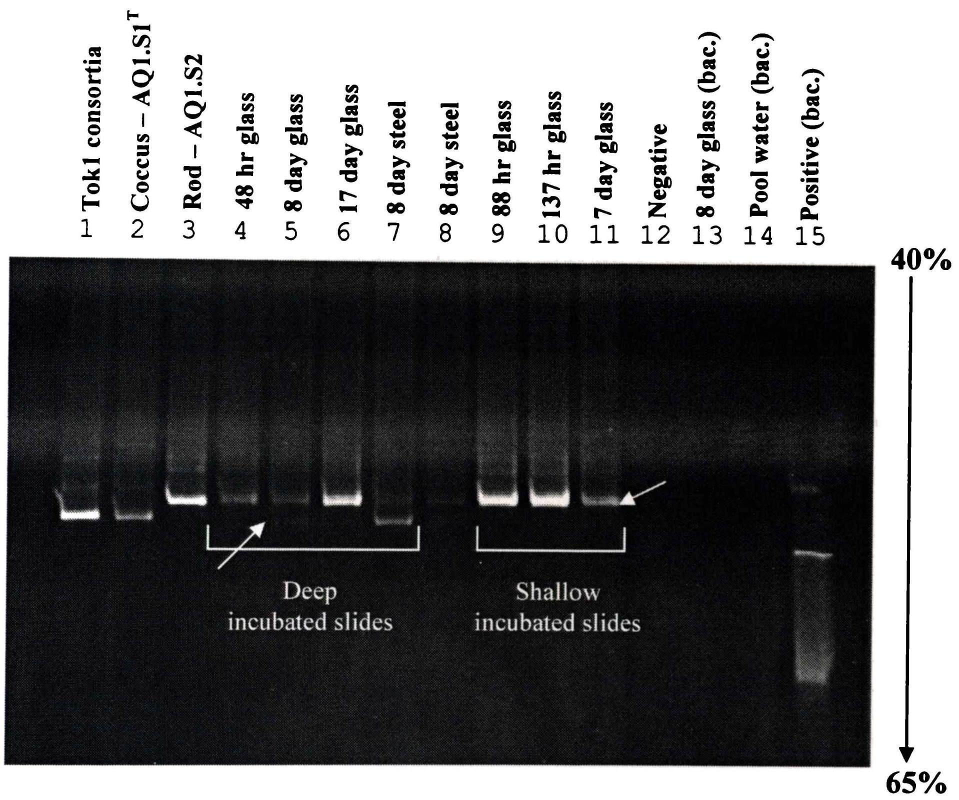


Figure 3.11 Archaeal DGGE of colonised slides, pool water and organisms isolated from AQ1. The lanes and the corresponding samples are: (1) Tokl consortium (see Section 3.5.4.1); (2) coccal isolate, AQ1.S1^T; (3) rod isolate, AQ1.S2; (4) 48 hour glass; (5) 8 day glass; (6) 17 day glass; (7) 8 day steel; (8) pool water; (9) 88 hour glass (10) 137 hours glass; (11) 7 day glass; (12) negative control; (13) 8 day glass (bacterial PCR); (14) pool water (bacterial PCR); (15) positive bacterial PCR control (*Thermotoga maritima*). Arrows indicate sequenced DGGE bands.

A dominant DGGE band was common to all colonisation samples which was in the same position as that of the rod isolate, AQ1.S2 (lane 3). A representative band as indicated by an arrow in lane 11, was extracted, purified and sequenced (see Appendix D for sequence) and was 100% identical (380 bp) to both *Pyrobaculum* *caldifontis* (AB078332) and the 16S rDNA from the rod isolate, AQ1.S2 (Section 3.5.4.2). A second lower migrating dominant band of the gel (indicated by an arrow in lane 5) was common to the slides incubated deep within the pool (lanes 4 to 7) and pool water (lane 8) and was only present in DGGE profiles when the coccal colonisers were observed on slides incubated deep within the pool (see Section 3.5.1.2), and was therefore assumed to be representative of this coccus. A representative band was sequenced (Appendix D), and its closest NCBI relative (408/420 bp; 97.1%) was an uncultured member of the Desulfurococcales order, clone SUBT-9 (AF361213) obtained from subterranean hot springs in Iceland with its closest cultured representative (407/421 bp; 96.7%) being *Aeropyrum pernix* strain K1^T (BA000002).

3.5.2 Molecular analyses of pool water

Approximately 2 litres of AQ1 pool water was filtered through a 0.45 µm filter and the filtrate ultrafiltered with an YM10 cut-off membrane and DNA extraction undertaken from the liquid concentrate. DNA was not detected following the extraction and both archaea (DGGE-based) and nanoarchaea (Eder *et al.*, 1999) were not detected by PCR.

Filtration was repeated using approximately 2 litres of AQ1 pool water and a 0.22 µm filter and DNA extracted from the filter surface was used for archaeal DGGE as shown in Figure 3.11. Bacteria were not detected in the pool water DNA with near-full length 16S rDNA or DGGE PCR. Positive nanoarchaeal PCR amplification was obtained from the DNA extracted from the filter (PCR assay by McCliment *et al.*, in press) as part of a survey of New Zealand hot pools as discussed in Section 4.5. The nanoarchaeal amplicon was sequenced and its closest relative was identified as an uncultured nanoarchaeote (89%, 261/293 bp), clone CU-1 (AJ458437) from the Uzon Caldera (Hohn *et al.*, 2002). Finally, the AQ1 pool water DNA was used to prepare an archaeal 16S rDNA clone library as discussed below.

The near-full length 16S rRNA gene was PCR amplified using primer pair RR149 (27F) and RR77 (1522R) from the DNA extracted from the 0.22 μm filter used to filter AQ1 pool water. The resulting amplicon was checked for size (1500 bp) by electrophoresis, purified and cloned into *E. coli*. A total of 101 clones containing presumed 16S rDNA (i.e. ~1500 bp insert) were grouped into 7 operational taxonomic units (OTUs) by restriction endonuclease digestion (*Eco*R I, *Hin*d III, *Hha* I and *Rsa* I). The *Hha* I digests of the clones provided complex profiles that were similar between all clones; therefore, *Hha* I digest profiles were not used in the grouping of OTU. Example restriction endonuclease patterns of some OTUs in a 2.5% agarose gel are presented in Figure 3.12.

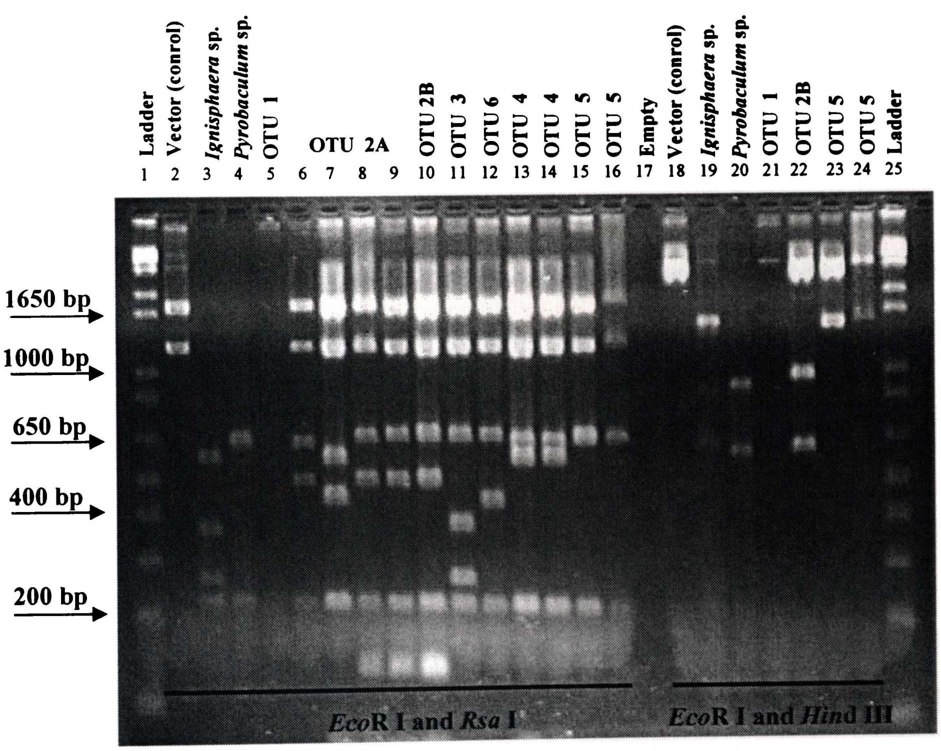


Figure 3.12 OTU from the cloning of archaeal 16S rRNA genes from AQ1 pool water. Lanes 3 to 16 are *Eco*R I and *Rsa* I digests and lanes 19 to 24 are *Eco*R I and *Hin*d III digests. The track numbers and corresponding samples are: (1) 1 kb ladder; (2) digested vector without insert; (3) *Ignisphaera aggregans*, AQ1.S1^T 16S rDNA; (4) *Pyrobaculum* sp. AQ1.S2 16S rDNA; (5) OTU1; (6) OTU2A; (7) OTU2A (short insert); (8 and 9) OTU2A; (10) OTU2B; (11) OTU3; (12) OTU6; (13 and 14) OTU4; (15 and 16) OTU5; (17) empty; (18) digested vector without insert; (19) *Ignisphaera aggregans*, AQ1.S1^T 16S rDNA; (20) *Pyrobaculum* sp. AQ1.S2 16S rDNA; (21) OTU1; (22) OTU2B; (23 and 24) OTU5; (25) 1 kb ladder.

A representative clone from each OTU was sequenced with the closest relative (NCBI BLASTn) and the identity of each OTU presented in Table 3.10 (the 16S rRNA gene sequences are listed in Appendix D).

Table 3.10 Closest relatives of sequenced clones and the abundance of each OTU from an AQ1 pool water 16S rDNA clone library.

OTU type	No. of clones	Closest NCBI relative	Identity (bp)	% Similarity	Accession no.
1	90/101	*Pyrobaculum caldifontis*	583/588	99.1%	AB078332
2A	3/101	*Pyrobaculum* sp.	88/88	100%	*
2B	2/101	Uncultured archaeon clone	474/488	97.1%	AB095128
3	1/101	*Pyrobaculum neutrophilus*	110/111	99.1%	AB178792
4	2/101	Uncultured archaeon clone	415/429	96.7%	AY672492
5	2/101	Unknown contaminant?	-	-	-
6	1/101	*Pyrobaculum* sp.	74/74	100%	*

***Includes: *Pyrobaculum aerophilum* (AE009843, AE009441), *P. calidifontis* (AB078332) and *P. neutrophilus* (AB178792).**

The clone library was dominated (95 of the 101 clones) by species of *Pyrobaculum* and included 4 clones (OTUs 2B and 4) that were most closely related to archaeal clones obtained from hydrothermal vent samples from the East Pacific Rise (EPR). Although OTUs 2A, 3 and 6 only provided short sequence read-lengths (<110 bp), their closest NCBI BLAST relatives were in the 99 to 100% range. The short sequencing reads may be due to the presence of proteins within the crude plasmid preparations. Interestingly, neither *Ignisphaera* detected by culture enrichments (see Section 3.5.4.1) nor the putative *Aeropyrum* signature detected on colonised slides (see Section 3.5.1.3) which have been detected in the pool were represented in the library.

3.5.3 Colonisation of surfaces incubated at varied water depths

From the initial AQ1 colonisation results, a transition from pioneer short rods to a thick biofilm consisting of filamentous rods was observed on slides incubated in the shallow depths of the pool. In contrast, colonisation rates for slides incubated at depths of ~ 1 meter were slower than the shallow slides and the colonising rods did not transform to the filamentous morphology and colonisation by cocci was also prevalent. To further investigate these differences in colonisation, slides were incubated at three different positions of the AQ1 water column: (1) 'shallow' - slides touching the top of the water surface (slides touching the water level); (2) 'mid' – slides incubated ~ 15 cm below the water level in glass racks positioned on top of the sediment layer; (3) 'deep' – slides suspended in plastic slide holders ~ 1 meter below the water level.

The temperature at the 'shallow' incubation depth was 95°C and the 'mid' and 'deep' sites were both 98°C. Slides were removed from each depth after 3.5, 16, 20, 24, 40 and 44 hours incubation and colonisation documented by ATP assay, phase-contrast microscopy and the DNA-based methods, DGGE and randomly amplified polymorphic DNA (RAPD).

3.5.3.1 ATP analyses

Slides were removed and ATP levels were measured on a single side of a slide's surface, with the results presented in Table 3.11.

Table 3.11 ATP levels measured on colonised slides incubated at differing depths in AQ1.

	50 µl	3.5 hours	16 hours	20 hours	24 hours	40 hours	44 hours
Shallow	180	92	Overload	95912	Overload	109766	Overload
Mid	-	131	3216	7764	4279	75868	32424
Deep	-	180	194	1104	1348	11945	30830

Overload relates to a measurement above ~ 180 000 RLU.

The ATP levels of colonised surfaces for 3.5, 20 and 40 hours from Table 3.11 (other values ignored due to overload values) for all incubation depths were plotted on a graph as presented below in Figure 3.13.

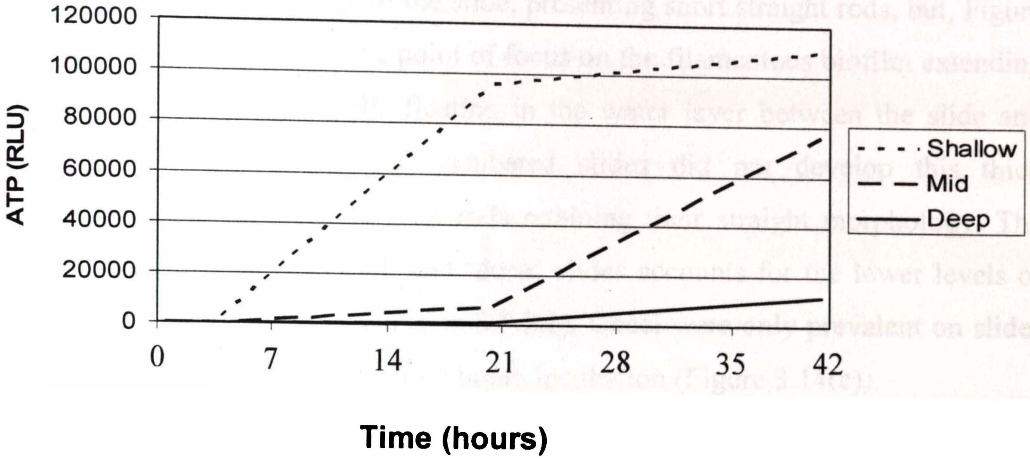


Figure 3.13 ATP (RLU) measured on colonised slides incubated at different depths of pool AQ1.

As indicated by the graph in Figure 3.13, colonisation rates were fastest for the shallow incubated slides with colonisation rates decreasing as the slides were incubated deeper within AQ1. These ATP levels are also reflected in the concentration of cells observed by microscopy as presented in Section 3.5.3.2, below.

3.5.3.2 Microscopic observation of colonised slides

All phase-contrast micrographs of colonised slides are contained on the supplementary compact disc, located inside the back cover of the thesis. Rods were observed colonising 'shallow' incubated slides after 3.5 hours incubation; however, colonisation of 'mid' and 'deep' positioned slides by rods was only observed after 16 hours and 40 hours, respectively. These differences in initial colonisation times are reflected by the slower rates of ATP development on the 'mid' and 'deep' incubated slides (Section 3.5.3.1). Furthermore, the 'shallow' incubated slides were silicified earlier than the 'mid' and 'deep' slides.

As was observed for the initial colonisation studies (Section 3.3), a thick biofilm consisting of filamentous rods eventually developed on the 'shallow' incubated slides. For example, Figure 3.14(a) presents a slide incubated for 44 hours where the point of focus is on the surface of the slide, presenting short straight rods, but, Figure 3.14(b) is the same slide with the point of focus on the filamentous biofilm extending from the slide surface (i.e. cells floating in the water layer between the slide and coverslip). The 'mid' or 'deep' incubated slides did not develop this thick filamentous containing biofilm, with rods retaining their straight morphology. The lack of a heavy biofilm for 'mid' and 'deep' slides accounts for the lower levels of ATP measured on these slides (Section 3.5.3.1). Cocci were only prevalent on slides incubated at the 1 metre depth after 44 hours incubation (Figure 3.14(c)).

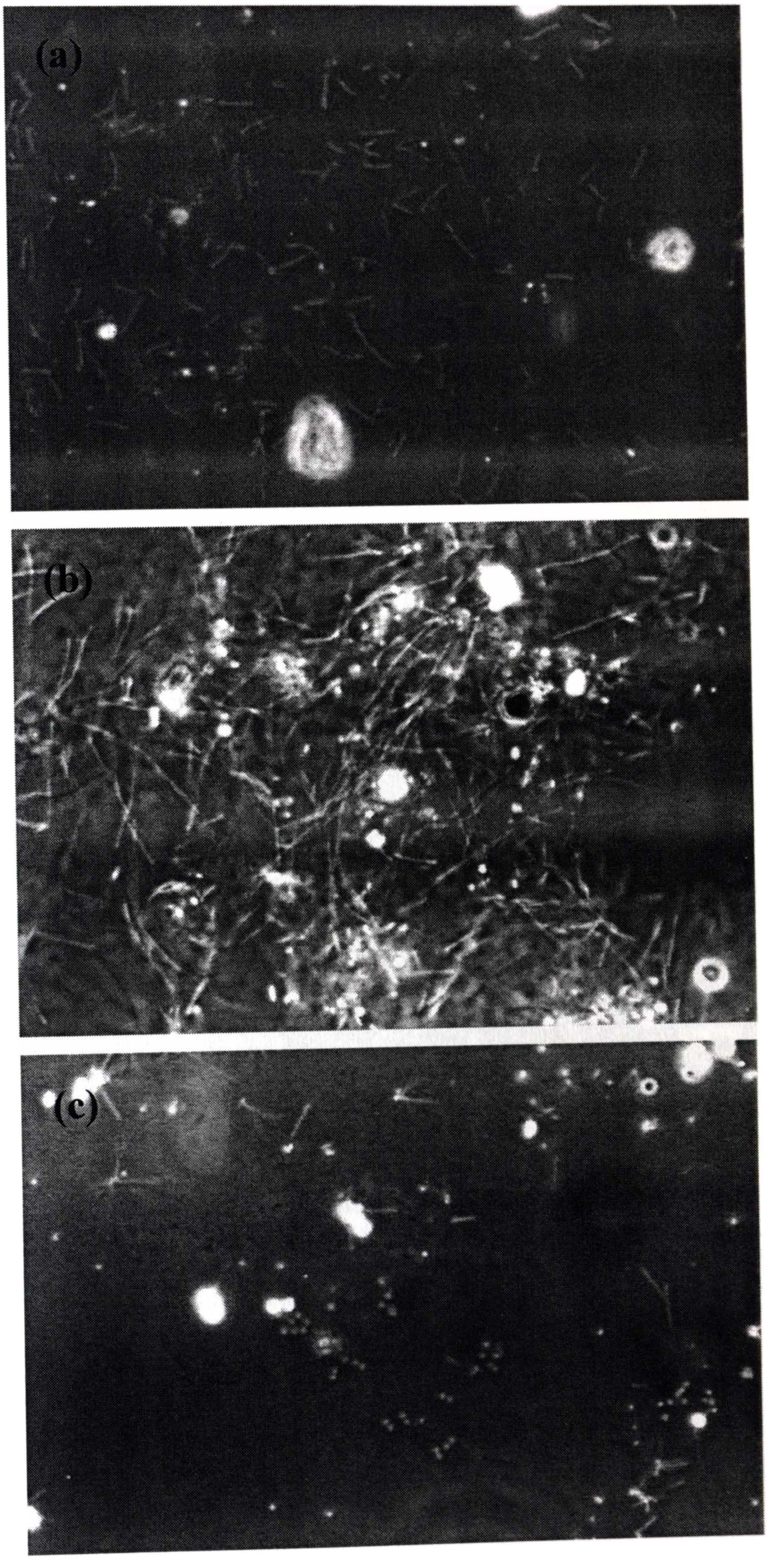


Figure 3.14 Colonisation micrographs of glass slides (phase-contrast; 1000X) incubated in AQ1 at differing depths: (a) 'shallow' - 44 hours incubation (focus on surface of slide); (b) 'shallow' - 44 hours incubation (focus off surface of slide); (c) 'deep' - 44 hours incubation.

3.5.3.3 Molecular analyses of slides

DNA was extracted from the surfaces of all slides with the exception of the 3.5 hour slides. However, the 'deep' incubated, 16, 20 and 24 hour slides did not provide detectable levels of DNA and consequently did not provide positive archaeal 16S rDNA PCR amplicons. Bacteria were not detected on any of the incubated slides by PCR amplification and an archaeal DGGE of DNA extracted from colonised slides is presented in Figure 3.15 (including the cultures isolated from pool AQ1, see Section 3.5.4).

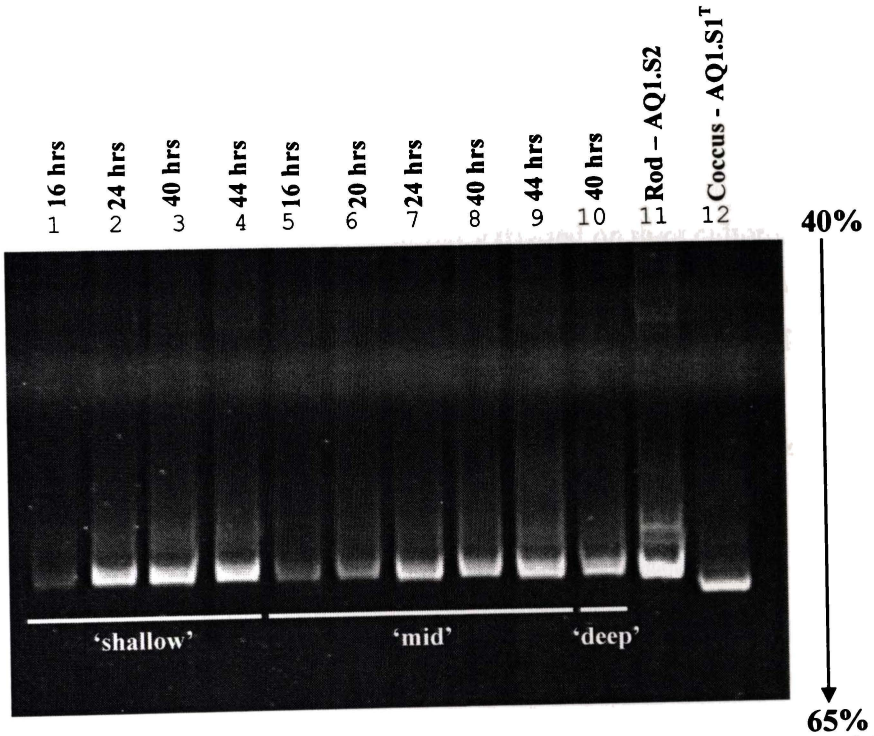


Figure 3.15 Archaeal DGGE from slides incubated at differing depths incubated in AQ1. The track numbers and corresponding samples are: (1) 16 hrs 'shallow'; (2) 24 hrs 'shallow'; (3) 40 hrs 'shallow'; (4) 44 hrs 'shallow'; (5) 16 hrs 'mid'; (6) 20 hrs 'mid'; (7) 24 hrs 'mid'; (8) 40 hrs 'mid'; (9) 44 hrs 'mid'; (10) 40 hrs 'deep'; (11) *Pyrobaculum* isolate, AQ1.S2; (12) coccal isolate, AQ1.S1^T.

All colonisation samples (lanes 1 to 10, Figure 3.15) had a dominant DGGE band residing in the same position as the *Pyrobaculum* isolate, AQ1.S2 (lane 11), therefore, it was assumed that this band was also the sequenced *Pyrobaculum* DGGE band as identified in Section 3.5.1.3, Thus, the different types of rods colonising slides of all three incubation depths, i.e. short pioneer rods and the filamentous rods of the 'shallow' slides and the straight rods of 'mid' and 'deep' slides are possibly identical members of the genus, *Pyrobaculum*. However, there maybe differing strains or species of *Pyrobaculum* colonising the slides that accounts for the differing rod morphologies. Therefore, the entire genome-based RAPD PCR assay (as opposed to single gene-based DGGE analysis) was undertaken in the expectation that any genetic differences below the genus level would be detected. These RAPD results are discussed below in Section 3.5.3.4. From Figure 3.15 it was apparent that the DGGE band associated with the coccus colonisers of slides incubated 'deep' in pool AQ1 was absent in all DGGE profiles and cocci were only detected by microscopy on the slides incubated at the 'deep' position after 44 hours incubation.

3.5.3.4 RAPD analysis

RAPD primers (OPR12 and OPR13) were initially trialled on pure cultures of archaea including the pure cultures isolated from pool AQ1, *Pyrobaculum* sp., AQ1.S2 and *Ignisphaera aggregans*, AQ1.S1^T (see Section 3.5.4). Therefore, the RAPD profile of the *Pyrobaculum* isolate, AQ1.S2 could be compared against the RAPD profiles of the slides colonised by *Pyrobaculum* sp. A 1.5% agarose gel with the result of the OPR12 and OPR13 RAPD trial is presented in Figure 3.16.

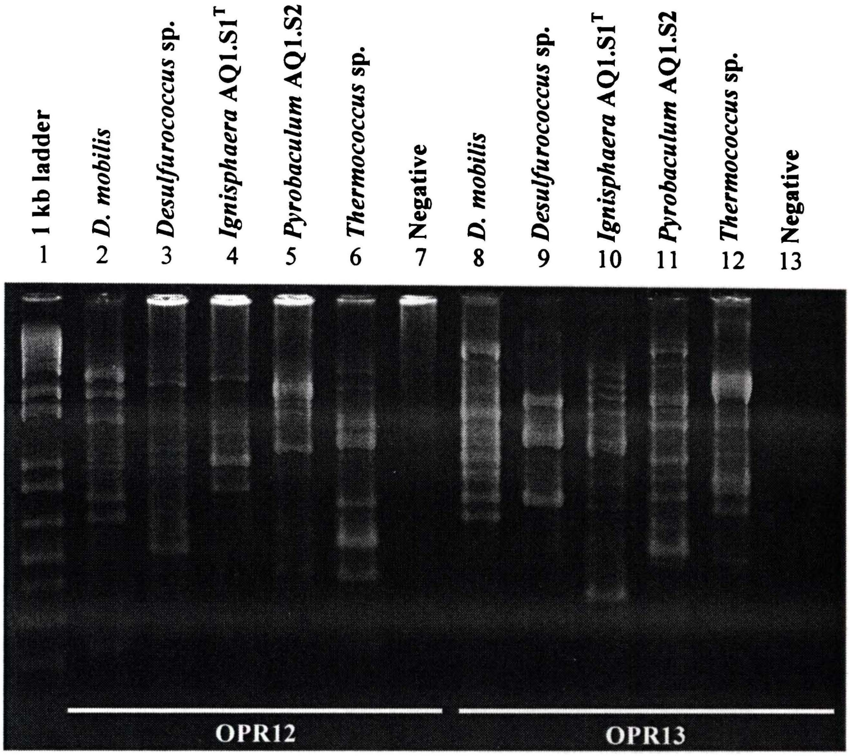


Figure 3.16 RAPD profiling of archaea including pure cultures isolated from pool AQ1. Samples in lanes (2) to (7) are screened with primer OPR12 and (8) to (13) with OPR13. The lane numbers and corresponding samples are: (1) 1 kb ladder; (2) *Desulfurococcus mobilis*; (3) *Desulfurococcus*-related isolate, TRUCC 321 (see Section 5.2.1); (4) *Ignisphaera* isolate, AQ1.S1^{T} (see Section 3.5.4.1); (5) *Pyrobaculum* isolate, AQ1.S2 (see Section 3.5.4.2); (6) *Thermococcus zilligii*, strain AN1; (7) negative control; samples in lanes (8) to (13) are the same as for (2) to (7).

The RAPD profile using OPR13 for the *Pyrobaculum* isolate, AQ1.S2 (lane 11, Figure 3.16) was more informative than the OPR12 profile (lane 5), due to the presence of more clearly defined bands. Therefore, primer OPR13 was used for a RAPD analysis of DNA from slides colonised for different time periods and at varying depths in pool AQ1, and compared to the RAPD profile of the pure culture *Pyrobaculum* isolate, AQ1.S2 which had been isolated from the pool. Results of the comparison are presented in Figure 3.17.

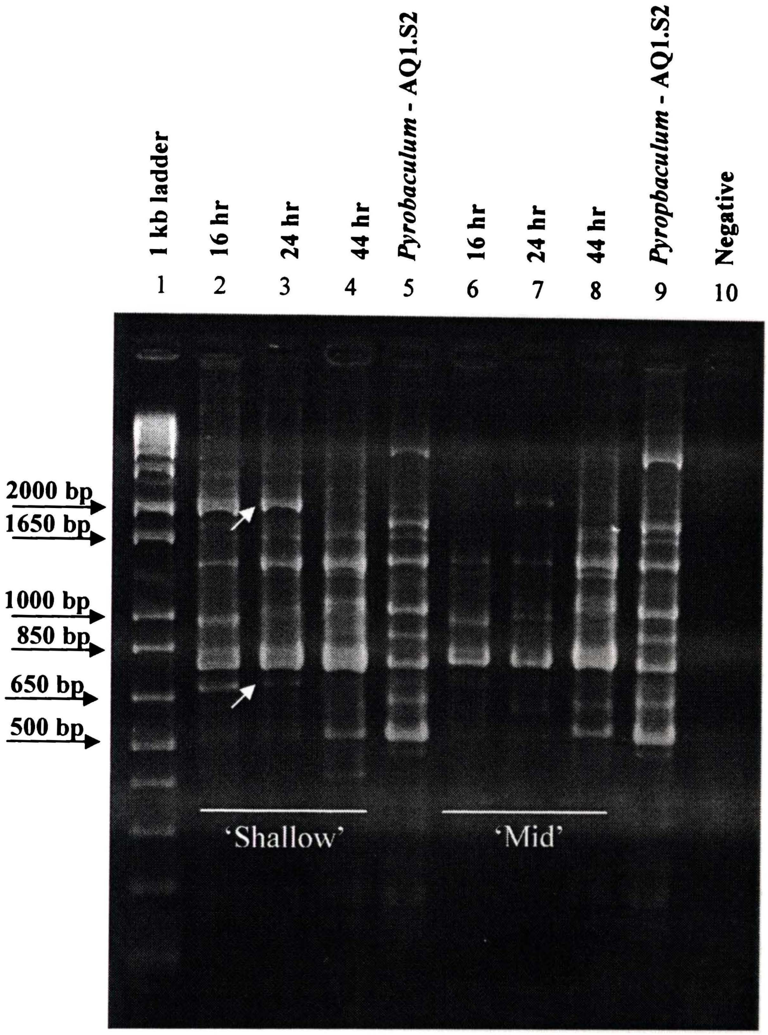


Figure 3.17 RAPD profiles of AQ1 colonised slides. The lane numbers and corresponding samples are: (1) I kb ladder; (2) 16 hours 'shallow'; (3) 24 hours 'shallow'; (4) 44 hours 'shallow'; (5) *Pyrobaculum* isolate, AQ1.S2; (6) 16 hours 'mid'; (7) 24 hours 'mid'; (8) 44 hours 'mid'; (9) *Pyrobaculum* isolate, AQ1.S2; (10) negative control.

The majority of dominant bands in each profile may indicate the presence of the *Pyrobaculum* isolate AQ1.S2 in each biofilm sample, but no two profiles were identical. The minor differences in banding pattern between the *Pyrobaculum* sp. and the environmental samples may be due to differing strains of *Pyrobaculum* or the presence of other DNAs from the pool environment, e.g. *Ignisphaera* sp. or the *Aeropyrum*-related deep-associated coloniser. The RAPD profiles from slides incubated in the shallower depths of the pool (lanes 2 to 4) exhibited slightly different patterns between early and late colonisation (bands indicated by arrows in Figure 3.17) which may be indicative of a change in the microbial population of the biofilm.

3.5.4 Culturing results

The compositions of all media used are listed in Appendix E. Various media were used in attempts to enrich for organisms from AQ1 pool water as listed in Table 3.12. A rod and a coccus were isolated into pure culture using Db+S medium from an enrichment of rods and cocci incubated at 90°C. This enrichment consortium was stable on successive transfers on the same medium. Initially, many of the rods had a golf-club morphology; however, the rods did not retain this golf-club morphology after serial dilution. Gelrite roll tubes (Db+S) were used in an attempt to isolate the rod and coccus into pure culture, however, growth was not observed. The stable consortium of rods and cocci grown on Db+S medium had a typical pattern of development when transferred to fresh medium. Only rods were observed over the first 24-48 hours, and as the increase of rods levelled off, cocci increased in abundance over the next 48 hours. Both the rod and coccus were isolated into pure culture by serial dilution; the rod was isolated by serial dilutions of a nascent consortia culture in Db+S medium incubated at 80°C and designated AQ1.S2, it was further characterised as outlined in Section 3.5.4.2, below. The coccus was isolated by serial dilutions from an older 90°C culture (where the cocci dominated) and designated AQ1.S1^T and was further characterised as discussed in Section 3.5.4.1, below.

MPN enumeration (Db+S medium in triplicate) of AQ1 pool water indicated densities of only 2.4×10^2 cells per ml for both rods and cocci.

, **Table 3.12 Enrichment attempts from AQ1 pool water.**

Medium	Solid (Gelrite) or Broth	Incubation temp. (°C)	Enrichments	Notes
AN1	Broth	90	Rods - low density	Black ppt. probably H_2S
		80	No growth	
Db	Broth	90 & 80	Rods – low density	
Db (aerobic)	Broth	90	No growth	
Db+S	Broth	90	Rods and cocci	Isolated: coccus (AQ1.S1) & rod (AQ1.S2)
	Broth	80	Rods	
	Roll tubes	90	No growth	
Db+Fe^{3+} citrate	Broth	90	Golf-clubs	
Arsenic-based (Huber *et al.*, 2000b)	Broth	90	Golf-clubs	
Thermoproteus medium	Broth	90	No growth	
Thermoproteus uzoniensis medium	Broth	90	No growth	
Db basal^2 + supplements^A	Plates^C	85	No growth	0.5 ml inocula.
Db basal^2 + supplements^B	Broth	85	Rods and cocci (no supplements) and rods (di-ammonium succinate)	
Db basal^2 + starch (2 g/L)	Roll tubes	90	No growth	0.5 and 0.1 ml inocula.

Media prepared anaerobically (N_2) and inoculated with 1 ml pool water unless otherwise stated. ^{A}Supplements (1% w/v) include: β-alanine, putrescine, sodium-β-glycerophosphate, glutamic acid and glycine. ^{B}Supplements (1% w/v) include: di-ammonium succinate, sodium pyruvate, β-alanine, sodium maleate, glutamic acid, glycine, sodium-β-glycerophosphate and putrescine. ^{C}CO_2 systems envelope, BBL GasPak, Becton-Dickson, USA.

3.5.4.1 Coccus isolate: AQ1.S1^T

As described in Section 3.5.4, a coccus designated AQ1.S1^T was isolated into pure culture from a co-culture with a rod. Both strands of the near-full length 16S rRNA gene from AQ1.S1^T were sequenced by PCR amplification as described in Chapter 5 (sequence contained in Appendix D). Sequence alignment indicated a close relationship (98%, 1191 nucleotides) to a previously identified archaeal coccus (designated, Tok1) in co-culture with a *Pyrobaculum* species obtained from a New Zealand hot pool (Götz, 1998). Attempts were made to isolate Tok1 using the Db+S medium used to isolate AQ1.S1^T, however, only the rod was obtained by serial dilution. Götz had devoted considerable time attempting to isolate the coccus as a pure culture from this consortium, without success, though the rod was easily obtained in pure culture by serial dilution. Given the previous attempts an exhaustive effort was not expended on this aspect. The closest characterised cultured relative of AQ1.S1^T (94.5%) was *Staphylothermus marinus* (X99560). The 16S rRNA nucleotide sequence grouped the isolate within the Crenarchaeota, with the most conservative phylogenetic position of AQ1.S1^T being a novel genus within the order Desulfurococcales (see Chapter 5). AQ1.S1^T was subsequently fully characterised and a manuscript was submitted to the International Journal of Systematic and Evolutionary Microbiology (IJSEM) proposing that strain AQ1.S1^T represents the type strain of a novel genus, *Ignisphaera aggregans*. This manuscript is reproduced and forms the basis of Chapter 5. As described in this manuscript *Ignisphaera* 16S rRNA gene sequences were obtained from four different thermal springs, namely: AQ1.S1^T, Tok1, Tok37.S1 and Tok10A.S1 (locations and descriptions of the springs and the isolation procedures are described in Chapter 5). Due to the phylogenetic grouping of these isolates being unclear, their sequences were aligned against crenarchaeotal 16S rRNA gene sequences (using the ARB software package) and the nucleotide positions to each phylogenetically distinct group of the Crenarchaeota were compared to the Desulfurococcales-specific nucleotides as presented in Table 3.13, (Table 3.13 modified from Itoh *et al.*, 2003).

Table 3.13 16S rRNA nucleotide positions specific to Crenarchaeota groups including *Ignisphaera* species.

Sequence position (*E. coli*)	***Acidilobus* group**	***Desulfurococcales***	***Ignisphaera* isolates (4 seqs. in total)**	***Thermoproteales/ Sulfolobales***
34	T	C	C	C
321:332	C:G	A:G	A:G	A:G
605:633	T:G	C:G	**A:T**	T:G
1308:1329	T:A	C:G	C:G	C:G
1335	C	G	G	G(A)
1393	T	C	C	C
Nucleotides specific to *Ignisphaera*				
59	G	G	A*	G
428	G	G	A	C
613	C	C	T	C
627	G	G	A	G
1112	C	C	T	C
1314	C	C	T (No Tok1 seq.)	C
1323	G	G	A (No Tok1 seq.)	G

***Only AQ1.S1^T and Tok1 sequence available. Table modified from Itoh *et al.*, (2003).**

From Table 3.13 the *Ignisphaera* isolates contain all the nucleotides specific to the *Desulfurococcales* with the exception of sequence positions 605 and 633 containing an adenine and thymine nucleotide, respectively. A number of other *Ignisphaera*-specific nucleotides are also presented in Table 3.13 which differ from those of all other Crenarchaeotal groups. Together, these differences might substantiate that the *Ignisphaera* group of organisms might not be members of the *Desulfurococcales*.

3.5.4.2 Rod isolate: AQ1.S2

As described in Section 3.5.4, a microorganism designated AQ1.S2 was isolated into pure culture from a co-culture of rods and cocci (AQ1.S1^T). The near-full length 16S rRNA gene for this pure culture was obtained (sequence contained in Appendix D) and its closest NCBI BLASTn relative (98.9%) was *Pyrobaculum caldifontis* (AB078332). Phylogenetically, AQ1.S2 grouped very closely with species of *Pyrobaculum,* and may represent a novel species/strain of the genus, although, further phenotypic characterization would be required to confirm this.

A nanoarchaeal amplicon was also obtained from DNA isolated from a culture of AQ1.S2 by 16S rDNA PCR assay (McCliment *et al.*, in press), as included in a nanoarchaeal survey of New Zealand hot pools (see Figure 4.15 in Section 4.5). Attempts were made to sequence this amplicon, but were unsuccessful. The primers (Hohn *et al.*, 2002) used in the nanoarchaeal PCR (McCliment *et al.*, in press) were tested for specificity and did not to amplify 16S rDNA from a *Pyrobaculum* sp. cloned into *E. coli*. Therefore, the positive nanoarchaeal amplification from the *Pyrobaculum* AQ1.S2 culture indicates the presence of nanoarchaea co-existing with the *Pyrobaculum* AQ1.S2 "pure culture". This must be a stable co-culture as it persisted through many transfers and serial dilutions. Attempts were made to isolate nanoarchaeal cells from the AQ1.S2 culture, including vortexing cultures to dislodge nanoarchaeal cells from the assumed *Pyrobaculum* host and then filtering the culture through a 0.45 μm filter and using the filtrate for nanoarchaeal 16S rDNA PCR. Cultured nanoarchaeal cells (*Nanoarchaeum equitans*) have a 400 nm diameter

(Huber *et al.*, 2002); therefore, it was assumed that nanoarchaeal cells could pass through the filter. However, positive nanoarchaeal amplicons were not obtained from the filtrate. In addition, the differential centrifugation separation of nanoarchaeal cells (*Nanoarchaeum equitans*) from its *Ignicoccus* host, as undertaken by Hohn *et al.*, (2002) was attempted; however, the *Pyrobaculum* cells were also isolated during the nanoarchaeal-specific separation centrifugation step.

3.5.5 Discussion and conclusion

Colonisation rates of surfaces incubated in AQ1 as measured by ATP levels were most rapid for slides incubated close to the surface. Furthermore, cell morphologies on colonised slides differed between incubation depths, i.e. colonisation of slides incubated near the surface involved a transition from short straight rods to a heavy filamentous biofilm (accounting for the high ATP levels) over a period of 44 hours, whereas slides incubated deeper within the pool did not develop this filamentous biofilm. Colonisation by coccal cells was observed by microscopy and DGGE only on the slides incubated at depths of circa 1 metre. By sequencing DGGE bands and RAPD testing it was shown that essentially the same *Pyrobaculum* species was present on all colonised surfaces including those where the morphology changed from rods to filaments. Minor changes in the RAPD profile of these colonising organisms could possibly indicate that different species or strains of *Pyrobaculum* were involved. Some *Pyrobaculum* species are able to undertake aerobic respiration; therefore, the thick biofilm developing only at the shallow depth may have been due to *Pyrobaculum* cells using the water-air interface to obtain atmospheric oxygen for metabolism (Völkl *et al.*, 1993; Amo *et al.*, 2002; Sako *et al.*, 2001) but attempts to grow *Pyrobaculum* strain AQ1.S2 aerobically or under microaerophilic conditions were unsuccessful. Likewise, attempts to isolate aerobic cultures of *Pyrobaculum* from AQ1 were unsuccessful. The cocci colonising slides incubated deep in the pool grouped phylogenetically to *Aeropyrum pernix*. This was surprising since *A. pernix* has only been isolated from marine hydrothermal vent ecosystems. In addition, *A. pernix* is an aerobic microorganism, therefore, if metabolism were to be inferred from a microorganism's phylogenetic relationship, such a coccus might have been predicted to reside close to the air-water interface rather than deep in the pool where available oxygen would be non-existent. As described in Chapter 4, DNA signatures closely related to the coccal coloniser of slides incubated deep within the water column of AQ1 were detected on slides incubated in other NZ pools of similar temperature and pH. Attempts were made to culture this coccus from these pools (including AQ1, see Section 4.3.2) using modified media (lowered salt composition) used to culture *Aeropyrum* species; however, these were unsuccessful.

The 16S rDNA clone library of AQ1 pool water also showed *Pyrobaculum* dominance, however, neither the coccal *A. pernix*-related coloniser nor the *Ignisphaera* isolate were detected. Similarly, culture-based enrichments were dominated by rods, with a *Pyrobaculum* species (AQ1.S2) isolated into pure culture as well as the novel coccus, *Ignisphaera aggregans* (AQ1.S1^T), both deposited in the TRUCC as TG 867 and 866, respectively. Therefore, the water and colonised surfaces of AQ1 are dominated by *Pyrobaculum* species with two types of cocci also being present within the pool. However, the *A. pernix*-related coccus was only detected by colonisation-based analysis and the *Ignisphaera* sp. by culture and serial dilution methods.

Furthermore, of interest was the detection of nanoarchaea in AQ1 pool water and the *Pyrobaculum* (AQ1.S2) culture with the implication that the *Pyrobaculum* culture might be the host for the nanoarchaeal cells. As is discussed in more detail in Chapter 6 (Section 6.1), nanoarchaea were discovered in 2002 with a single nanoarchaeal species (*Nanoarchaeum equitans*) being characterised with its *Ignicoccus* host (Huber *et al.*, 2002). *N. equitans* was isolated from a submarine hydrothermal habitat; however, subsequent ecological studies using PCR-based screening by Hohn *et al.*, (2002) have detected nanoarchaeal signatures in other 'hot' environments of Yellowstone National Park, (USA) the Uzon Caldera, (Kamchatka, Russia) and an abyssal vent system (East Pacific Rise). Therefore, nanoarchaea may also be present in terrestrial high-temperature habitats, including New Zealand based on the above data.

3.6 Champagne Pool

Throughout the sampling period the temperature of this pool remained fairly constant from 70 to 75°C with the pH between 5.3 and 5.5. Due to the various problems encountered studying the colonisation of glass slides placed in Champagne Pool, the study was only conducted for a short period of time.

3.6.1 Colonisation results

Glass, Teflon and stainless steel surfaces were incubated in the pool and removed periodically over a period of weeks and microbial colonisation documented by ATP assay, phase-contrast and fluorescent microscopy (DAPI staining), and DGGE analysis on extracted DNA.

3.6.1.1 ATP analyses

The levels of ATP on colonised surfaces were measured and the results presented in Table 3.14, below.

Table 3.14 ATP levels measured on colonised surfaces incubated in Champagne Pool.

Incubation period	ATP (RLU)
4 day glass	294/286
8 day glass	1031/835
8 day steel	1040
17 day glass (1 side)	479:326
17 day Teflon (1 side)	648:491

3.6.1.2 Microscopic observation of colonised surfaces

Typically, cells were difficult to locate on surfaces incubated in Champagne Pool; therefore, the micrographs taken of the colonised surfaces over-represent the actual concentrations of cells observed on the surfaces. Colonisation was typified with rods attaching to debris adsorbed to the surface, as shown in Figure 3.18(a). The slides with the highest amounts of debris or crystal formation on their surfaces also had the highest concentrations of cells. This was also shown to be the case with an increased presence of cells at the Teflon-glass interface of Teflon slides, whereby, it seemed that the rods used the Teflon material to adhere onto the slide. Figure 3.18(b) shows this phenomenon, with the Teflon part of the slide on the left part of the image and the glass on the right, with rods present at the interface. All micrographs (fluorescent and phase-contrast) of surfaces incubated in Champagne Pool are included on a supplementary compact disc located inside the back cover of the thesis.

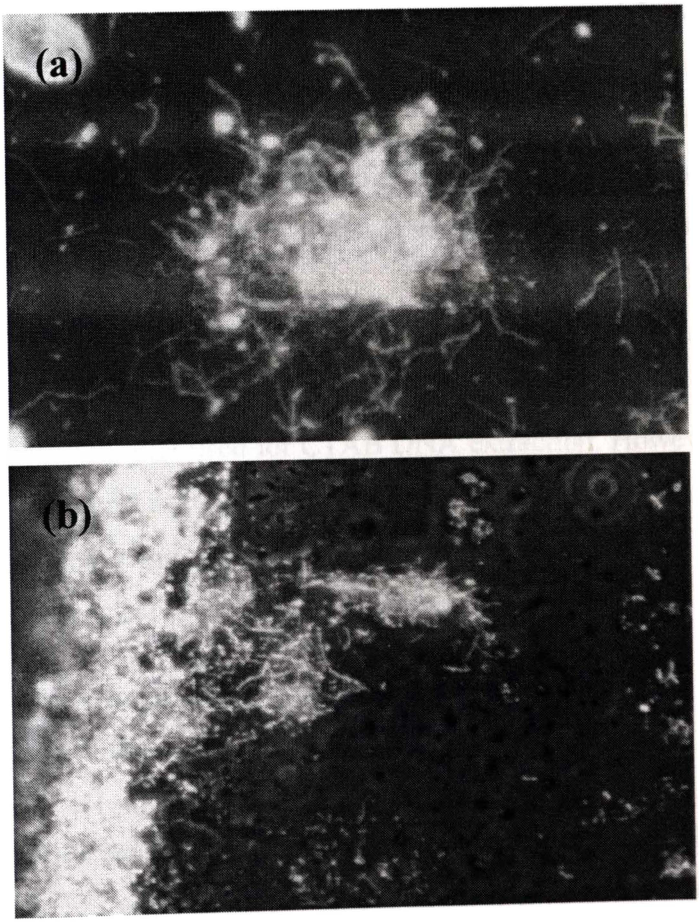


Figure 3.18 Colonisation phase-contrast micrographs (1000X) of glass slides incubated in Champagne Pool: (a) 8 days incubation (glass surface of a Teflon slide); (b) 17 days incubation (Teflon-glass interface).

3.6.1.3 Molecular analyses

Various attempts were made to isolate DNA from surfaces incubated in Champagne Pool; however, the extraction protocols did not yield quantifiable DNA and positive bacterial or archaeal amplifications were not obtained from any DNA extract. Typically, the CTAB method was used to isolate DNA from colonised slides, but the GIT-based method was also attempted; however, an orange precipitate was obtained with the GIT-based method and positive bacterial PCR amplicons were not obtained. Attempts were also made to extract DNA from Champagne Pool water by way of filtering Champagne Pool water and using the filter for DNA extraction. However, due to the large amount of suspended orange-coloured flocculent (largely antimony sulphide) precipitates within Champagne Pool water, it proved impossible to filter sufficient water to obtain detectable amounts of DNA. Therefore, differing methods were employed for DNA extraction including: (1) pool water was centrifuged at 1000 *g* for 20 minutes (thereby avoiding precipitation of most of the orange floccular material) and the pellet used for CTAB DNA extraction; (2) pool water was centrifuged at 100 *g* for 20 minutes and the pellet used for CTAB DNA extraction; (3) the supernatant from step 2 was filtered through a 0.45 μm filter and the filter used for CTAB DNA extraction; (4) the supernatant from step 2 was filtered through a 0.22 μm filter and the filter used for CTAB DNA extraction. However, quantifiable amounts of DNA were not obtained with any protocol and amplification was similarly not obtained using either bacterial or archaeal 16S rDNA primers (either DGGE or near full-length 16S rDNA) for any extract.

3.6.2 Culturing results

Attempts to enrich for organisms from Champagne Pool involved CMD agar medium (see Appendix E for composition) and solidified (agar) Champagne Pool water supplemented with 1.0 and 0.1 g/L yeast extract and trypticase peptone, respectively. No enrichment or colony development occurred on any medium used when incubated at 70°C. Due to the high concentrations of arsenic (4.7 mg/L) in Champagne Pool water (see Section 3.2.2), Champagne Pool water was also used to inoculate arsenic-based broth medium (Huber *et al.*, 2000b) and arsenic-supplemented agar plates (Gihring *et al.*, 2001) which were prepared with and without arsenic, but with no positive results.

3.6.3 Discussion and conclusion

Due to the various problems encountered whilst attempting to document colonisation for Champagne Pool, the studies were ultimately suspended. ATP yields from the colonised slides were very low even after 17 days incubation. There was little increase in ATP on surfaces between slides incubated for 4 or 17 days, and this correlates with the paucity and microscopic appearance of the cells on the surface. Cell concentration reflected more the deposition of crystallised deposits on the surface than the time of incubation. The observation of low biomass on the slides (and the distorted appearance of many of the cells) is also supported by low or non-existent yields of DNA, and the resultant lack of amplification by PCR. PCR inhibition was shown not to be a problem as positive PCR amplification was obtained from filter and slide DNA extractions which were supplemented with exogenous DNA. Moreover, no positive enrichments were obtained from Champagne Pool samples for any medium used. In conclusion, it was unclear whether the surfaces were actually being colonised, as the appearance of the cells adhering and the very low ATP values were indicative of the random adsorption of small numbers of live cells, the larger numbers of cells observed under microscopy reflecting adsorption of largely dead cells. Further culture-based experimentation using Champagne Pool water has been undertaken within the laboratory by a Ph.D. student. Preliminary results indicate Champagne Pool water is inhibitory to growth of a thermophilic *Bacillus* sp. which may due to high concentrations of heavy metals and/or dissolved gases which may explain the apparent low microbial biomass within Champagne Pool (pers. comm.).

3.7 Final discussion and conclusion

Colonisation was detected on all surfaces incubated in both KP1 and AQ1, by ATP measurement and high concentrations of cells being observed on incubated surfaces. Cells were also observed on slides incubated in Champagne Pool, but due to very low numbers of cells and low ATP yields on slides, it was not clear whether colonisation was taking place.

Colonisation rates correlated to the amount of water movement, for example, colonisation was slower on slides incubated in AQ1 after the bubbling of the pool had discontinued. In addition, colonisation was also observed to be slower for slides that were relocated to the near-stagnant KP1 pool itself, rather than the KP1 runoff water stream due to a drop in water level. This may be linked to the constant input of nutrients into the hot pool, fuelling cell growth and the removal of metabolic waste products.

It is difficult to draw comparisons of colonisation to other studies, as the studied colonisation parameters have not been followed in such detail in other thermal environments. Pioneer work undertaken by Brock, (1978) has demonstrated that colonisation of glass slides in Yellowstone pools (5 pools studied) is rapid, with cell generation times (on the glass slides) of between 3.0 and 6.0 hours at temperatures above 90°C and pH 8.5 to 9.0. Most microbial colonisation studies of thermal environments have involved the removal of biofilms from surfaces incubated in hot pools with subsequent DNA-based molecular analysis being undertaken (Blank *et al.*, 2002; Spear *et al.*, 2005). Both, Blank *et al.*, (2002) and Spear *et al.*, (2005) documented the microbes colonising surfaces, including glass slides incubated in high-temperature pools of Yellowstone National Park. A total of four pools of similar temperature and pH as AQ1 were studied by Blank *et al.*, (2002) with temperatures from 89 to 98°C and pH ranges from 7.8 to 8.9. However, in direct contrast to the AQ1 results, archaea were not detected by PCR and surfaces were dominated by the members of the bacterial order of the Aquificales. Interestingly, Boulder Spring was included in the study by Blank *et al.*, (2002) which shares a pool water chemistry similar to that of AQ1 (Brock, 1978). Although, archaeal lipids were detected in the pool, archaea were not detected by PCR on the surface of subaqueous geyserite in Boulder Spring. However, the colonisation survey described in Chapter 4 has demonstrated the presence of archaea colonising glass slides incubated in Boulder Spring (see Section 4.4.3). The study of Spear *et al.*, (2005) has also showed that members of the Aquificales are universal to high-temperature pools of Yellowstone National Park, which dominate the archaeal members of the community, including colonised glass slides.

At the present time, no colonisation of surfaces incubated in hot pools of similar temperature and pH to that of KP1 have yet been published. However, DGGE and 16S rDNA cloning experiments have been used to characterise microbial members of thermophilic biofilms, e.g. microbial mats of hot springs located in Japan, 72-80°C, pH 6.3-8.8 (Nakagawa and Fukui, 2003), Iceland, 60-80°C, pH 6.7 (Skirnisdottir *et al.*, 2000) and Octopus Spring, YNP, 72°C (Ferris *et al.*, 1996b). As was established for KP1, these studies indicate that these biofilms are complex microbial communities dominated by bacterial signatures.

Also, of interest is the detection of a nanoarchaeal signature in the pool water of AQ1 and in the *Pyrobaculum* culture (AQ1.S2). Therefore, *Pyrobaculum* may be the assumed host for nanoarchaea cells, and as presented in Chapter 4, Section 4.5, a nanoarchaeal survey of New Zealand pools indicates that nanoarchaea may be ubiquitous members of New Zealand high-temperature (>83°C) hot pools.

In conclusion, methods were successfully developed to follow the succession of biofilm development on surfaces in New Zealand hot pools, with the hottest pool of AQ1, unexpectedly having the greatest microbial biomass on colonised surfaces of all three pools. These methods were subsequently applied to a survey of colonisation in a number of high-temperature pools located in New Zealand and Yellowstone National Park, as outlined in Chapter 4.

Chapter 4 : Colonisation of surfaces incubated in high-temperature hot pools

4.1 Introduction

The development of methods which were successful in tracking the microbial colonisation of glass slides incubated *in situ* in hot pools (Chapter 3) facilitated a survey to compare and contrast microbial colonisation of a number of near-neutral (pH 5-9) high-temperature (>80°C) pools in New Zealand. It was assumed that the higher temperatures used would facilitate the study by limiting microbial diversity, thus, making comparisons between habitats easier. An invitation to sample pools in Yellowstone National Park (WY, USA) by Dr. Melanie Holland and Prof. Everett Shock (Arizona State University, AZ, USA), provided an opportunity to compare colonisation between two geographically distinct thermal habitats. Slides were also incubated in high-temperature, acidic hot pools on White Island (NZ), and the results included in this Chapter, providing comparison to acidic pools. The colonisation of surfaces in New Zealand pools and results of enrichment culture are included in Sections 4.2 and 4.3, respectively, followed by the results colonisation of slides incubated in pools from Yellowstone National Park (YNP) in Section 4.4. PCR-based screening of both New Zealand and YNP pools for the 16S rRNA genes of nanoarchaea are detailed in Section 4.5. Finally, the results from the White Island studies are presented in Section 4.6 with the final discussion and comparison between all environments in Section 4.7.

4.2 Colonisation of surfaces in New Zealand pools

Clean glass microscope slides were incubated *in situ* (suspended deep within the water column by plastic slide holders) for the periods stated in the pools listed in Table 4.1. When removed, colonisation was documented by ATP measurement (Section 4.2.1), phase-contrast and DNA staining (DAPI) microscopy (Section 4.2.2) and DGGE (Section 4.2.3). The DGGE results based on DNA extracted from colonised slides are compared to DGGE analyses of DNA isolated from the water column (Section 4.2.3). Slides were also incubated in the sediment layer of pools for 9 days. Only pools; AQ1; AQ2; AQ4; WH1; WH2; OH1 and TK2 contained accessible sediment, however, colonisation was not apparent by microscopy and DNA yield was not detectable or amplifiable by PCR.

4.2.1 New Zealand hot pools and ATP analyses

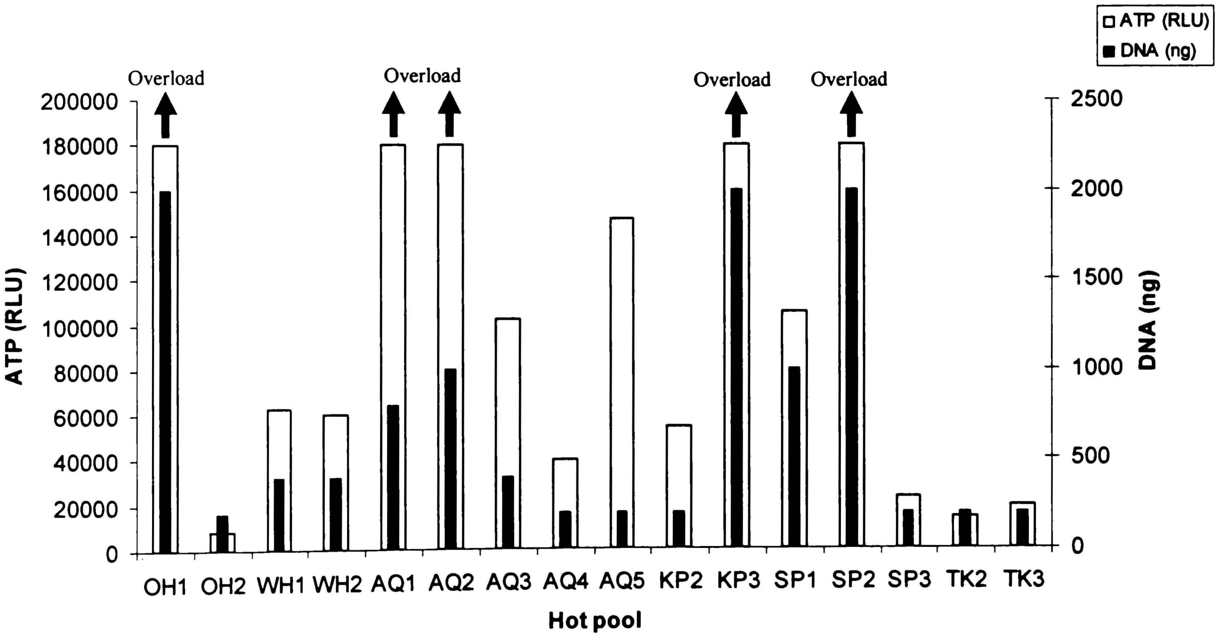
The New Zealand hot pools and their salient characteristics are listed in Table 4.1. The temperature and pH range for each pool refers to the values measured at the initial and final day of incubation. Photographs of the pools and their respective GPS coordinates are contained in Appendix A. The amount of ATP on the surface of a glass slide was used as an indicator of microbial biomass, i.e. biofilm development. Table 4.1 includes the ATP yield and the amount of DNA extracted from the surface of a colonised slide for each pool and background ATP levels as determined by directly measuring levels of ATP in 50 µl of pool water. Table 4.1 also includes the cell densities observed by microscopy on the colonised slides as graded from dense cell concentrations (+++) to low cell concentrations (+), including the dominant cell morphologies. Examples micrographs of colonised slides are presented and discussed further in Section 4.2.2.

Table 4.1 Colonisation parameters of glass slides incubated in New Zealand hot pools.

Geographical area	Hot Pool	Incubation time (days)	Temp. range (°C)	pH range	ATP (RLU) 50 µl pool water	ATP (RLU) entire slide surface on day of collection	DNA yield approx. (ng)	Colonisation cell density	Colonising cell morphologies
Rotorua, Ohinemutu	OH1	11	81-82	8.35-8.98	9447	Overload	2000+	+++	At least 4 morphologies
	OH2	11	92-93	9.37-9.70	321	8341	200	+	Rods
Rotorua, Whakarewarewa	WH1	11	95-95.5	8.71-9.09	466	62813	400	+	Rods & cocci
	WH2	11	83.5-86	6.68-6.65	369	60351	400	+	Rods & cocci
Rotorua, Kuirau Park (near Aquatic centre)	AQ1	11	95-96	8.11-8.24	1919	Overload	800	+++	Rods & cocci
	AQ2	11	96-100	8.46-8.4	252	Overload	1000	+++	Rods & cocci
	AQ3	11	88	6.38-6.34	2380	102458	400	+++	Long thin & short rods & cocci
	AQ4	11	83-85	6.48-6.67	1148	39530	200	++	Long thin & short rods
	AQ5	11	88-93	8.12-8.48	2536	146669	200	+	Rods
Rotorua, Kuirau Park	KP2	11	89-90	5.51-6.10	1082	54261	200	++	Rods
	KP3	11	95-96	5.70-5.55	931	Overload	2000+	+++	Cocci
Rotorua, Sulphur Point	SP1	11	88-90	6.31-6.50	2633	105471	1000	++	Rods
	SP2	11	85.5-90	5.95-6.0	1817	Overload	2000+	+++	Rods & cocci
	SP3	11	90-93	6.61-7.12	811	23097	200	++	Rods
Tokaanu Thermal Park	TK2	7	85-86	6.75-7.41	238	14367	200	+	Rods & cocci
	TK3	7	84	7.18	606	18916	200	+	Rods & cocci

From Table 4.1, ATP levels on the colonised slides were well above background levels and are indicative of the exponential increase of cells growing as a biofilm rather than random continuous adsorption of cells to the slide surface. In fact, after 5 hours of *in situ* incubation, ATP levels on slides for pools AQ2 and AQ3 were 3923 and 4276 RLU, respectively, which are already above their background levels. The ATP levels on the colonised slides generally correlated with the amount of DNA extracted from the slide and the cell densities observed on the slides by microscopy. Although this did not hold true for all cases, e.g. high ATP levels were detected on slides incubated in AQ1, AQ2 and AQ5. However, low levels of DNA were obtained from the slide surface and low densities of cells were documented by microscopy. A graph of ATP and DNA yields from the colonised slides incubated in the New Zealand pools provides a clearer perspective of this relationship in Figure 4.1.

Figure 4.1 ATP (RLU) and DNA (ng) yields from colonised slides incubated in NZ hot pools.



4.2.2 Microscopy of colonised slides

The colonisation of the slides was documented using both phase-contrast microscopy and DNA staining (DAPI) by fluorescent microscopy. The dominant cell morphologies of the colonised slides have been listed previously in Table 4.1, but are described in greater detail, below. Typical colonisation micrographs are presented in Figure 4.2(a) through (f); with a fuller representation of micrographs contained on the supplementary compact disc located inside the back cover of the thesis. The following descriptions can only be a subjective summary of the more salient features of colonisation in the different pools, since microbial diversity can only be assumed from morphology, and colonisation of slide surfaces was frequently uneven making comparison difficult. However, readily observed differences were apparent and are briefly outlined. Slides immersed in the lowest temperature pool, OH1 (Figure 4.2(a)), were colonised with a diverse array of cell morphologies including long filaments, several types of short rods and cocci. The colonisation profiles for pools AQ3 and AQ4 were similar and were typically dominated by long filaments and fewer shorter, more phase-dense rods (Figure 4.2(b), AQ3). A similar pattern of colonisation was observed in the two Tokaanu pools (TK2 and TK3), although the cell density observed was less than that for AQ3 and AQ4. Pools AQ5 and OH2 also had low cell densities on colonised slides, with few rod-shaped cells observed (although this does not correlate with ATP values which suggest high cell density for AQ5). The slides incubated in the pools located at Sulphur Point, Rotorua (SP1, SP2 and SP3) were difficult to observe under microscopy (both phase-contrast and fluorescent) due to the presence of crystalline precipitates on the slides with few rod-shaped cells documented for SP1 and SP3 (Figure 4.2(c), SP3) and large numbers of similar shaped rods for SP2. The slides for the two higher temperature pools of AQ1 and AQ2 had high levels of cells present dominated by both cocci and long rod-shaped cells (Figure 4.2(d), AQ2). Rod morphotypes dominated in both Whakarewarewa pools (WH1 and WH2), (Figure 4.2(e), WH1), as was the acidic KP2 pool, however, rods were not observed for the other acidic pool KP3, which possessed only irregular cocci (only by DAPI staining) (Figure 4.2(f)).

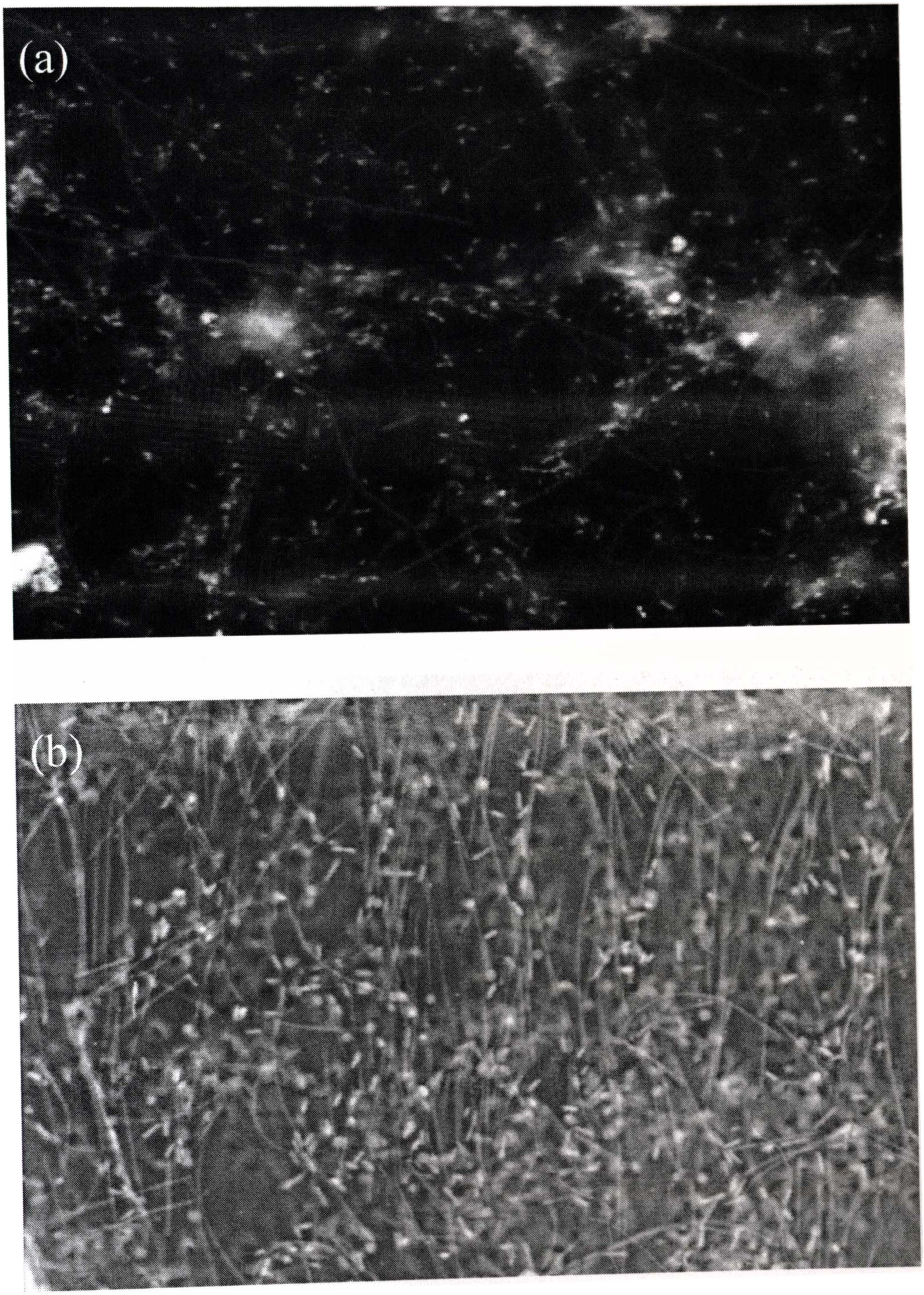


Figure 4.2 Colonisation micrographs (1000X) for New Zealand's hot pools. The samples and the corresponding micrographs are: (a) OH1 (DAPI); (b) AQ3 (phase-contrast); (c) SP3 (phase-contrast); (d) AQ2 (phase-contrast); (e) WH1 (DAPI); (f) KP3 (DAPI).

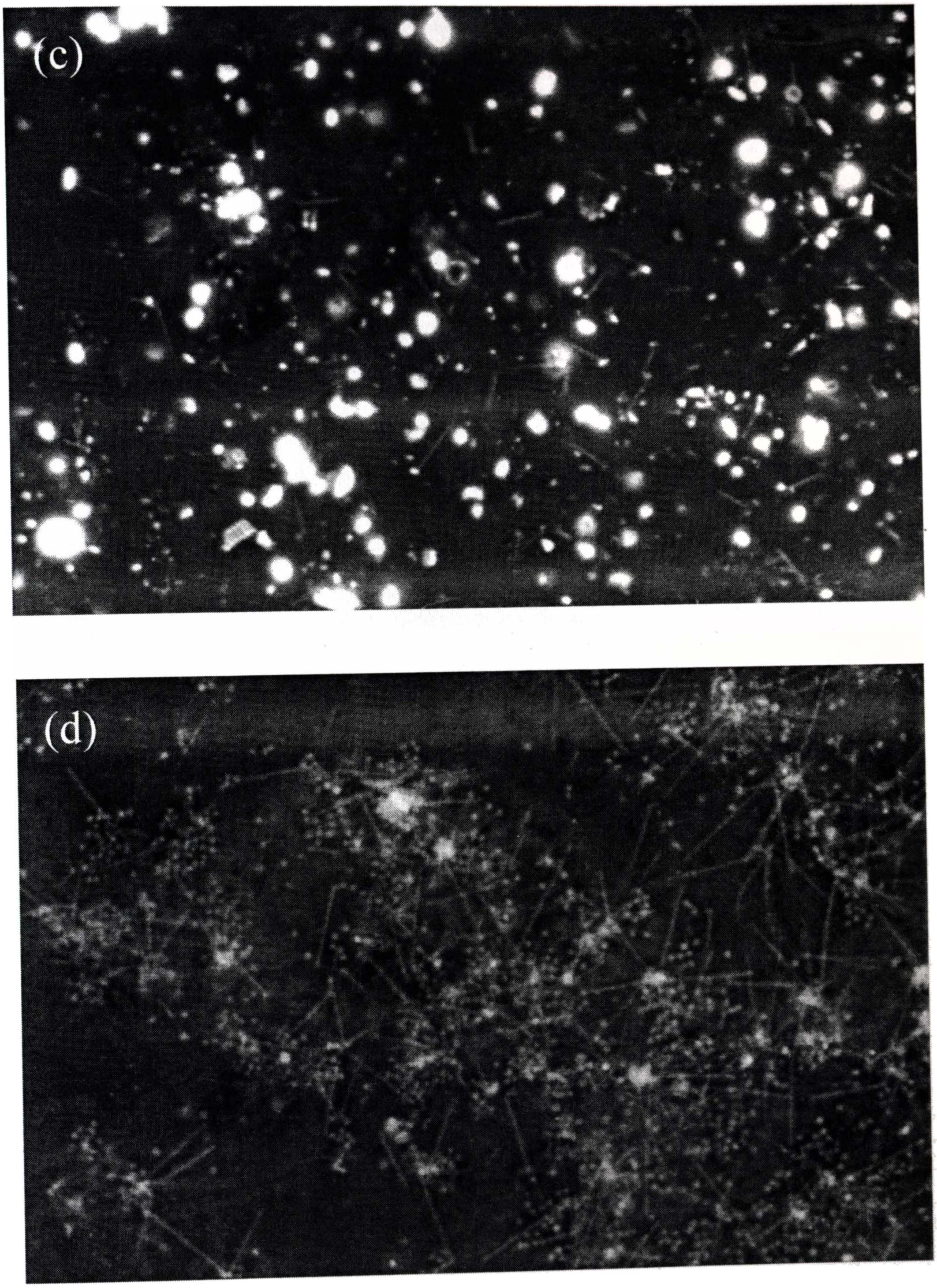


Figure 4.2 Colonisation micrographs (1000X) for New Zealand's hot pools. The samples and the corresponding micrographs are: (c) SP3 (phase-contrast); (d) AQ2 (phase-contrast).

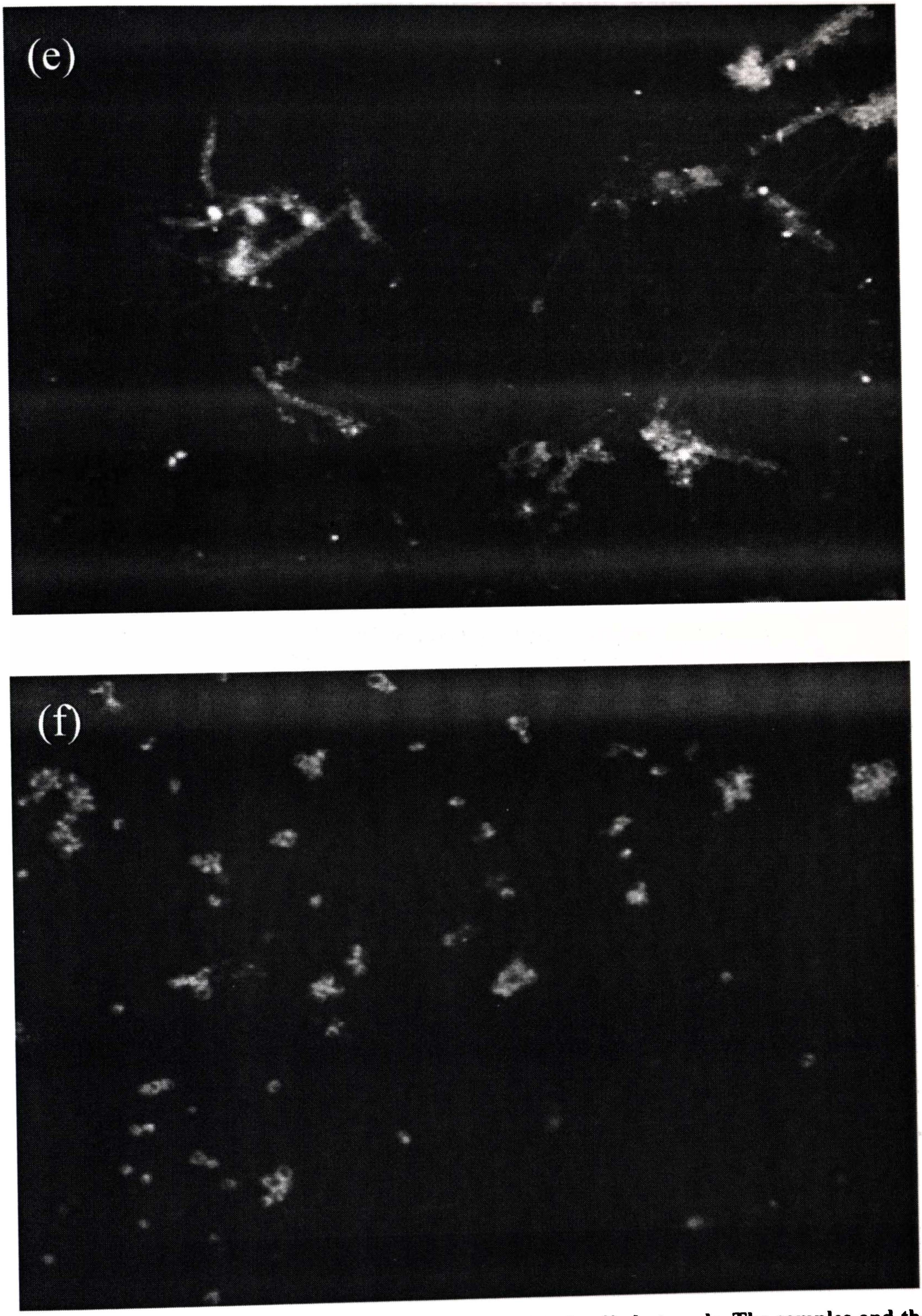


Figure 4.2 Colonisation micrographs (1000X) for New Zealand's hot pools. The samples and the corresponding micrographs are: (e) WH1 (DAPI); (f) KP3 (DAPI).

4.2.3 DGGE analysis of colonised slides and pool water

DNA was obtained from all colonised slides and was amplified by PCR with primer pairs specific for either bacteria or archaea targeting the 16S rRNA gene; however, inhibition of archaeal DNA amplification was commonly encountered. This was normally overcome by the addition of 5% DMSO to the PCR reaction, but for DNA from the slide in pool AQ2, a further phenol extraction procedure was required. DNA from two pure cultures isolated from pool AQ1 (*Ignisphaera aggregans*, strain AQ1.S1^T, and *Pyrobaculum* sp., isolate AQ1.S2, see Chapter 3) were used as references to compare DGGE band positions for all archaeal DGGE gels.

The DGGE results from the colonisation of glass slides were also to be compared to the microbes residing in the water column and sediment of the pools. Therefore, DNA extracted from a filter used to filter pool water from some of the pools was used for DGGE. SEM micrographs were undertaken of filter surfaces used to filter both AQ2 and SP2 pool water, with the images being contained on the supplementary compact disc located in the back cover of the thesis. Also, sediment was collected from pools AQ1, AQ2, AQ4, WH1, WH2, OH1 and TK2 for DNA extraction and subsequent DGGE analysis. Initially, the CTAB method of DNA extraction was utilised, however, as discussed in the Materials and Methods (Chapter 2), this did not provide a quality of DNA capable of being amplified (or inhibitors of the reaction were not removed). Therefore, a bead-beating extraction method was trialled which gave variable success with few clearly defined DGGE profiles obtained using bacterial primers. Archaeal DGGE results were also less than ideal with the profiles containing smeary and/or faint DGGE profiles. Therefore, both the bacterial and archaeal DGGE profiles from hot pool sediment were not considered worthy of including in the thesis.

4.2.3.1 Bacterial DGGE of pool waters

The DNA extracted from slides colonised in two YNP pools (Obsidian Pool and Steep Cone) was included in DGGE gels with the DNA extracted from NZ pools for reference. The DNA from the YNP pools contain the three bands commonly found in all YNP samples, presumably representing the three species of dominant bacteria in these pools (Section 4.4.3.1). No bacterial PCR amplification (and therefore DGGE pattern) was detected with DNA extracted from slides incubated in pools with an average *in situ* temperature above 88°C, i.e. OH2, WH1, AQ1, AQ2, AQ5, KP2, KP3, SP1 and SP3. For those pools where amplification with bacterial primers was successful, three distinct DGGE profiles were evident (Figure 4.3). Profile 1 was from pool OH1, profile 2 from pools WH2, AQ4, AQ3, SP2 and profile 3 from pools TK2 and TK3 (as indicated on Figure 4.3). Moreover, none of the dominant New Zealand DGGE bands resided in the same position as the comparative YNP samples (lanes 1 and 2 in Figure 4.3) indicating differences in the dominant bacterial species. All three DGGE profiles were relatively simple indicating the presence of very few dominant species present on the slide surfaces.

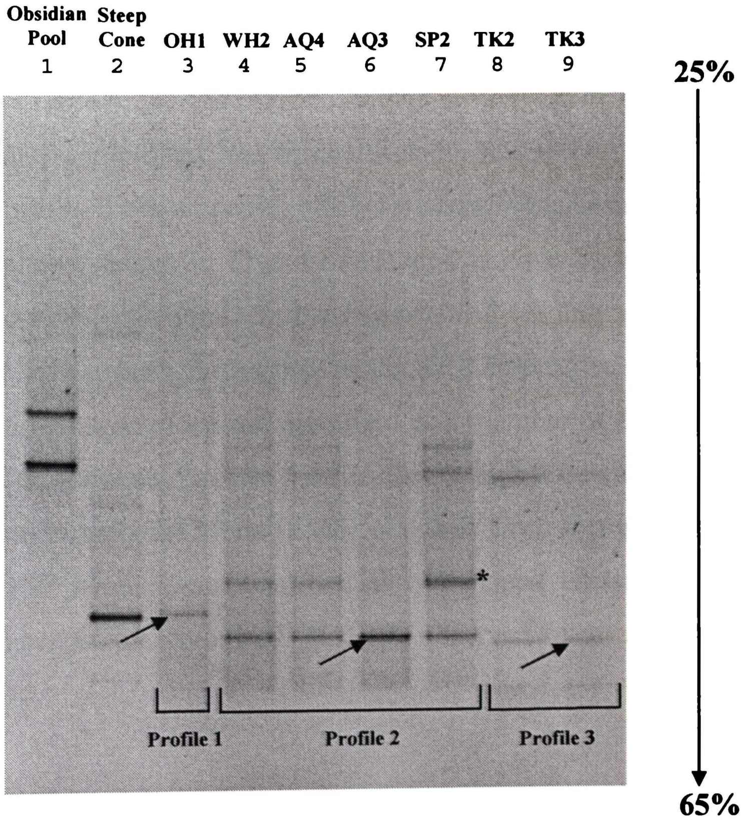


Figure 4.3 Bacterial DGGE of DNA from slides colonised in New Zealand hot pools. The track numbers and corresponding samples are: (1) Obsidian Pool (slide); (2) Steep Cone (slide); (3) OH1; (4) WH2; (5) AQ4; (6) AQ3; (7) SP2; (8) TK2; (9) TK3. Arrows indicate sequenced bands. *Band identified as a member of the Aquificales as discussed for the pool water samples.

The dominant band from each of the three NZ bacterial DGGE profiles (indicated by arrows in Figure 4.3) were extracted and sequenced. The resulting sequences were BLASTn and their closest relatives (two closest relatives for OH1, due to differing comparative nucleotide lengths) are listed in Table 4.2 (all sequences are presented in Appendix D).

Table 4.2 Closest NCBI relatives of bacterial DGGE sequences obtained from DNA of slides colonised in New Zealand hot pools.

Bacterial DGGE Band	Profile # (Figure 4.3)	Sequence length (bp)	Closest BLASTn relatives	Similarity	Accession
OH1 glass slide	1 – Lane 3	40	*Thermotoga maritima* MSB8	95%	AE001703.1
		54	*Thermotogales* sp. SRI-25	90%	AF255593.1
AQ3 glass slide	2 – Lane 6	116	*Thermodesulfobacterium hydrogeniphilum*	95-98%	AF332514.1
TK3 glass slide	3 – Lane 9	115	*Eubacteria* EM 3	92%	U05660

The sequences of all bacterial DGGE bands were most closely related to sequences from other thermophilic bacteria. From Figure 4.3, the dominant band in profile 1 (pool OH1) was a member of the *Thermotogales*, with the closest relatives, i.e. strains MSB8 and SRI-25 (Table 4.2) originating from geothermally heated marine sediment at Vulcano, Italy and a microbial mat in an Icelandic hot spring, respectively. The dominant band of profile 2 was a species of *Thermodesulfobacterium* with the closest NCBI BLASTn relative being an organism isolated from a hydrothermal vent at Guaymas Basin. An additional dominant band of profile 2 (as indicated by an asterisk in Figure 4.3) was also present in the pool water DGGE profiles of these pools. A representative band was sequenced and identified as a member of the Aquificales, as discussed below. The dominant band in profile 3 was a signature closely related to an uncultured dominant eubacterial clone (EM3) obtained from Octopus Spring, YNP. All sequenced DGGE bands were identified as rod-shaped bacteria, reflecting the rod-shaped dominance of the colonised slides as observed by microscopy (Section 4.2.2).

The DGGE profiles of amplified DNA from pool water are presented in Figure 4.4. A representative of each of the three DGGE profiles observed from colonised slides (Figure 4.3) is also included; i.e. from pools, OH1 (profile 1), WH2 (profile 2) and

TK2 (profile 3) as well as the two YNP profiles from Obsidian Pool and Steep Cone. Interestingly, a bacterial signature was detected in pool water from WH1, although, bacteria were not detected on the slides colonised in the pool.

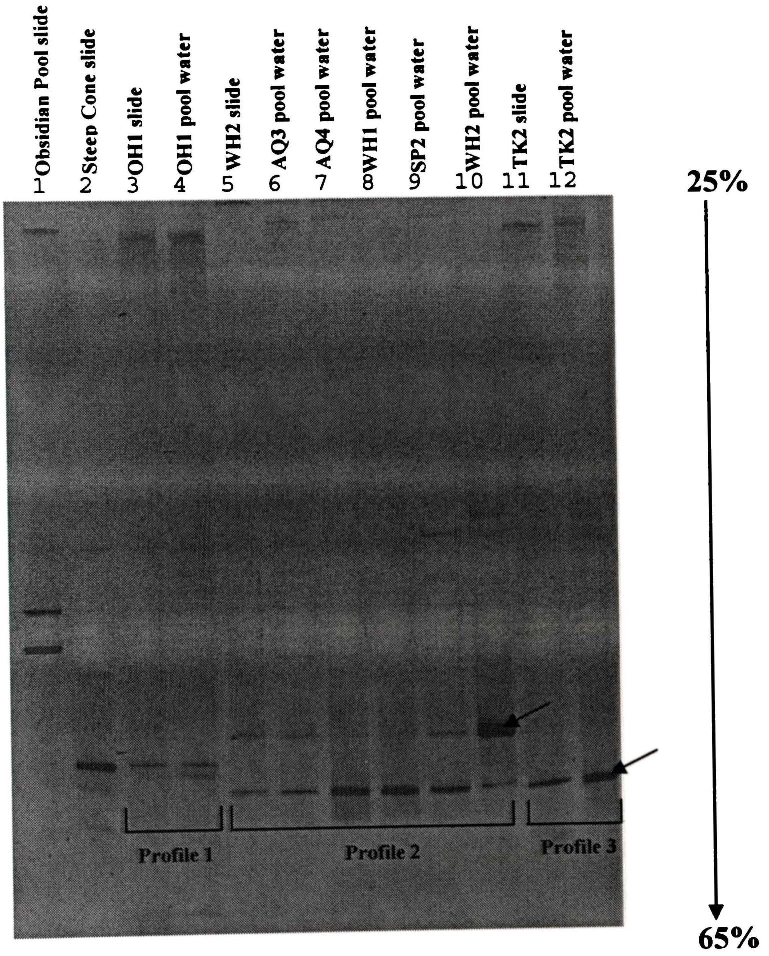


Figure 4.4 Bacterial DGGE of DNA from New Zealand pool water. The track numbers and corresponding samples are: (1) Obsidian Pool (slide); (2) Steep Cone (slide); (3) OH1 (slide); (4) OH1; (5) WH2 (slide); (6) AQ3; (7) AQ4; (8) WH1; (9) SP2; (10) WH2; (11) TK2 (slide); (12) TK2. Arrows indicate sequenced bands.

Two of the dominant bands as indicated by arrows in Figure 4.4 were extracted and sequenced with their closest NCBI BLASTn relatives listed in Table 4.3 (sequences included in Appendix D).

Table 4.3 NCBI BLASTn results of bacterial DGGE sequences obtained from New Zealand's hot pool water.

Bacterial DGGE Band	Sequence length (bp)	BLASTn closest relatives	Similarity	Accession
WH2 pool water	152	*Aquificales* str. CIR3017HO90	91%	AF393377.1
	152	*Aquifex* sp. Aq-365-85-1%O2	91%	AB095173.1
TK2 pool water	44	*Eubacteria* EM 3	97%	U05660
	44	*Aquifex* sp. Ob6	95%	AJ320224

The bacterial DGGE profiles from the pool water samples were not significantly different from their corresponding colonised slide profiles and also segregated into the three DGGE profiles as observed for the colonised slides in Figure 4.4. Only the OH1 pool water profile (lane 4, Figure 4.4) contained an extra over the equivalent slide DNA (residing below its dominant band) (lane 3, Figure 4.4). Attempts were made to sequence this band; however, this band was inconsistently obtained by bacterial DGGE PCR. A dominant band of the pool water profile 2 (labelled by an arrow in lane 10, Figure 4.4) was identified as a relative of the Aquificales (Table 4.3). This band was also dominant in profile 2 of the colonised slides as marked by an asterisk in Figure 4.3.

4.2.3.2 Archaeal DGGE

Archaeal DGGE of amplified DNA obtained from colonised slides was undertaken and results presented in Figure 4.5 and Figure 4.6. Amplicons of the archaeal 16S rRNA gene were obtained from all slides with the exception of OH1 which had the lowest *in situ* temperature of the pools investigated (81-82°C). The DGGE profiles obtained were all relatively simple indicating a low diversity of archaea colonising the slide surfaces and could be segregated into three profile types dependant on the pH of the pools. Pools with a pH above 7.0 (the exception being AQ4) had a similar DGGE profile. The DGGE profiles of the colonised slides for these pools are presented in Figure 4.5, which were typified by a dominant band residing in the same position to that obtained with DNA extracted from the *Pyrobaculum* sp. isolate AQ1.S2 (lane 1, Figure 4.5) with some pools containing an additional band just below that of the reference band for *Ignisphaera aggregans*, strain AQ1.S1^T sample (lane 2, Figure 4.5).

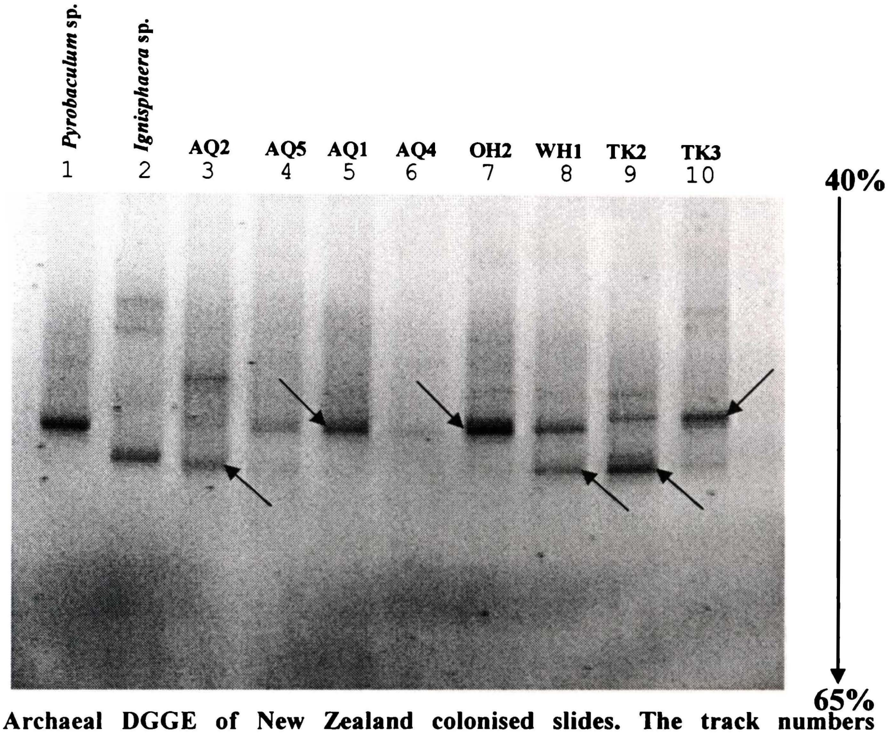


Figure 4.5 Archaeal DGGE of New Zealand colonised slides. The track numbers and corresponding samples are: (1) *Pyrobaculum* sp. AQ1.S2; (2) *Ignisphaera aggregans*, strain AQ1.S1^T; (3) AQ2; (4) AQ5; (5) AQ1; (6) AQ4; (7) OH2; (8) WH1; (9) TK2; (10) TK3. Arrows indicate sequenced bands.

Pools of pH between 6.0 and 7.0 (WH2, SP3, SP2, SP1, and AQ3) shared a similar DGGE profile as presented in Figure 4.6 (lanes 3 to 7). Both the KP pools (KP2 and KP3) with a pH of between 5.0 and 6.0 had a different DGGE profile (lanes 1 and 2 in Figure 4.6) to that of the near-neutral pools.

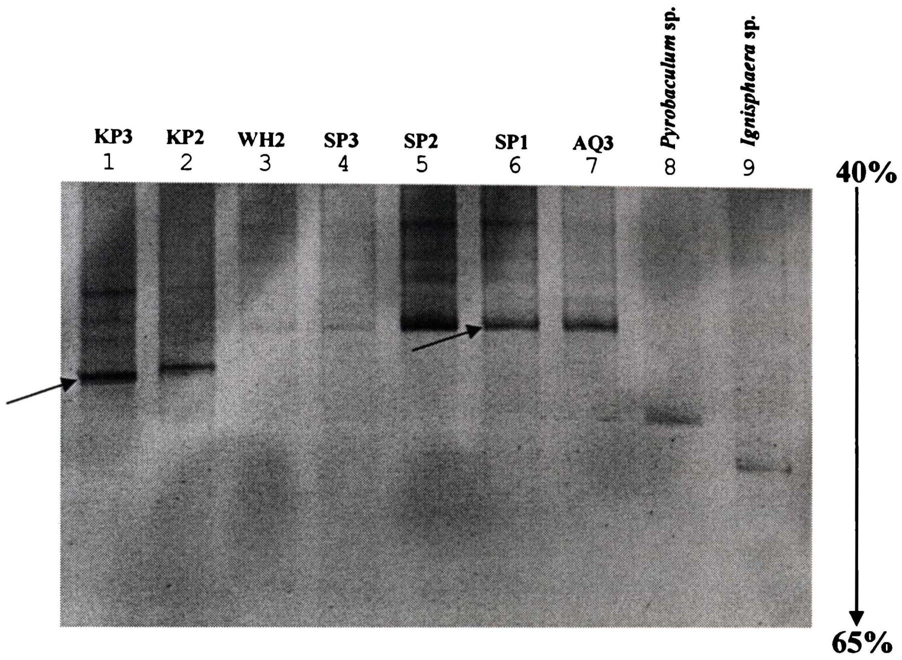


Figure 4.6 Archaeal DGGE of New Zealand colonised slides. The track numbers and corresponding samples are: (1) KP3; (2) KP2; (3) WH2; (4) SP3; (5) SP2; (6) SP1; (7) AQ3; (8) *Pyrobaculum* sp. isolate AQ1.S2; (9) *Ignisphaera aggregans*, strain AQ1.S1^T. Arrows indicate sequenced bands.

Dominant DGGE bands from representative profiles were sequenced, as indicated by arrows on Figures 4.5 and 4.6 and their closest NCBI BLASTn relatives are listed in Table 4.4 below (sequences are included in Appendix D).

Table 4.4 Closest NCBI relatives of archaeal DGGE bands of New Zealand colonised slides.

Archaeal DGGE Band	Corresponding Figure and Lane #	Sequence length (bp)	BLASTn closest relatives	Similarity	Accession
AQ1 slide	Figure 4.5, Lane 5	429	*Pyrobaculum calidifontis*	99%	AB078332
		429	*Pyrobaculum neutrophilus*	99%	X81886
OH2 slide	Figure 4.5, Lane 7	414	*Pyrobaculum calidifontis*	99%	AB078332
		414	*Pyrobaculum neutrophilus*	99%	X81886
TK3 slide	Figure 45, Lane 10	377	*Pyrobaculum calidifontis*	99%	AB078332
		377	*Pyrobaculum neutrophilus*	99%	X81886
AQ2 slide	Figure 4.5, Lane 3	322	*Aeropyrum pernix*	96%	AP000062
		322	*Stetteria hydrogenophila*	95%	Y07784
WH1 slide	Figure 4.5, Lane 8	227	*Thermodiscus maritimus*	95%	X99554
		227	*Aeropyrum pernix*	95%	AP000062
TK2 slide	Figure 4.5, Lane 9	383	*Aeropyrum pernix*	96%	AB078016 AB078015
		378	*Stetteria hydrogenophila*	95%	Y07784
KP3 slide	Figure 4.6, Lane 1	433	*Sulfolobus* sp.	96%	AB022438
		433	*Sulfolobus tokodaii* str. 7	96%	AP000985
		433	*Sulfurisphaera ohwakuensis*	96%	D85507
SP1 slide	Figure 4.6, Lane 6	448	*Thermofilum pendens*	97%	X14835
		425	*Thermofiliaceae* str. SRI-325	97%	AF255607

All sequenced bands were closely related (>95%) to cultured hyperthermophilic Crenarchaeaota (Table 4.4). From the sequencing data in Table 4.4 it is evident that the dominant band for pools pH above 7.0, i.e. the band in a similar position to the *Pyrobaculum* sp. isolate AQ1.S2 in Figure 4.5 (as sequenced for AQ1, OH2 and TK3) was as expected, closely related to *Pyrobaculum* sp., but, aligning the AQ1, OH2 and TK3 DGGE sequences showed that they were not identical. This difference in nucleotide sequence may account for the observation that the band identified as *Pyrobaculum* for both Tokaanu pools migrates slightly higher in the DGGE gel than that of the other pools presented in Figure 4.5. *Pyrobaculum* was in fact readily isolated from these pools (see Section 4.3). The second dominant band, (that of AQ2, WH1 and TK2 in Figure 4.5) which migrates below the *Ignisphaera aggregans*, strain AQ1.S1^T reference was closely related to members of the Desulfurococcales such as *Aeropyrum pernix, Stetteria hydrogenophila* and *Thermodiscus maritimus*. Again the sequences obtained from pools AQ2, WH1 and TK2 were not homologous indicating the presence of different species or strains. Organisms related to *Aeropyrum pernix, Stetteria hydrogenophila* and *Thermodiscus maritimus* have not previously been isolated from these pools, and indeed these results are unexpected since the species listed are all associated with marine hydrothermal vent ecosystems.

The dominant DGGE band for the pools with an *in situ* pH of 6.0 to 7.0 was related to *Thermofilum* sp. and the KP3 pool dominant coloniser was a species of *Sulfolobales* (Figure 4.6). Attempts were made to indicate the phylogenetic affiliations of all the sequenced DGGE bands by producing a 16S rRNA gene based phylogenetic tree. However, when all sequences were aligned only a short (172 columns of aligned sequence) region could be used for comparison which produced spurious results. Therefore, individual trees (FITCH, Jukes-Cantor distance analysis) were produced for each sequence which showed that all the sequenced DGGE bands also grouped with their closest NCBI BLASTn relatives in Table 4.4 (results not shown).

Archaeal DGGE undertaken on DNA from pool water samples is presented in Figure 4.7. For reference, the profiles for pure cultures of *Pyrobaculum* and *Ignisphaera* and that from slides colonised in pool SP1, which contains the common band for the *Thermofilum* signature commonly found in the lower pH pools were included in Figure 4.7.

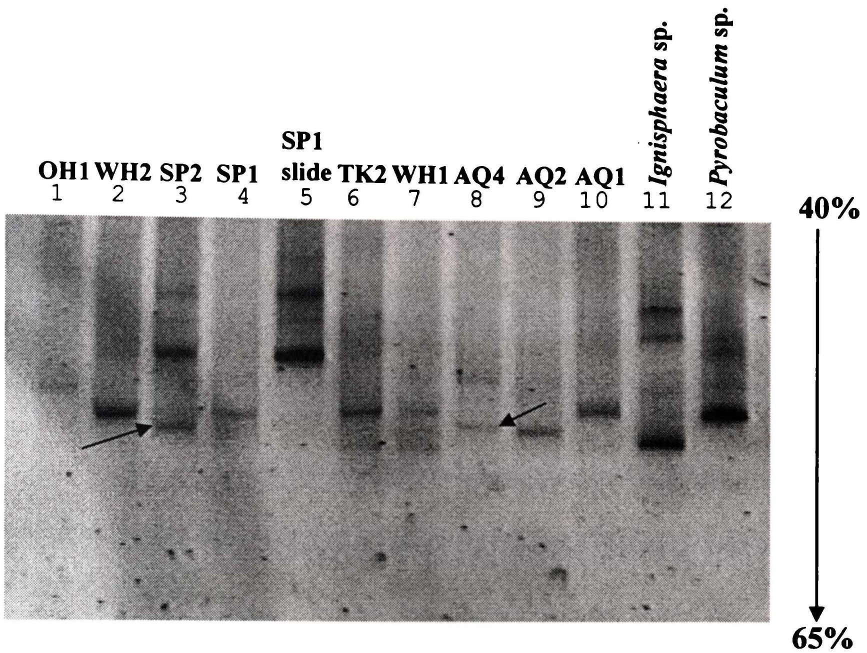


Figure 4.7 Archaeal DGGE of New Zealand pool water. The track numbers and corresponding samples are: (1) OH1; (2) WH2; (3) SP2; (4) SP1; (5) SP1 (slide); (6) TK2; (7) WH1; (8) AQ4; (9) AQ2; (10) AQ1; (11) *Ignisphaera aggregans* strain AQ1.S1^T; (12) *Pyrobaculum* sp. isolate AQ1.S2. Arrows indicate sequenced bands.

Interestingly, in contrast to the slides colonised in OH1, an archaeal signature was obtained in OH1 pool water (lane 1). Only the SP2 water sample (lane 3) contains a strong *Thermofilum* signature. The *Thermofilum* signature is absent from pool water from pools WH2 (lane 2) and SP1 (lane 4) although it was prominent on slides colonised in these pools. In both these pools the dominant bands are the same as for the *Pyrobaculum* isolate (lane 12). This presence of *Pyrobaculum* in these pools is not surprising as *Pyrobaculum* cells have been enriched from these pools (Section 4.3). Interestingly, both AQ4 (indicated by an arrow in lane 8) and the *Thermofilum* containing SP2 sample (indicated by an arrow in lane 3) contained another DGGE band that was not present for their corresponding colonised slides. Both bands were sequenced and their closest NCBI BLASTn relatives are presented in Table 4.5 (sequences are included in Appendix D).

Table 4.5 Closest NCBI relatives for archaeal DGGE bands from New Zealand pool water.

Archaeal DGGE band	Sequence length (bp)	BLASTn closest relatives	Similarity	Accession
SP2 water	284	*Thermococcus acidaminovorans*	99%	Y15935
	284	*Thermococcus waiotapuensis*	99%	AY099187
AQ4 water	340	*Thermococcus acidaminovorans*	96%	Y15935
	340	*Thermococcus waiotapuensis*	96%	AY099187

From the sequencing data in Table 4.5 it appears that *Thermococcus* species reside in some of the pools, but predominantly in the water column and are not detectable in DGGE profiles of DNA extracted from colonised slides.

4.3 Culturing and enumeration of thermophiles from New Zealand pools

4.3.1 Most Probable Number (MPN) enumeration

Pool water and sediment (sediment was obtained from pools AQ1, AQ2, AQ4, OH1, WH1 and WH2) was used to inoculate MPN tubes (dilutions series from 10^{-1} to 10^{-5} in triplicate) according to standard protocols. A range of different media were used to cover the diversity of likely organisms present from the sequencing of the dominant bands of the DGGE profiles. Media and conditions of use are listed in Table 4.6 and media compositions are listed in Appendix E. Table 4.6 also lists the pools and their respective pH and temperature measured when pool water and sediment was collected for MPN testing. Medium 182 (used to cultivate *Sulfolobus* sp.) was also tested (aerobically/anaerobically and with and without sulfur) for the two acidic pools, KP2 and KP3, however, growth was not detected. MPN enrichments were followed by phase-contrast microscopy and incubated for up to 10 days. The results and observed cell morphologies of each of the MPN dilutions are listed in Table 4.6, below.

Table 4.6 MPN results (approximate cells/ml) from New Zealand hot pools.

Pool	Temp. (°C)	pH	Lab. pH^{A}	Incubation temp. (°C) and pH of medium	*Thermofilum librum* medium	Db+S medium	*Thermococcus zilligii*, AN1 medium
OH1	80	8.5	8.71	80°C, pH 8.0	N.D.	4.3 x10^{2} P	3.6 x10^{0} F
OH1 (sed.)	N.D.	N.D.	8.89	80°C, pH 7.0	N.D.	2.4 x10^{3} F	2.3 x10^{1} F (unhealthy)
OH2	93	9.0	9.33	92°C, pH 8.0	N.D.	-	-
WH1	85	8.5	8.72	85°C, pH 8.0	N.D.	9.3 x10^{1} P (golf clubs)	-
WH1 (sed.)	89	N.D.	8.11	85°C, pH 7.0	N.D.	2.4 x10^{3} P	-
WH2	81	8.0	7.20	80°C, pH 6.0	4.3 x10^{1} F (few T)	1.5 x10^{2} C & F	9.3 x10^{1} C
WH2 (sed.)	82	N.D.	8.11	80°C, pH 7.0	N.D.	> 2.1 x10^{4} D 2.4 x10^{3} F* 2.1 x10^{3} C*	2.1 x10^{4} C
AQ1	88	8.0	8.10	85°C, pH 8.0	N.D.	2.4 x10^{2} P	3.6 x10^{0} P (unhealthy)
AQ1 (sed.)	N.D.	N.D.	8.24	85°C, pH 7.0	N.D.	2.1 x10^{3} P 4.3 x10^{1} C*	-
AQ2	90	8	7.96	92°C, pH 8.0	N.D.	4.3 x10^{1} P 3.6 x10^{0} C*	-
AQ2 (sed.)	N.D.	N.D.	7.99	92°C, pH 7.0	N.D.	9.3 x10^{3} P & C	-
AQ3	87	7.0	7.00	85°C, pH 8.0	3.6 x10^{0} P	9.3 x10^{1} P (golf club rods)	-
AQ4	86	7.0	6.80	85°C, pH 6.0	N.D.	4.3 x10^{2} P 2.4 x10^{2} C*	-
AQ4 (sed.)	N.D.	N.D.	7.24	85°C, pH 7.0	N.D.	2.4 x10^{4} P	-
AQ5	88	8.0	7.925	85°C, pH 8.0	N.D.	2.4 x10^{3} P 3.6 x10^{0} C*	-
KP2	85	5.0	5.67	85°C, pH 6.0	N.D.	-	-
KP3	85	5.0	4.54	85°C, pH 6.0	N.D.	-	-
SP1	86	6.0	6.17	85°C, pH 6.0	9.3 x10^{1} P & C & T	9.3 x10^{1} P & C & T	-
SP2	89	6.0	6.16	85°C, pH 6.0	2.4 x10^{3} P & C & T	2.4 x10^{3} P (golf club rods) 4.3 x10^{1} C*	2.3 x10^{1} P
SP3	93	7.5	7.00	92°C, pH 6.0	-	-	-
TK2	84	6.5	6.89	85°C, pH 6.0	N.D.	-	-
TK3	85	6.5	6.90	85°C, pH 6.0	N.D.	-	-

'P' = *Pyrobaculum* morphology (short thick rods); 'F' = *Fervidobacterial* morphology (motile and containing spheroids); 'D' = *Dictyoglomus* morphology (filamentous cells forming conglomerates); 'T' = *Thermofilum* morphology (long thin straight rods). 'C' = coccus morphology. *Not 'true' MPN values due to the influence of other dominant morphologies. ^{A}Pool water was transported to the laboratory and pH determined as detailed in the Materials and Methods (Chapter 2). N.D. = Not determined. - = No growth.

Pure cultures presumed to be *Fervidobacterium* and *Dictyoglomus* were obtained from OH1 (pool water) and WH2 (sediment) MPN tubes by serial dilution and stored at -70°C in cryoprotectant. The details of these isolates and their culture conditions are listed in Table F.1 (Appendix F). Consortia from WH2 sediment MPN tubes and pool water MPN tubes from SP1, AQ4, AQ3, WH1 and SP2 were also stored at -70°C in cryoprotectant as listed in Table F.1 of Appendix F.

From the MPN results (Table 4.6) growth was not detected for the KP pools (KP2 and KP3), the Tokaanu pools (TK2 and TK3) pools and SP3. Furthermore, WH2 was the only pool that showed healthy growth of cells in the AN1 (*Thermococcus zilligii*) medium. Typically, consortia of *Pyrobaculum*-shaped rods and cocci were present in both Db+S and *Thermofilum* media at 85 or 92°C, with few *Thermofilum*-type rods present in the *Thermofilum* medium. MPN tubes incubated at 80°C from pools WH2 and OH1 were typically dominated by *Fervidobacterium*-like cells. The MPN tubes inoculated with sediment (although sediment samples will also include pool water) showed growth of the same morphologies, with the exceptions pool AQ1 containing cocci in sediment but not the water column and WH2 which had *Dictyoglomus*-like cells in sediment only. From the MPN numbers obtained, the sediment appears to contain one or sometimes two orders more bacteria than the corresponding water column.

4.3.2 Isolation attempts

Attempts were made to culture microbes colonising the slides incubated in the pools. A *Thermotogales* signature was detected on slides for pool OH1; therefore, a modified *Thermotoga* MSM medium (0.5 g/L NaCl and 2 g/L maltose) was trialled in an attempt to isolate this microorganism (Appendix E contains the medium composition). A rod was isolated in pure culture (at 75°C) by incubating the tubes containing media with the caps loosened (i.e. in the presence of atmospheric oxygen). The isolate (designated Oh.A3) was added to the Thermophile Research Unit Culture Collection (TRUCC), as listed in Table F.1 of Appendix F. The isolate was assumed to be a species of *Thermus* due to its rod morphology, growth temperature and aerobic growth. No typical *Thermotoga* isolates were obtained under any culture conditions.

The presence of a *Thermofilum*-related organism dominating the colonised slides in pools, SP1 SP2, SP3 and AQ3 and a *Sulfolobales*-related coloniser in pools KP2 and KP3 led to attempts to trial *Thermofilum librum* medium and *Sulfolobales*-based medium, (medium 88) to isolate organisms from these pools. Results are presented in Table 4.7; all medium compositions are listed in Appendix E.

Table 4.7 Isolation attempts from some New Zealand hot pools.

Pool	*In situ* temp. (°C)	*In situ* pH	Lab. pH	Medium and incubation temp.
SP1	84	6.0-6.5	6.01	*Thermofilum librum* – 85°C
SP2	86	5.5-6.0	6.04	*Thermofilum librum* – 85°C
SP3	91	6.5-7.0	6.40	*Thermofilum librum* – 85°C
AQ3	87	7.0	6.76	*Thermofilum librum* – 85°C
KP2	85	5.0	5.31	Medium 88* - 85°C
KP3	92	5.0	4.40[A]	Medium 88* - 93°C

***Medium 88 was trialled both aerobically and anaerobically. [A]KP3 had a recent explosion event prior to sampling and perhaps as a consequence, the pH was lower than that of the previous colonisation study.**

For all the *Thermofilum librum* medium enrichments, a total of three cell morphologies were obtained; long thin rods (probable *Thermofilum* species); shorter thicker rods (probable *Pyrobaculum* species) and cocci. Dilution series (1:10) were undertaken in fresh media in an attempt to isolate the *Thermofilum*-shaped cells into pure culture, but with all the dilution attempts the *Pyrobaculum* cells dominated. The SP1 enrichment seemed to contain more *Thermofilum* cells than the other pool enrichments, therefore, dilution series (1:10) in fresh media were undertaken at both 80°C and 93°C, however, both the *Pyrobaculum*-like rods and cocci dominated these isolation attempts. Medium solidified with Gelrite was used at 93°C with no growth detected after 12 days incubation.

For the medium 88 enrichments, no growth was observed for KP3 (aerobically or anaerobically) and the KP2 aerobic enrichment contained very few unhealthy cocci that were not transferable. Similarly, the anaerobic KP2 enrichments contained *Pyrobaculum*-like rods which only achieved low cell densities.

This study was extended in 2005 on all pools which retained their original pH and temperature values. Unfortunately, the pH and temperature of pools WH1 and WH2 had dropped significantly (36°C, pH 3.9-3.5 and 52°C, pH 3.0-3.5, respectively) and AQ2 had completely dried up. Attempts were made to enrich for *Thermodesulfobacterium* and *Aeropyrum/Thermodiscus/Stetteria*-related organisms, since signature sequences for these organisms had been obtained from colonised slides (Section 4.2.3). These organisms were targeted using both modified medium used to culture *Aeropyrum* species (O_2, N_2 and N_2:CO_2 gas phases were trialled) and modified SME medium used for culturing members of the genera *Aeropyrum*, *Pyrodictium*, *Staphylothermus* and *Thermodiscus* (compositions of all media are listed in Appendix E). The subsequent enrichments obtained are listed in Table 4.8.

Table 4.8 New Zealand hot pools and their corresponding enrichments.

Pool	*In situ* temp. (°C)	*In situ* pH	Incubation temp. (°C)	Medium 206*	Medium 63*	*Aeropyrum* medium (O_2)	*Aeropyrum* medium (N_2)	*Aeropyrum* medium (N_2:CO_2)
AQ1	83	7.5-8.0	85	Low density *Pyrobaculum*-like rods	-	-	*Pyrobaculum*-like rods	Large irregular cocci
AQ3	87	7.0-7.5	85	-	-	Motile slightly curved rods	-	-
AQ4	83	7.0-7.5	85	*Pyrobaculum*-like rods	*Pyrobaculum*-like rods	-	*Pyrobaculum*-like, golf clubs	-
SP1	84	6.0-6.5	85	-	-	-	-	-
SP2	90	6.0-6.5	90	-	-	-	*Pyrobaculum*-like rods & cocci	*Pyrobaculum*-like rods & cocci
SP3	87	7.0-7.5	85	-	-	-	-	-

Growth was not observed in modified SME medium for all pools tested. - = No growth. *Used to culture species of *Thermodesulfotobacteria*.

Two of the enrichments (Table 4.8) were of interest and used for further isolation attempts, namely, the coccal culture from AQ1 in *Aeropyrum* medium (N_2:CO_2) and the motile, slightly curved, rod culture from AQ3 in *Aeropyrum* medium (O_2). The coccus was thought to be a species of *Thermococcus*; but, growth at 85°C was atypical. It showed good growth when inoculated into AN1 medium and incubated at 80°C, supporting its likely *Thermococcus* identity. Finally, in a comparison of DGGE profiles with type strains (Figure 4.8) the identity of this isolate as *Thermococcus* was also supported. DNA from the motile-rod culture from AQ3 was included in the archaeal DGGE (Figure 4.8), together with a reference *Pyrobaculum* strain. The profile in Figure 4.8 supports the conclusion that the rod is a species of *Pyrobaculum*.

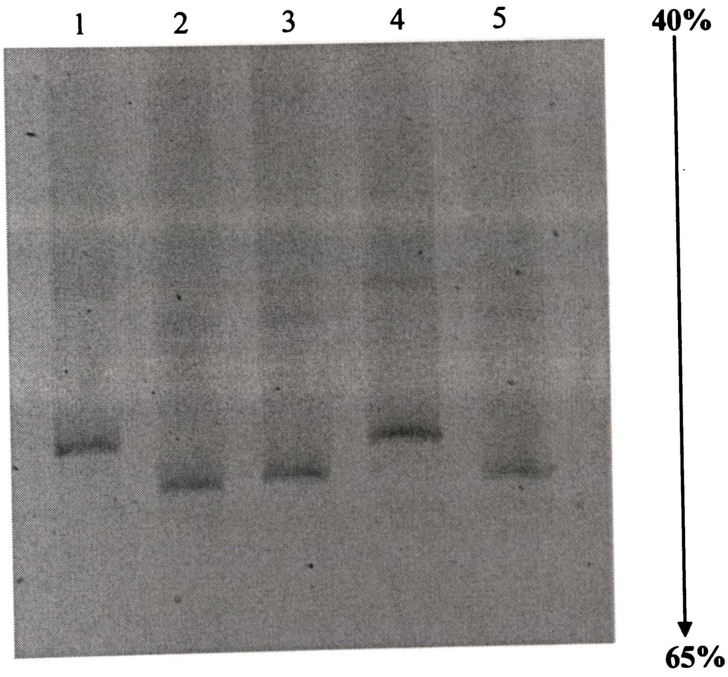


Figure 4.8 Archaeal DGGE of isolates from New Zealand hot pools. The samples and the corresponding lanes are: (1) *Pyrobaculum* sp. isolate AQ1.S2; (2) *Thermococcus zilligii* strain AN1; (3) *Thermococcus* sp. isolate TRUCC 95; (4) AQ3 motile rod culture; (5) AQ1 coccus culture.

4.4 Colonisation of surfaces in Yellowstone National Park hot pools

4.4.1 Yellowstone National Park (YNP) hot pools involved in the study

The YNP hot pools involved in the study are listed in Table 4.9, including the length of time glass slides were incubated in each pool and their *in situ* temperature and pH. Table 4.9 also includes the amount of DNA extracted from the surface of the slides and the dominant cell morphologies and cell densities, graded from high (+++) to low (+) as viewed by microscopy (see Section 4.4.2). Photographs of the pools are presented in Appendix B.

Table 4.9 Yellowstone National Park hot pools involved in the study.

Geographical area	Hot Spring	Incubation time (days)	Temp. range (°C)	pH range	DNA yield approx. (ng)	Cell densities and morphologies
Boulder	Boulder Spring	5	86.4	8.0	+2000	++ Rods
Sentinel Meadows	Steep Cone	8	91	7.68	+2000	+++ Rods & filaments
	Evil Mouth	8	81-85	7.0-8.0	500	++ Rods
	Bison Pool	8	87-92.5	7.0-7.4	+2000	+++ Rods & filaments
GOPA[A]	OB1 Heim	7 and 11	75-85	5.0-6.0	1000/200	++ Rods, at least two types
	South Obsidian Pool	11	75.5-80	6.0	600	+ Rods & cocci
	Obsidian Pool	7	73.5-76	6.0	<200	++ Rods & cocci
	Happy Harfer[B]	N.A.	66-70	5.0-6.0	N.A.	N.A.
Sylvan	Sylvan Spring	8	75-78	5.0-6.0	1800	+++ Rods
	Avocado Pool[B]	N.A.	70-72	6.0-7.0	N.A.	N.A.
South Sylvan	Jan's Tide Pool[B]	N.A.	89	6.6	N.A.	N.A.
	Frog Lips[B]	N.A.	81	6.0	N.A.	N.A.

[A] Greater Obsidian Pool Area. [B] Slides were not incubated in these pools; water from the pools was filtered for subsequent DNA extraction from the filters. N.A. = Not Applicable.

The incubated glass slides were removed and stored in pool water for later microscopical analysis (Section 4.4.2). Biofilm from colonised slides was removed while in the field and stored on ice in CTAB buffer. Filters used to filter pool water were also stored on ice in CTAB buffer. On returning to the base camp, all CTAB samples were stored at -20°C and finally transported back to the laboratory on dry ice for DNA extraction and DGGE analysis (Section 4.4.3).

4.4.2 Microscopy of colonised slides

Micrographs of the biofilms on the colonised slides were taken by differential interference contrast (DIC) microscopy for all samples with the exception of Obsidian Pool and South Obsidian Pool which were photographed under phase-contrast microscopy. Typically, all slides were dominated by rod-shaped microorganisms, with examples of colonisation shown by the micrographs in Figure 4.9(a) through 4.9(f), with all micrographs contained on the supplementary compact disc located inside the back cover of the thesis. The dominant cell morphologies and cell densities of the colonised slides are also listed in Table 4.9.

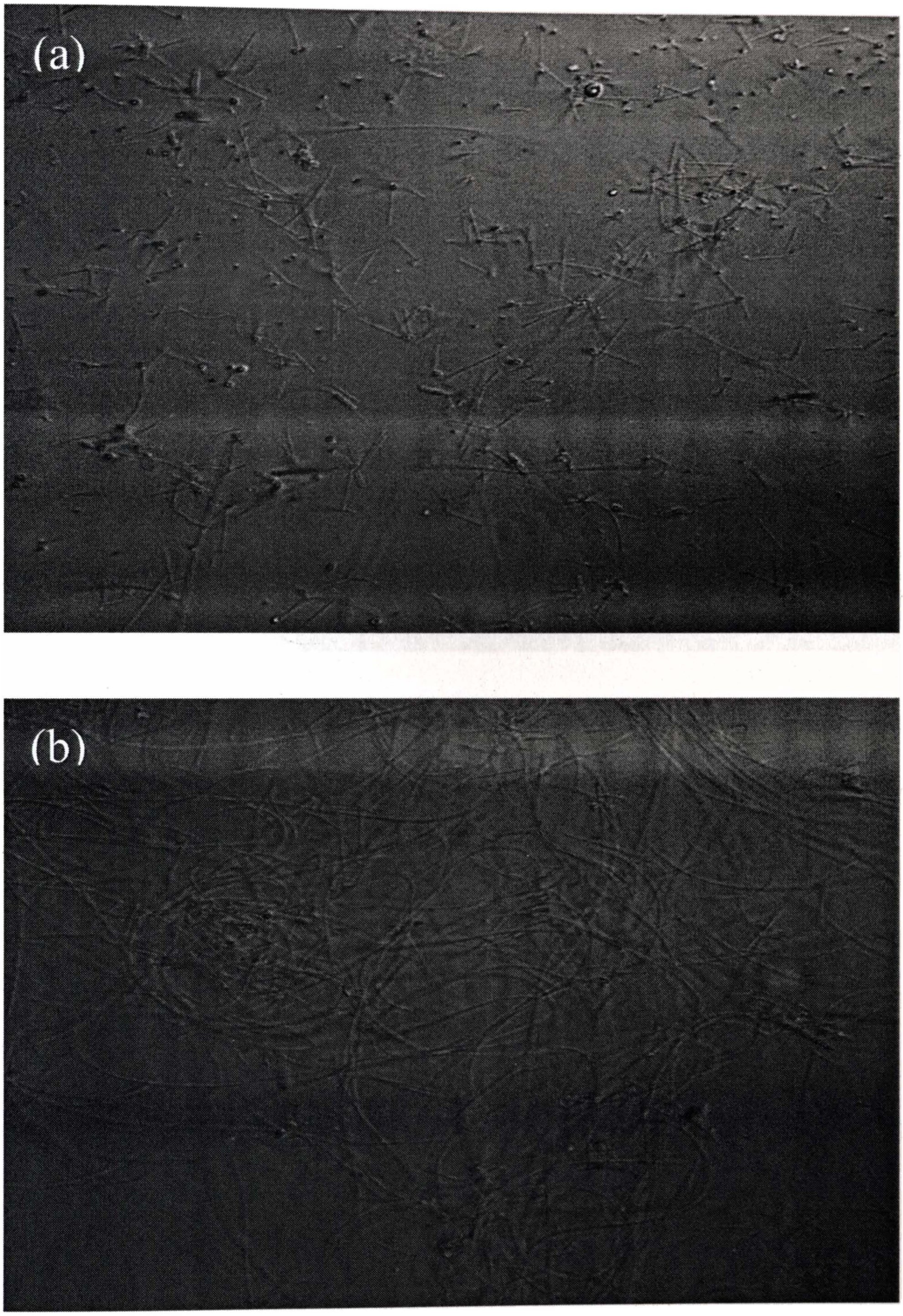


Figure 4.9 Colonisation micrographs of YNP hot pools (DIC, 630X) and the respective samples are: (a) Bison Pool (surface of slide); (b) Bison Pool; (c) Evil Mouth; (d) Boulder Spring; (e) OB1 Heim; (f) South Obsidian Pool (phase-contrast, 1000X).

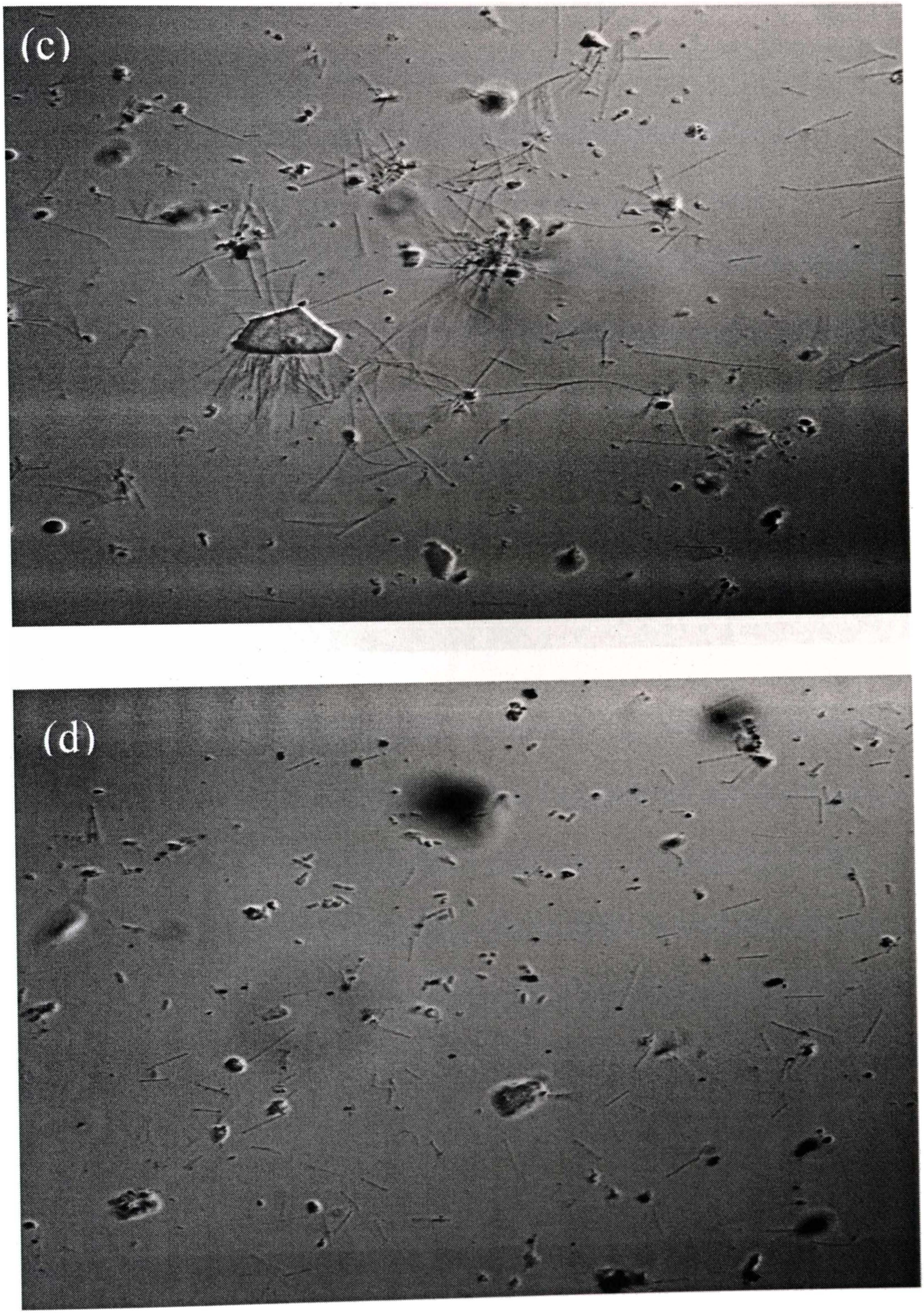


Figure 4.9 The micrographs (DIC, 630X) and the respective samples are: (c) Evil Mouth; (d) Boulder Spring.

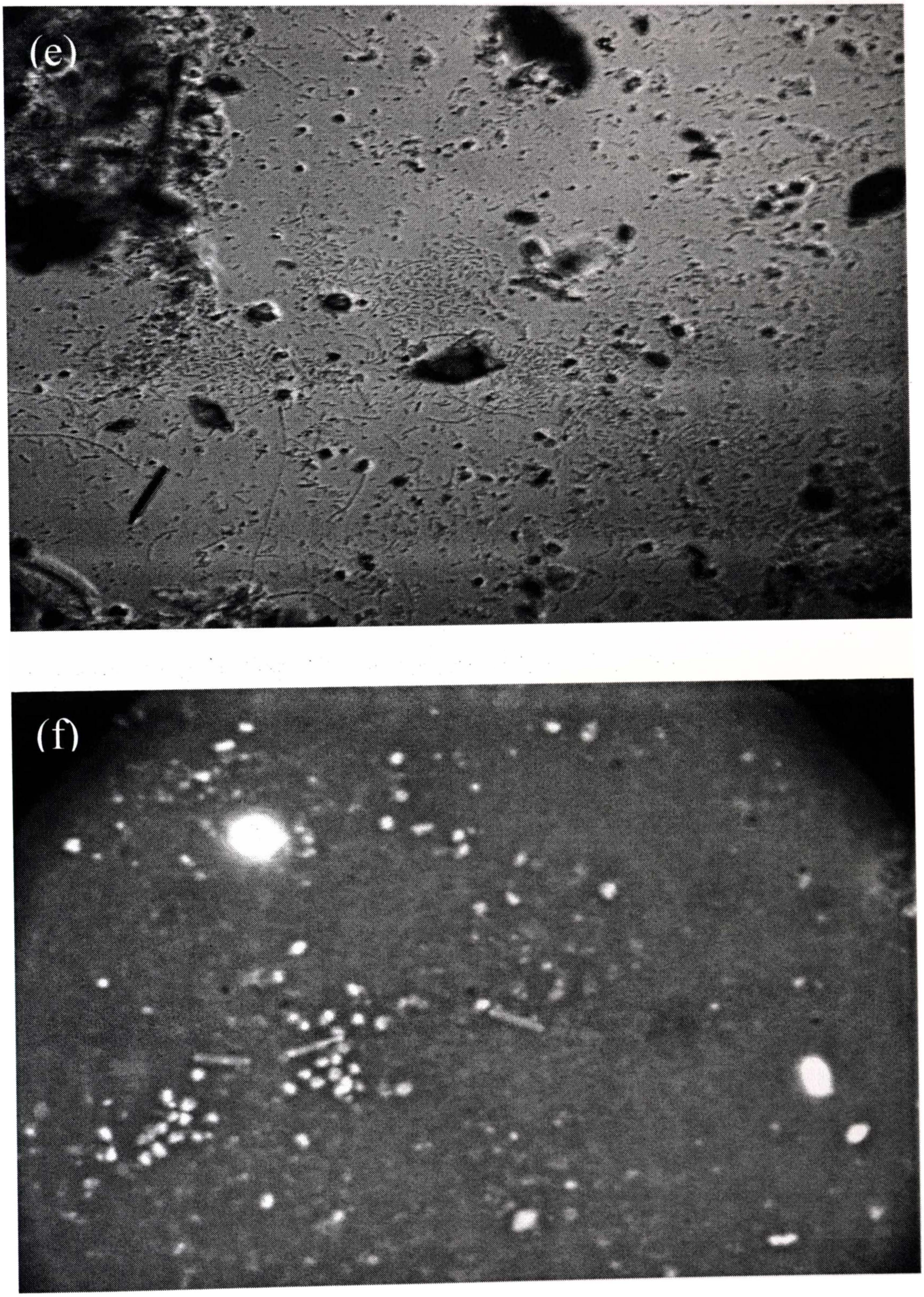


Figure 4.9 The micrographs (DIC, 630X) and the respective samples are: (e) OB1 Heim; (f) South Obsidian Pool (phase-contrast, 1000X).

4.4.3 DGGE of colonised slides and pool water

DNA was obtained from all colonised slides and amplified by PCR. DNA extracted from slides incubated in Obsidian Pool and Steep Cone was used as representative of YNP samples and included for comparison in the New Zealand bacterial DGGE results as presented in Section 4.2.3.1. DNA from *Ignisphaera aggregans*, strain AQ1.S1^T and *Pyrobaculum* sp., isolate AQ1.S2 was also used as reference to compare profiles for the archaeal DGGE gels.

4.4.3.1 Bacterial DGGE

Amplification of DNA using bacterial DGGE primers was obtained with DNA extracted from all slides colonised in YNP pools and presented in Figure 4.10.

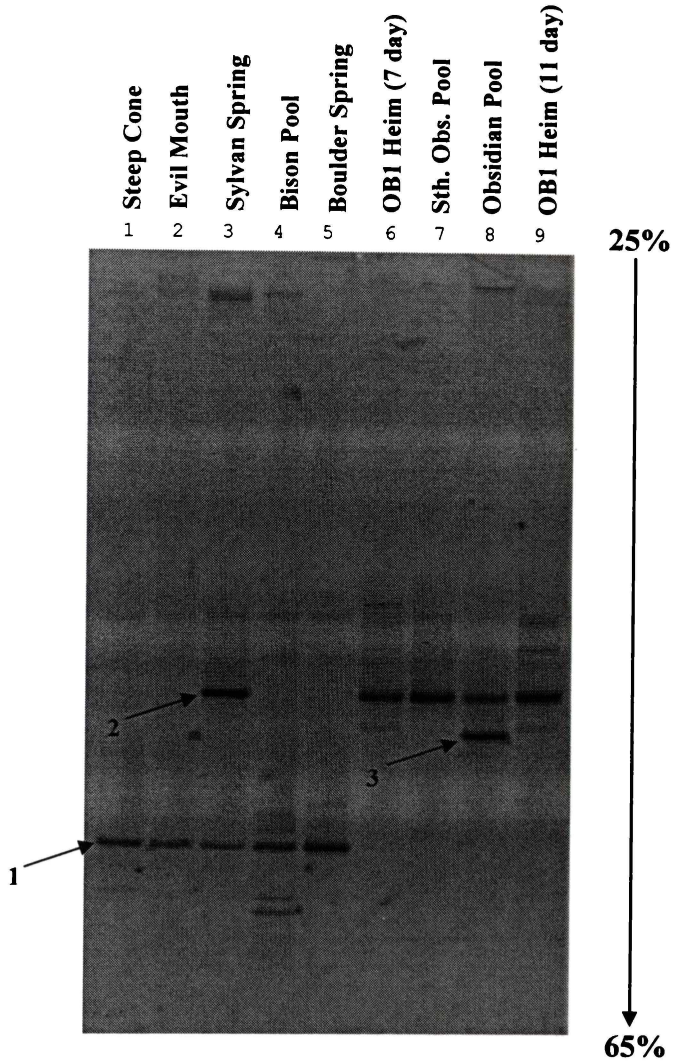


Figure 4.10 Bacterial DGGE of YNP colonised slides. The track numbers and the corresponding samples are: (1) Steep Cone; (2) Evil Mouth; (3) Sylvan Spring; (4) Bison Pool; (5) Boulder Spring; (6) OB1 Heim (7 day); (7) South Obsidian Pool (11 day); (8) Obsidian Pool (7 day); (9) OB1 Heim (11 day). Arrows indicate sequenced bands.

Three common dominant bands were apparent from the DNA for all the YNP colonised slides (Figure 4.10) and one of each was sequenced (indicated by the arrows in Figure 4.10). The closest NCBI BLASTn relatives of the sequenced bands are listed in Table 4.10. Bacterial DGGE was also undertaken on DNA extracted from pool water, with DNA extracted from slides of Obsidian Pool and Steep Cone included for comparison (Figure 4.11).

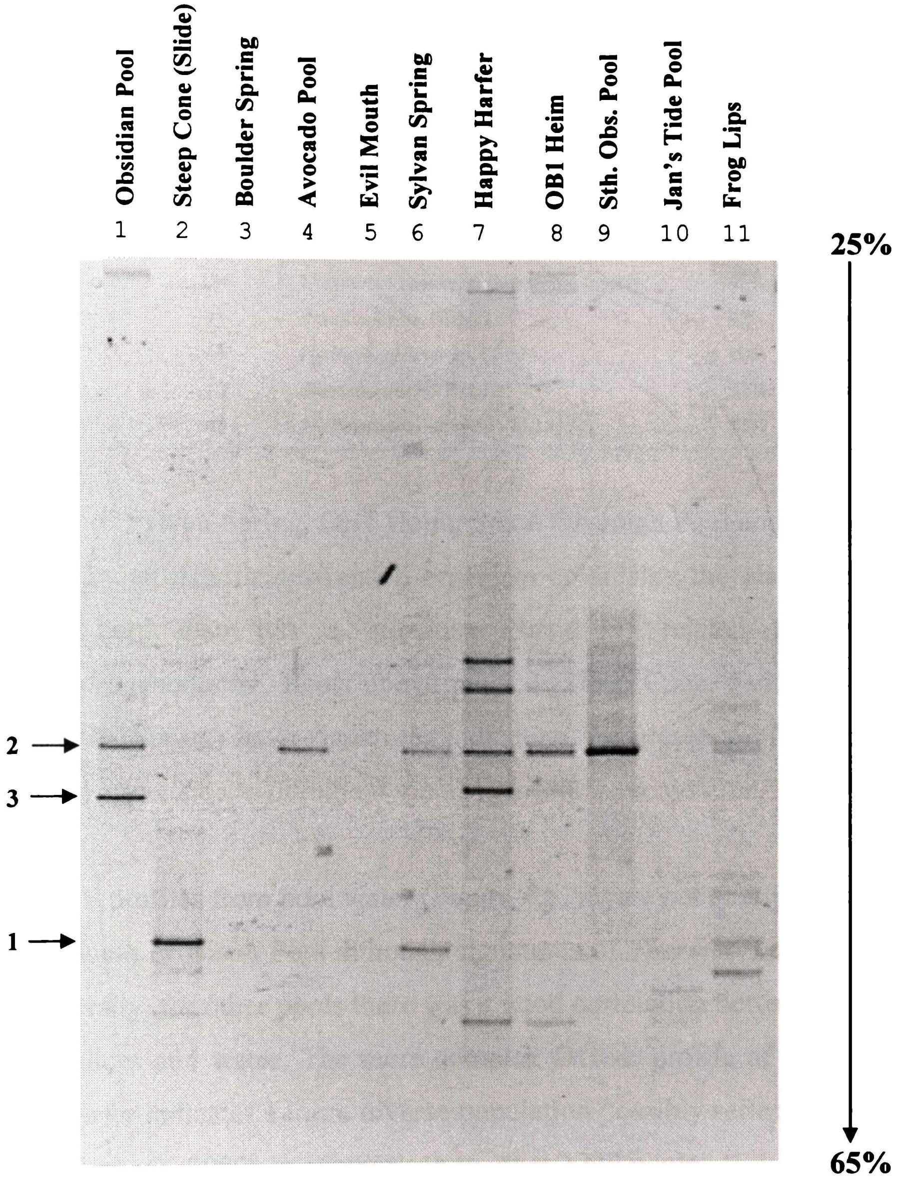


Figure 4.11 Bacterial DGGE of YNP pool water. The track numbers and the corresponding samples are: (1) Obsidian Pool (slide); (2) Steep Cone (slide); (3) Boulder Spring; (4) Avocado Pool; (5) Evil Mouth; (6) Sylvan Spring; (7) Happy Harfer; (8) OB1 Heim; (9) South Obsidian Pool; (10) Jan's Tide Pool; (11) Frog Lips. Arrows indicate the three dominant bands sequenced for colonised slides in Figure 4.10.

Table 4.10 Closest NCBI BLASTn relatives of sequenced bacterial DGGE bands of YNP slides.

Bacterial DGGE Band (Figure 4.10)	Sequence length (bp)	Closest NCBI BLASTn relative	Similarity	Accession
Band 1 - Steep Cone	77	*Thermocrinis ruber*	96-97%	AJ005640
	51	*Eubacteria* EM 17	90%	U05661
	32	*Thermocrinis* sp. P2L2B	96%	AJ320219
Band 2 - Sylvan Spring	134	*Aquificales* bacterium clone Lig060299-6.8	96%	AY293406
	134	*Aquificales* bacterium YNP-SS1	96%	AF507961
	134	Uncultured bacterium (hot spring Japan)	96%	AB081528
Band 3 - Obsidian Pool	41	*Aquificales* str. OIB-21	92%	AF311358
	41	*Hydrogenobacter* sp. IT-7242	92%	AY007593
	40	*Thermocrinis* sp. P2L2B	92%	AJ320219
	40	*Hydrogenobacter* sp. GV8L3A	92%	AJ320218

DGGE profiles of Sylvan Spring, OB1 Heim, South Obsidian Pool and Obsidian Pool are dominated by an Aquificales-related organism colonising the glass slides (band 2). Obsidian Pool also has a signature (band 3) related to species of *Aquificales/Hydrogenobacter*. Pools above pH 7.0 (Steep Cone, Evil Mouth, Bison Pool and Boulder Spring) have signatures indicating the presence of *Thermocrinis*-related (band 1) organisms as dominant members of their ecosystems.

Bacterial DGGE profiles from pool water (Figure 4.11) were not obtained for Boulder Spring, Evil Mouth or Bison Pool although signatures of *Thermocrinis* were evident on slides. Generally, for other pools there was a good correlation between the profiles for colonised slides and water. The more complex DGGE profile of the pool water from Happy Harfer indicates a more diverse population possibly reflecting the lowest *in situ* temperature (66-70°C) in comparison to other YNP pools.

4.4.3.2 Archaeal DGGE

Archaeal DGGE was undertaken on DNA extracted from colonised slides as presented in Figure 4.12. The bands indicated by an arrow in Figure 4.12 were extracted for sequencing; however, the band from Boulder Spring could not be sequenced due to multiple sequences. The closest NCBI BLASTn relatives of the sequenced bands are list in Table 4.11.

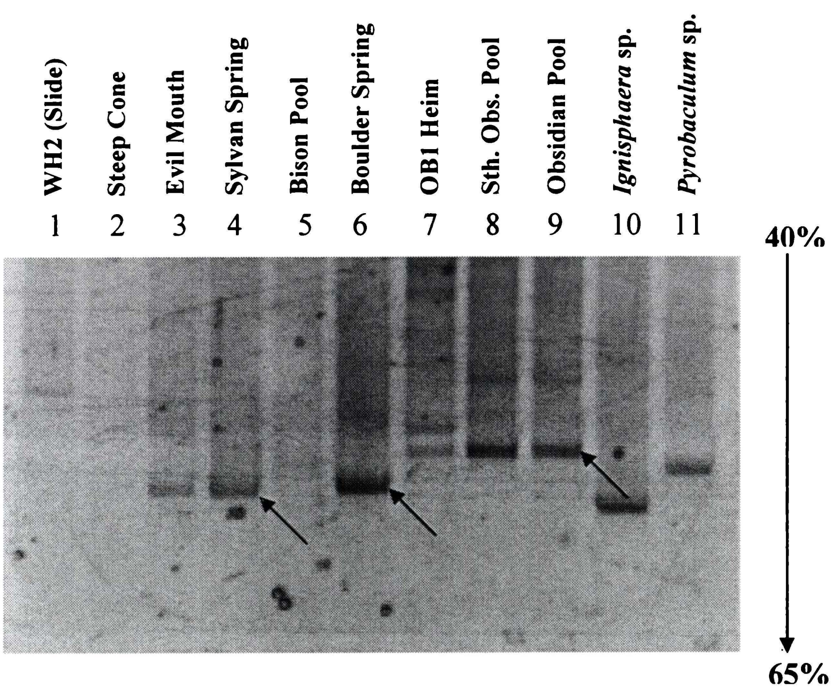


Figure 4.12 Archaeal DGGE of YNP colonised slides. The track numbers and corresponding samples are: (1) WH2 (slide); (2) Steep Cone; (3) Evil Mouth; (4) Sylvan Spring; (5) Bison Pool; (6) Boulder Spring; (7) OB1 Heim; (8) South Obsidian Pool; (9) Obsidian Pool; (10) *Ignisphaera aggregans* strain AQ1.S1^T; (11) *Pyrobaculum* sp. isolate AQ1.S2. Arrows indicate bands extracted for sequencing.

Archaeal DGGE was also performed on DNA obtained from filtered pool water and presented in Figure 4.13. Bands indicated by arrows were sequenced with the closest NCBI BLASTn relatives shown in Table 4.11.

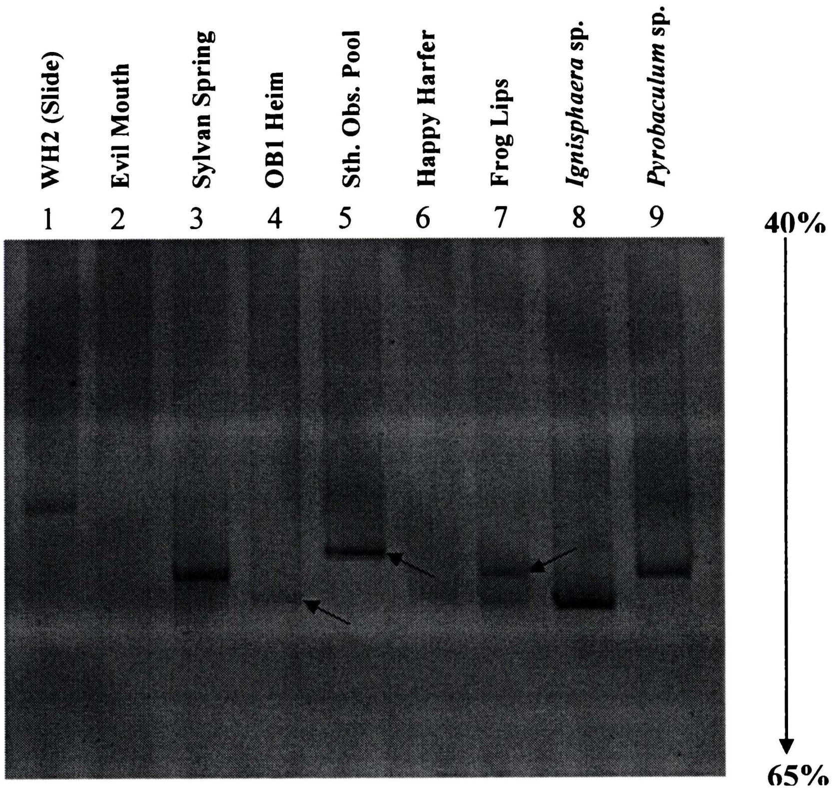


Figure 4.13 Archaeal DGGE of YNP pool water. The track numbers and corresponding samples are: (1) WH2 (slide); (2) Evil Mouth; (3) Sylvan Spring; (4) OB1 Heim; (5) South Obsidian Pool; (6) Happy Harfer; (7) Frog Lips; (8) *Ignisphaera aggregans* strain AQ1.S1^{T}; (9) *Pyrobaculum* sp. isolate AQ1.S2. Arrows indicate bands extracted for sequencing.

Table 4.11 Closest NCBI relatives of archaeal DGGE bands of YNP slides and water.

Archaeal DGGE band	Sequence length (bp)	BLASTn closest matches	Similarity	Accession #
Sylvan Spring slide	216/133 (intron)	Uncultured archaeon clone pEPR863	94/95%	AF526968
	215/134 (intron)	Uncultured archaeon clone pEPR954	94/96%	AF526969
	215/132 (intron)	*Staphylothermus marinus*	94/94%	Y07963
Obsidian Pool slide	365	*Sulfolobus acidocaldarius* *Stygiolobus azoricus*	92%	U05018 D85520
	371	*Sulfolobus solfataricus/islandicus*	91%	AE006720, AE006641 AY247894
South Obsidian Pool water	349	*Sulfolobus solfataricus* *Sulfolobus acidocaldarius*	92%	X03235 U05018
OB1 Heim water	406	4 uncultured Desulfurococcaceae	99%	AY882768, AY882767, AY861992, AY861909
	406	Uncultured archaea clones pEPR863 and pEPR954	90%	As above
Frog Lips water	62	2 uncultured crenarchaeotes	100%	U63340, U63343
	62	Various uncultured archaeons (Thailand hot spring)	100%	AY555831, AY555824 and more

The archaeal DGGE of the colonised slides (Figure 4.12) and the sequenced DGGE bands (Table 4.11) show that the band sequenced from Sylvan Spring (lane 4, Figure 4.12) was also present in the profile of Evil Mouth and was 94-96% similar to uncultured archaeal clones that were obtained from *in situ* samplers deployed on hydrothermal vents at the East Pacific Rise. This sequence contained a 30 bp intron, as can be observed in bold font in the sequence alignment against the *Desulfurococcus mobilis* 16S rRNA gene (M36474), below. The intron sequence was used in a BLASTn search against the NCBI database, however, no matches were found amongst any other 16S rDNA sequences. The band sequenced from the Obsidian Pool profile (lane 9, Figure 4.12) was also present in the profiles of OB1 Heim and South Obsidian Pool, indicating they were dominated by a distantly (<92%) related member of the Sulfolobales.

```
Desulfurococcus mobilis    TCAACGCCGGGAATCTCACCGGGGGAGACAGCAGGATGACGGCCAGGTTA 1050
Sylvan Spring slide        --------------CTCACCGGGGGCGACAGCAGGATTACGGCCAGGCTA 36
                                         *********** *********** ********* **
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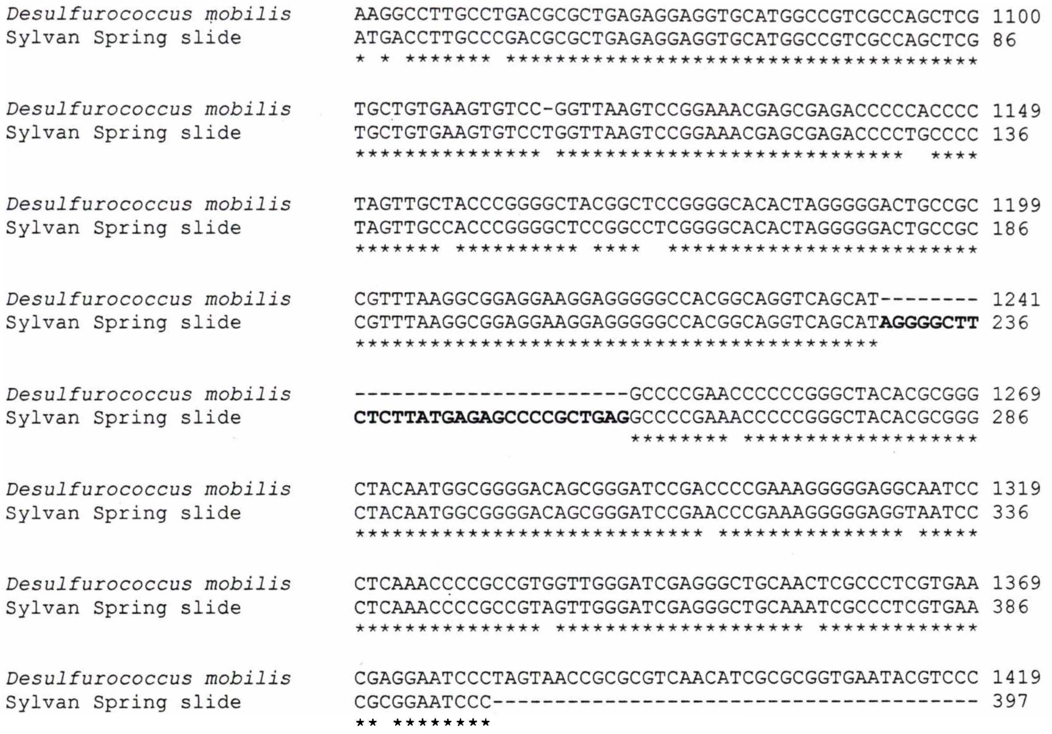

```
Desulfurococcus mobilis    AAGGCCTTGCCTGACGCGCTGAGAGGAGGTGCATGGCCGTCGCCAGCTCG 1100
Sylvan Spring slide        ATGACCTTGCCCGACGCGCTGAGAGGAGGTGCATGGCCGTCGCCAGCTCG 86
                           * * ******* **************************************

Desulfurococcus mobilis    TGCTGTGAAGTGTCC-GGTTAAGTCCGGAAACGAGCGAGACCCCCACCCC 1149
Sylvan Spring slide        TGCTGTGAAGTGTCCTGGTTAAGTCCGGAAACGAGCGAGACCCCTGCCCC 136
                           *************** ****************************  ****

Desulfurococcus mobilis    TAGTTGCTACCCGGGGCTACGGCTCCGGGGCACACTAGGGGGACTGCCGC 1199
Sylvan Spring slide        TAGTTGCCACCCGGGGCTCCGGCCTCGGGGCACACTAGGGGGACTGCCGC 186
                           ******* ********** ****  *************************

Desulfurococcus mobilis    CGTTTAAGGCGGAGGAAGGAGGGGGCCACGGCAGGTCAGCAT-------- 1241
Sylvan Spring slide        CGTTTAAGGCGGAGGAAGGAGGGGGCCACGGCAGGTCAGCATAGGGGCTT 236
                           ******************************************

Desulfurococcus mobilis    ----------------------GCCCCGAACCCCCCGGGCTACACGCGGG 1269
Sylvan Spring slide        CTCTTATGAGAGCCCCGCTGAGGCCCCGAAACCCCCGGGCTACACGCGGG 286
                                                 ******** *******************

Desulfurococcus mobilis    CTACAATGGCGGGGACAGCGGGATCCGACCCCGAAAGGGGGAGGCAATCC 1319
Sylvan Spring slide        CTACAATGGCGGGGACAGCGGGATCCGAACCCGAAAGGGGGAGGTAATCC 336
                           **************************** *************** *****

Desulfurococcus mobilis    CTCAAACCCCGCCGTGGTTGGGATCGAGGGCTGCAACTCGCCCTCGTGAA 1369
Sylvan Spring slide        CTCAAACCCCGCCGTAGTTGGGATCGAGGGCTGCAAATCGCCCTCGTGAA 386
                           *************** ******************** *************

Desulfurococcus mobilis    CGAGGAATCCCTAGTAACCGCGCGTCAACATCGCGCGGTGAATACGTCCC 1419
Sylvan Spring slide        CGCGGAATCCC--------------------------------------- 397
                           ** ********
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The sequences from the DGGE profiles for YNP (with the exception of Frog Lips due to its short sequence length) were included in a 16S rRNA gene-based phylogenetic tree to indicate phylogenetic affiliations (Figure 4.14).

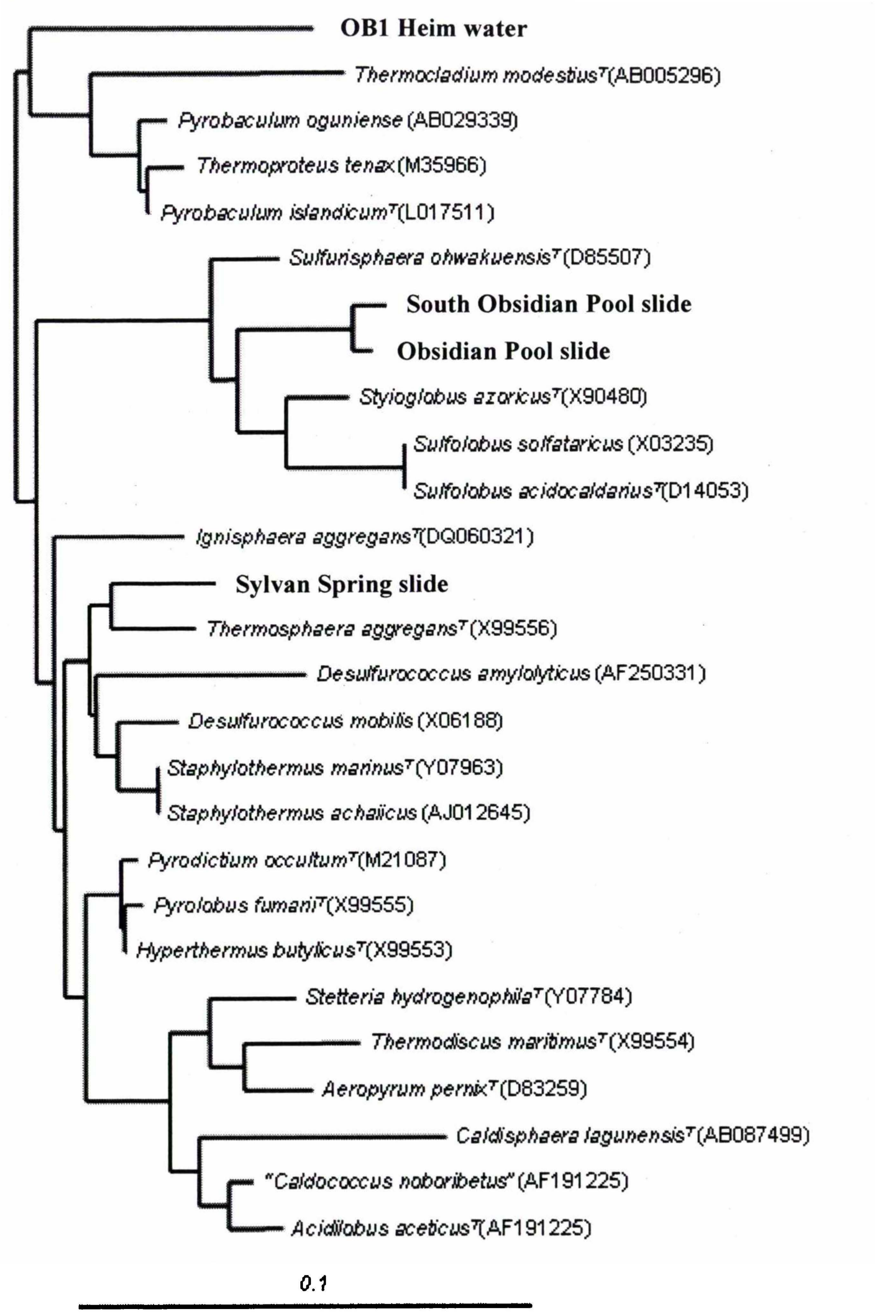

Figure 4.14 A 16S rRNA gene-based phylogenetic tree showing positions of the YNP sequenced DGGE bands. The dendogram was constructed using 231 nucleotides of aligned sequence with FITCH (Jukes-Cantor) distance analysis. The tree was rooted to *Thermococcus celer* DSM 2476^T (M21529). Bar = 10% sequence divergence.

4.4.4 Culturing from YNP pools

Culturing was only undertaken from the YNP pools in an attempt to isolate *Ignisphaera*-related microorganisms, as discussed in Chapter 5.

4.5 Screening for nanoarchaea in New Zealand and YNP hot pools

Initially, the New Zealand hot pools were screened for the presence of nanoarchaea using the protocol and primers as described by Hohn *et al.*, (2002). The expected 1500 bp PCR product was obtained from the amplification of some of the DNA of the colonised slides as presented in Figure 4.15. However, it was not certain how specific the primer pair was to nanoarchaeal 16S rDNA; moreover, a positive control DNA was not available.

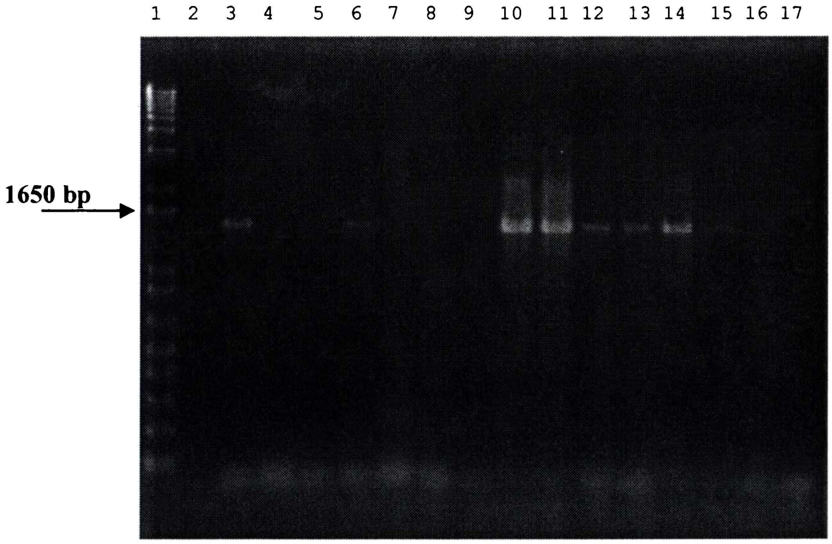

Figure 4.15 Nanoarchaeal 16S rDNA PCR amplification of New Zealand hot pools. The track numbers and corresponding samples are: (1) 1 kb ladder; (2) AQ2; (3) AQ3; (4) AQ4; (5) AQ5; (6) KP2; (7) KP3; (8) OH1; (9) OH2; (10) SP1; (11) SP2; (12) SP3; (13) WH1; (14) WH2; (15) TK2; (16) TK3; (17) Negative control.

During the visit to YNP, a nanoarchaeal 16S rRNA gene-specific PCR protocol, including a primer pair and positive DNA (nanoarchaeal 16S rDNA obtained from a hydrothermal vent within a vector) became available from McCliment *et al.*, (in press). The primers were tested for specificity by using archaeal DNA as templates for the PCR assay. PCR products were not obtained from the following cultures: *Ignisphaera* sp. isolate Tok37.S1 (see Chapter 5); *Thermococcus zilligii* (AN1); *Ignisphaera aggregans* (AQ1.S1^T); *Sulfolobus solfataricus* (DSM 1616); a *Pyrobaculum* 16S rDNA clone obtained from AQ1 (see Section 3.5.2). Subsequently, a nanoarchaeal PCR survey was undertaken (Figure 4.16) on DNA obtained from the filter used to filter pool water of YNP and New Zealand pools and included the pure culture isolated from AQ1 (*Pyrobaculum* sp., isolate AQ1.S2, see Chapter 3).

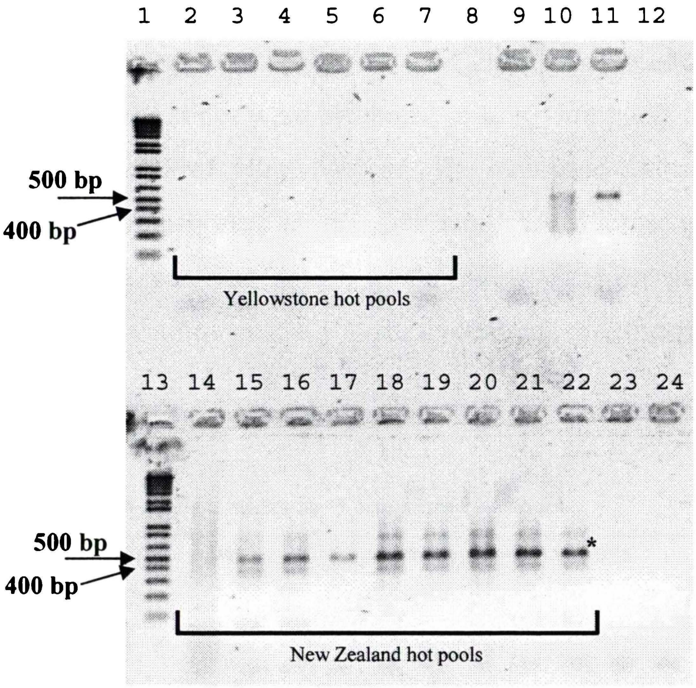

Figure 4.16 Nanoarchaeal 16S rRNA gene amplification from New Zealand and YNP pools. The track numbers and corresponding samples are: (1) 1 kb ladder; (2) Evil Mouth; (3) Sylvan Spring; (4) OB1 Heim; (5) South Obsidian Pool; (6) Happy Harfer; (7) Frog Lips; (8) empty; (9) negative control; (10) positive control (nanoarchaeal 16S rDNA clone); (11) *Pyrobaculum* sp. isolate AQ1.S2; (12) empty; (13) 1 kb ladder; (14) OH1; (15) WH2; (16) SP2; (17) AQ3; (18) TK2; (19) WH1: (20) AQ4; (21) AQ2; (22) AQ1; (23) empty; (24) empty. *Band was extracted and sequenced.

Nanoarchaea were not detected in any of the YNP samples, lanes 2 to 7 in Figure 4.16. All the New Zealand pools (lanes 14 to 22 in Figure 4.16) gave positive amplification, including the pure culture of *Pyrobaculum*, isolate AQ1.S2 (lane 11 in

Figure 4.16). The pool water from which this culture was isolated also gave a positive result for nanoarchaeal amplification. The AQ1 PCR product (indicated by an asterisk in Figure 4.16) was extracted and sequenced and was most closely related to the 16S rRNA genes from an uncultured nanoarchaeote, clone CU-1 (AJ458437) from the Uzon Caldera (89%, 261/293 bp) and an uncultured nanoarchaeote, clone OP-9 (AJ458436) from Obsidian Pool, YNP (87%, 246/280 bp) (Hohn *et al.*, 2002).

4.6 White Island

4.6.1 Colonisation analyses

In February 2005 glass slides were incubated in seven hot pools on White Island for a period of two days by Prof. Hugh Morgan. The incubated slides were removed from the hot pools and transported to the laboratory for microscopical and molecular analysis. Table 4.12 contains details of the pools used for glass slide incubation including the observations made by phase-contrast microscopy of the colonised slides.

Table 4.12 Colonisation of glass slides in White Island hot pools.

Pool #	Temp. (°C)	pH	Slide microscopy observations	DNA yield approx. (ng)
1	94	3.3	Coccal colonisation	200
2	63	3.8	Coccal colonisation	400
3	89	3.0	Dark iron precipitate on surface	300
4	77	3.0	Dark iron precipitate on surface	Below detection
5	72	1.5	Colonisation not evident	Below detection
6	82	1.5	Colonisation not evident	Below detection
7	85	1.9	Colonisation not evident	Below detection

DNA was extracted from a total of two slides for each pool and bacterial and archaeal DGGE undertaken. Bacteria were not detected on the colonised slides by bacterial DGGE PCR, this was repeated with 5% DMSO included in the PCR and again bacterial signatures were not detected. Finally, a full length bacterial 16S rDNA PCR was undertaken on all colonised slides however, bacterial signatures were not detected.

Archaea were detected by PCR on slides incubated in pools 1, 2 and 3. The resulting archaeal DGGE is presented in Figure 4.17, below.

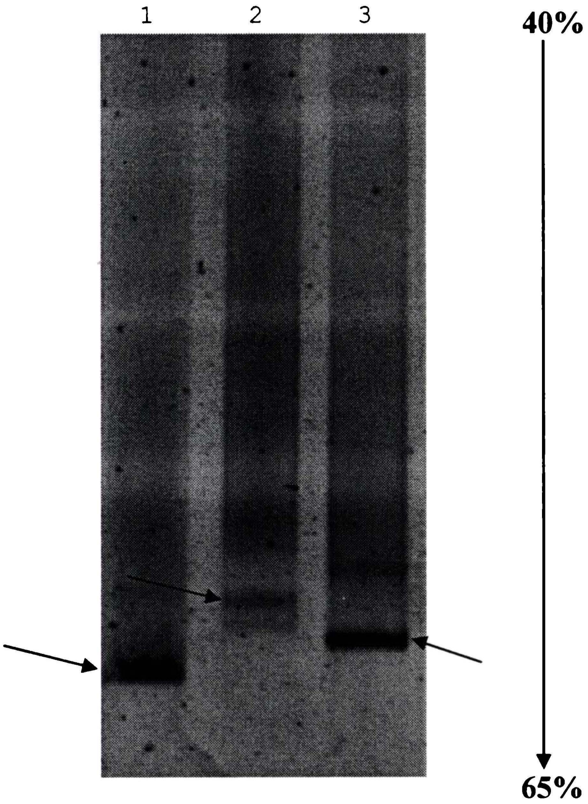

Figure 4.17 Archaeal DGGE of White Island colonised slides. The track numbers and corresponding samples are: (1) Pool 1; (2) Pool 2; (3) Pool 3. Arrows indicate bands extracted for sequencing.

The major DGGE band obtained from DNA of colonised slides from each of the three pools (indicated by arrows in Figure 4.17) was extracted for sequencing. The sequences are contained in Appendix D, however, the band from pool 2 did not sequence due to multiple sequences being present. The closest NCBI BLASTn relatives (96% similar) for the sequences from pool 1 and pool 3 were species of *Sulfolobus* and *Sulfurisphaera* (363/347 bp and 368/381 bp, respectively).

4.7 Discussion and conclusion

The New Zealand pools involved in the colonisation survey were between 80 and 100°C and pH 5.5 to 9.0 with microbial colonisation observed for all slides incubated *in situ*, including YNP slides. However, as is discussed below, colonisation was not detected for all slides incubated in White Island pools.

Colonisation was rapid for the near-neutral New Zealand hot pools, with high levels of ATP measured on slide surfaces as compared to background water levels. Also, ATP levels and the amount of DNA extracted from the surfaces of the slides generally reflected the concentration of cells observed on slide surfaces by microscopy. Typically, pools with the highest concentration of cells on slide surfaces (observed by microscopy) overloaded the ATP meter, and DNA yields of greater than 2 μg were obtained. ATP has also been used as an indicator of microbial biomass, or biological activity, in hot pools of Monserrat Island where it was shown that biological activity decreased as acidity of the water samples increased (Atkinson *et al.*, 2000). The differences in the amount of biofilm development on the slides between all pools generally reflected the amount of water movement in the pool either because of extensive bubbling (AQ1, AQ2, KP2, KP3, SP1, SP2 and SP3), or because slides were placed in pool water runoff (OH1, AQ5) compared to the stagnant pools (OH2, TK2, TK3, AQ3, AQ4, WH1 and WH2). This may be due to the constant input of nutrients and/or gases and the mixing of these energy sources into the hot spring (and removal of waste products) promoting biomass increase within the developing biofilm (although Champagne Pool has proven to be an exception, see Chapter 3).

Microscopic examination of the New Zealand slides revealed a dominance of rod-shaped cells for all pools (with exception of KP3) which was reflected in the dominance of DNA signatures relating to rod morphotypes of sequenced DGGE bands, i.e. *Thermodesulfobacteria*, *Thermotogales*-related, *Aquificales*-related organisms, *Pyrobaculum* species and *Thermofilum* species. The coccal colonisation for some pools was also accounted for by sequenced DGGE bands, i.e. the

Desulfurococcales-related and *Sulfolobus*-related organisms. When bacterial primers for DGGE analysis were used, positive amplification were only obtained from slides colonised in pools with *in situ* temperatures below 88°C for the New Zealand pools. For archaea, only pools with an average *in situ* temperature above 83°C gave positive amplification. However, it cannot be assumed that bacteria and archaea are not present outside these temperature limits, their omission could be due to a variety of factors including population density being below the detection threshold, PCR bias or PCR inhibition. Archaea have been shown to be present in other near-neutral terrestrial thermophilic environments with temperatures below 83°C (Reysenbach *et al.*, 2000a; Barns *et al.*, 1994). Also as is discussed in further detail below, bacteria have been detected by PCR in hot pools of other thermal habitats with temperatures above 88°C. However, the maximum growth temperatures of characterised bacteria isolated from NZ hot pools is approximately 78 to 82°C (Patel *et al.*, 1985; Patel *et al.*, 1987; Hudson *et al.*, 1987).

Besides temperature the other major determinant of archaeal colonisation of surfaces in New Zealand pools was pool pH, with similar DGGE profiles obtained for pools of similar pH. Pools with a pH between 6.0 and 7.0 had a dominant *Thermofilum* signature; pools with a pH above 7.0 were dominated by *Pyrobaculum* and a coccus related to the *Desulfurococcales* and the acidic pools were dominated by a *Sulfolobales*-related species which was also the case for the White Island pools. Not surprisingly, organisms cultured from pools over the above pH ranges had pH growth optima that reflected the pool pH, e.g. *Thermofilum* (*T. pendens* and *T. librum),* approximately pH 5.0-6.0 (Zillig *et al.*, 1983a), *Pyrobaculum* species being typically near-neutral (Völkl *et al.*, 1993; Sako *et al.*, 2001; Huber *et al.*, 2000b) and the members of the *Sulfolobales* being acidophiles (Johnson, 1998). It was also noted that the *Desulfurococcale*-related coloniser (closest cultured relative being *Aeropyrum pernix*) as detected on slides incubated in the pools with a pH above 7.0 was very closely related to the coccal coloniser of the slides incubated deep within the water column of AQ1, as discussed in detail in Chapter 3 (2 bp difference in 261 bp aligned sequence between AQ1, WH1 and TK2 DGGE band sequences).

The bacteria detected on colonised slides included the dominant signatures of *Thermodesulfobacteria* and *Aquificales* species. Other international studies of terrestrial geothermal ecosystems have also shown a *Thermodesulfobacterial* and *Aquificales* bacterial dominance. For example, *Thermodesulfobacterial* and *Aquificales* 16S rDNA signatures were dominant in clone libraries of a sulfur mat hot spring in Iceland (Skirinsdottir *et al.*, 2000) and geyserite of near-neutral silica depositing pools of Boulder Spring and Queens Laundry hot pools located in YNP (Blank *et al.*, 2002). Similarly, *Aquifex* and *Thermodesulfobacterial* signatures were obtained from DGGE profiles of microbial biofilm of springs (72-80°C) situated in Nakabusa and Yumata, Japan (Nakagawa and Fukui, 2003). However, isolations of these organisms from New Zealand's hot pools were not obtained using media that have been used to isolate and characterise the original cultured representatives of each genera.

Although, the geographically distinct pools at Tokaanu shared similar archaeal DGGE profiles to the pools of similar pH and temperature of Rotorua, i.e. having a *Pyrobaculum* and *Desulfurococcale* dominance on colonised slides, the *Pyrobaculum* band resided slightly higher in DGGE gels. This may be due strain/species variation in 16S rRNA gene sequences between *Pyrobaculum* colonisers. In addition, the Tokaanu bacterial DGGE profiles were different to the Rotorua pools of similar pH and temperature with the dominant coloniser being related to the EM3 clone obtained from the hyperthermophilic pink filament community in Octopus Spring, YNP (Reysenbach *et al.*, 1994). Similarly, Blank *et al*, (2002) showed that clones most closely related to EM3 dominated 16S rDNA libraries for both Octopus Spring (near the source vent) and Abyss Pool sediment of YNP. These differences in both archaeal and bacterial colonisation for the Tokaanu pools (TK2 and TK3) as compared to the Rotorua pools of similar pH and temperature may be due to differences in pool water chemistry, therefore, leading to altered populations of microorganisms due to different metabolic opportunities. A study by Whitaker *et al.*, (2003) has in fact shown that the geographical isolation of *Sulfolobus* populations is primarily responsible for genetic divergence rather than hot spring characteristics.

In conclusion, the bacterial and archaeal colonisers as discussed above show the presence of novel uncultured organisms in New Zealand geothermal ecosystems that are related to both cultured hyperthermophiles and uncultured microbial signatures obtained from other hot spring habitats. Attempts to culture these organisms using media that have been used to isolate and characterise the original cultured representatives of each genera from the hot pools were unsuccessful, with the exception of *Pyrobaculum* and probable *Thermofilum* cells (although attempts to obtain pure cultures of *Thermofilum* were unsuccessful). This is most probably due to the New Zealand species possessing different metabolic requirements than the already isolated and characterised cultured type strains. The unsuccessful culturing attempts were also paralleled by the low diversity of cell enrichments obtained from isolation and MPN results, whereby tested media were typically dominated by similar consortia of rod-shaped cells (probably *Pyrobaculum* cells) and cocci, with the lower temperature (80°C) incubated enrichments being dominated by *Fervidobacteria*. The type strain of the *Fervidobacterium* genus (*F. nodosum*) was originally isolated from a New Zealand hot pool, fully characterised, and shown to have a maximum growth temperature of 80°C (Patel *et al.*, 1985). Moreover, pools KP2, KP3, TK2, TK3, OH2 and SP3 did not provide any cell enrichments. As also discussed below for the YNP samples, it has been shown that the major Aquificales mode of metabolism is H_2 oxidation, i.e. utilizing H_2 as an electron acceptor under micro-aerophilic conditions (Eder and Huber, 2002). Therefore, if time had permitted it would have been interesting to attempt to culture the Aquificales-related coloniser from New Zealand hot pools using an H_2-containing Aquificales-based medium.

In comparison to the colonisation results, the pool water DGGE profiles did differ in some respects to their respective colonisation profiles, i.e. *Thermococcus* species were detected in pool water but not on colonised slides, and the *Thermofilum* dominance of the slides incubated in pH 6.0-7.0 pools was not seen in their respective pool water DGGE profiles. For the pool water MPN results, levels of up to approximately $10^{2\text{-}3}$ cells/ml for pool water were obtained and sediment MPN cell counts were typically 1 to 2 orders of magnitude higher. This is most probably due cells being able to adsorb to the available surfaces within the sediment and remain within the environment, thus, supporting growth.

The YNP pools included in the colonisation study were in the temperature and pH ranges of 73-92.5°C and pH 5.0-8.0, respectively, and colonisation was detected on all incubated slides by DGGE and microscopy. Also, high concentrations (over 2 μg) of DNA were isolated from the densely colonised YNP slides. Likewise, Hugenholtz *et al.*, (1998) has also shown colonisation micrographs of glass slides incubated in Obsidian Pool that were dominated by rods and filamentous cells, however, they were not analysed by molecular methods. The dominant bacterial and archaeal signatures detected on colonised slides in YNP were different from those found on slides in NZ pools. The YNP slides were dominated by bacteria, with bacterial signatures detected in high-temperature pools of Steep Cone (91°C) and Bison Pool (87-92.5°C), whereas, in NZ pools bacterial amplification was only detected at pool temperatures up to 88°C. Archaea were absent from YNP pools for which DNA was obtained, but present in all NZ pools above 83°C. Other studies of YNP geothermal habitats confirm a bacterial dominance and the absence of archaeal DNA signatures. For example, archaea were not found by 16S rRNA gene PCR cloning in seven high temperature (85-98°C) near-neutral hot pools (including Boulder Spring) with samples including both colonised glass slides and geyserite (Blank *et al.*, 2002). Similarly, archaeal PCR amplicons were not obtained from the hyperthermophilic pink filament community in Octopus Spring (Reysenbach *et al.*, 1994) and Obsidian Pool sediment (Hugenholtz *et al.*, 1998) of YNP. However, various other studies of YNP hot springs have shown a diversity of both bacteria and archaea in YNP hot pools (Barns *et al.*, 1994; Barns *et al.*, 1996a; Spear *et al.*, 2005; Reysenbach *et al.*, 2000a). Moreover, our study shows a presence of archaeal colonisers in YNP hot pools, including Boulder Spring which was studied by Blank *et al.*, (2002). These contradictions in the detection of archaea by PCR-based methods within YNP hot pools may be due archaeal population densities being below detection thresholds, e.g. Blank *et al.*, (2002) did not detect archaea in seven high-temperature YNP hot pools by PCR, however, archaeal lipids were detected in some pools. Also, PCR bias or

PCR inhibition may be an influence as various DNA extraction procedures and PCR primers were used in the above mentioned studies.

In this study, all the bacterial signatures found colonising the glass slides in YNP pools were related to the Aquificales phylogenetic division, which has been shown to be the case in other thermal habitats by a number of other studies, i.e. YNP (Spear *et al.*, 2005; Blank *et al.*, 2002; Reysenbach *et al.*, 1994; Reysenbach *et al.*, 2000a), Iceland (Takacs *et al.*, 2001; Hjorleifsdottir *et al.*, 2001) and Japan (Yamamoto *et al.*, 1998). Moreover, the dominance of rod morphotypes on colonised slides would be in accordance with the presence of Aquificales. As was the case for the NZ pools, colonisation was pH dependent, with similar DGGE profiles for pools of similar pH ranges. Pools with a pH below 7.0 were dominated by *Aquifex*-related signatures and pools with a pH above 7.0 were dominated by *Thermocrinis,* with an additional dominant *Hydrogenobacter*-related signature detected in Obsidian Pool. This may in part be due to increased levels of H_2 availability because slides were incubated in an 'active' bubbling position of the pool. The pH-based grouping of the bacterial signatures on the slides is also in accordance with the optimal growth pH of the cultured representatives of these genera, i.e. the cultured *Thermocrinis* species (*T. ruber* and *T. albus*) both grow optimally at approximately pH 7.5 to 8.0, and the cultured *Aquifex* species (*A. pyrophilus* and *A. aeolicus*) at approximately pH 6.5 (Eder and Huber, 2002). As mentioned earlier, the cultured representatives of the Aquificales undertake micro-aerophilic H_2-based metabolism, and thus, it has been theorised that this is the major source of energy for primary production in the YNP ecosystem (Spear *et al.*, 2005; Blank *et al.*, 2002).

For White Island, colonisation was apparent by microscopy and DGGE on slides incubated in pools of 63 to 94°C and pH 3.0 to 3.8. However, colonisation was not detected in pools of 72 to 85°C and pH 1.5 to 1.93 by either DGGE or microscopy. This lack of colonisation maybe due to the short incubation periods (i.e. low biomass accumulation) or due to the combination of high temperature (72-88°C) and low pH (1.5-3.0) inhibiting microbial colonisation. Thermophilic, acidophilic microorganisms such as the two cultured representatives of the *Picrophilus* genera have been isolated from such extreme temperature and pH habitats (below pH 2.0) although the upper temperature for their growth at such extreme pH is 65°C (Schleper *et al.*, 1996). Attempts to isolate such extreme acidophiles from White Island hot pools proved unsuccessful (results not shown). As may have been predicted, *Sulfolobales* were the dominant microbial signatures on the colonised slides for the White Island pools. The *Sulfolobales*-related signature colonising slides in White Island pool 1 was closely related (2 bp difference in a total aligned sequence of 372 bp) to the *Sulfolobales* coloniser of the pool of KP3 pool with the White Island *Sulfolobales* coloniser of pool 3 being distantly related to both the KP3 and White Island pool 1 (11 bp differences to both sequences of 372 bp aligned sequence).

Finally, positive nanoarchaeal 16S rDNA PCR amplicons were obtained from pool water for all New Zealand pools (with the exception of OH1, and significantly archaea were also not detected in this pool) but not for the YNP samples. Due to the recent discovery of nanoarchaea in an *Ignicoccus* culture isolated from a submarine hydrothermal vent system (see Section 6.1 for a background on nanoarchaea); new PCR primers were developed to target the nanoarchaeal 16S rRNA gene (Huber *et al.*, 2002). Hohn *et al.*, (2002) consequently utilised these primers to screen a number of high-temperature (70-98°C) environments and found the presence of nanoarchaeal signatures in the Obsidian Pool of YNP (in contrast the present study did not detect nanoarchaea in Obsidian Pool), the Uzon Caldera, Kamchatka, Russia and an abyssal vent system at the East Pacific Rise (EPR). Therefore, it was concluded that nanoarchaea are present in other high-temperature habitats including terrestrial high-temperature hydrothermal systems. The PCR-based detection of nanoarchaea in New Zealand pools was thought to be a true indication of nanoarchaeal presence, as the primers were shown to be specific to nanoarchaea, and the AQ1 amplicon was sequenced and shown to be closely related to a nanoarchaeal 16S rDNA clone obtained from the study by Hohn *et al.*, (2002). Related to this, as discussed in Chapter 3, a presumed pure culture of *Pyrobaculum* from the AQ1 pool may contain nanoarchaea. Therefore, if the *Pyrobaculum* cells within this consortium are the host cells for the nanoarchaea, then the detection of *Pyrobaculum* in the pools screened for nanoarchaea, i.e. *Pyrobaculum* DGGE band in WH2 pool water; *Pyrobaculum* cultured from pH 6.0-7.0 pools SP2 and AQ3; dominant *Pyrobaculum* coloniser for pools with pH above 7.0, TK2, WH1, AQ4, AQ2 and AQ1, may explain the widespread occurrence of nanoarchaea in New Zealand hot pools.

Chapter 5 : Isolation and characterisation of novel coccal-shaped archaea

5.1 Introduction

A paper has been submitted to the International Journal of Systematic and Evolutionary Microbiology (IJSEM), describing the isolation and characterisation of a novel coccal-shaped archaeum isolated from a New Zealand hot pool (pool AQ1 as outlined in Chapter 3). This paper has been published online (doi:10.1099/ijs.0.63899-0) and is reproduced below and forms the basis of this Chapter. All experimental work as described in the manuscript was undertaken by T. Niederberger, the Tok1 16S rDNA sequence was obtained by D. Götz and I. McDonald helped with the ARB software package. Supplementary data that is related to this study is discussed following the article in Section 5.2 below.

IJSEM MANUSCRIPT: *Ignisphaera aggregans* gen. nov., sp. nov., a novel hyperthermophilic crenarchaeote isolated from hot springs in Rotorua and Tokaanu, New Zealand

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Running title: *Ignisphaera aggregans*, a novel archaea from N. Z.

Subject Category: New Taxa, *Archaea*

The GenBank accession numbers for the 16S rRNA gene sequences of *Ignisphaera aggregans* AQ1.S1^T, and strains Tok10A.S1, Tok37.S1 and Tok1 are DQ060321, DQ060322, DQ060323 and DQ060320, respectively.

ABSTRACT

Consortia containing a novel coccus-shaped anaerobic heterotroph together with *Pyrobaculum* rods were cultivated from geothermal environments in New Zealand. Pure cultures of the cocci were only obtained from one such consortium, despite extensive attempts. Cells of this strain (AQ1.S1^T) were regular to irregular cocci in morphology, and occasionally formed large

aggregates, especially when utilizing polysaccharides such as konjac glucomannan as a carbon source. AQ1.S1^T is a hyperthermophile, with an optimal temperature for growth between 92 and 95 °C (range of 85 to 98 °C), and a moderate acidophile, with optimal growth at pH 6.4 (range of 5.4 to 7.0). Growth was inhibited by the addition of sulfur and NaCl (optimal growth was without the addition of NaCl) and an electron acceptor was not necessary. AQ1.S1^T utilized starch, trypticase peptone, lactose, glucose, konjac glucomannan, mannose, galactose, maltose, glycogen, and β-cyclodextrin as carbon sources. The G+C content is 52.9 mol%. Based on 16S rRNA gene sequence analysis and physiological features we propose that isolate AQ1.S1^T (= DSM 17230^T = TG 866^T) represents the type strain of a novel species of a new genus within the *Crenarchaeota*, *Ignisphaera aggregans* gen. nov., sp. nov.

INTRODUCTION

According to 16S rRNA gene analysis and phenotypic characteristics, the cultured representatives of the *Crenarchaeota* within the archaeal domain group into three orders, the *Thermoproteales, Sulfolobales* and *Desulfurococcales* (Burggraf *et al.*, 1997). The *Thermoproteales* are rod-shaped organisms that group into the families of *Thermoproteaceae* and *Thermofilaceae*, whereas the *Sulfolobales* are coccoid-shaped, lobed acidophiles. The *Desulfurococcales* are all coccoid to disc-shaped, neutrophilic or weakly acidophilic and strict anaerobes with fermentative metabolism or anaerobic respiration, with the exception of *Aeropyrum pernix* and *Aeropyrum camini* (Sako *et al.*, 1996; Nakagawa *et al.*, 2004). Phenotypic and 16S rRNA gene phylogenetic analysis divides the members of the *Desulfurococcales* into two families, the *Pyrodictiaceae* and the *Desulfurococcaceae*. The *Pyrodictiaceae* form a distinct group (genera of *Pyrodictium* (Stetter *et al.*, 1983), *Hyperthermus* (Zillig *et al.*, 1991) and *Pyrolobus* (Blöchl *et al.*, 1997)) within 16S rRNA phylogenetic trees, whereas, the *Desulfurococcaceae* consist of a diverse range of cultured representatives (Burggraf *et al.*, 1997). These two families can also be distinguished phenotypically by growth temperatures, where the *Pyrodictiaceae* consist of organisms with optimal temperatures above 100 °C, and the *Desulfurococcaceae* are characterized by maximal growth temperatures of up to 100 °C. The *Desulfurococcaceae* consist of cocci to disc-shaped micro-organisms of the genera *Desulfurococcus* (Zillig *et al.*, 1982), *Aeropyrum* (Sako *et al.*, 1996; Nakagawa *et al.*, 2004), *Ignicoccus* (Huber *et al.*, 2000), *Staphylothermus* (Fiala *et al.*, 1986; Arab *et al.*, 2000), *Stetteria* (Jochimsen *et al.*, 1997), *Sulfophobococcus* (Hensel *et al.*, 1997), *Thermodiscus* (Stetter, 2003), *Thermosphaera* (Huber *et al.*, 1998), and the distantly related 'Acidilobus group' consisting of *Acidilobus aceticus* (Prokofeva *et al.*, 2000), *Caldisphaera lagunensis* (Itoh *et al.*, 2003) and strain NC12 ("*Caldococcus noboribetus*", Aoshima *et al.*, 1996).

As part of a project investigating the microbial ecology of New Zealand's high temperature geothermal habitats, a novel coccoid-shaped archaeum (isolate AQ1.S1^T) was isolated into pure culture from co-culture with a strain of *Pyrobaculum*. The isolated coccus was similar in morphology and 16S rRNA gene sequence to a previously identified archaeon, which was also in co-culture with a strain of *Pyrobaculum* and obtained from a New Zealand hot spring (Götz, 1998). Other thermal habitats of New Zealand and Yellowstone National Park, (WY, USA) were screened for the presence of these novel cocci by culturing methods. In this study, we describe the novel isolate, AQ1.S1^T and based on 16S rRNA nucleotide sequence and phenotypic analysis propose that it represents a novel genus and species, *Ignisphaera aggregans* within the *Crenarchaeota*.

MATERIALS AND METHODS

Enrichment and culture conditions.

Samples from hot springs and mud pools situated in the Rotorua and Tokaanu thermal areas of New Zealand were collected in sterile containers and transported back to the laboratory under ambient conditions. The medium used for enrichment and isolation contained (g l^{-1}): $(NH_4)_2SO_4$, 1.3; $CaCl_2$, 0.074; $MgSO_4\cdot 7H_2O$, 0.28; KH_2PO_4, 0.28; yeast extract, 0.1; trypticase peptone, 2.0; soluble starch, 2.0 and cystine, 0.6. Resazurin (0.1% w/v), $FeCl_3$ (0.28 g l^{-1}), and trace elements (mg l^{-1}: $MnSO_4$, 2.2; $ZnSO_4\cdot 7H_2O$, 0.5; H_3BO_4, 0.5; $CuSO_4$, 0.016; $Na_2MoO_4\cdot 2H_2O$, 0.025

and $CoCl_2 \cdot 6H_2O$, 0.046) were added at 1 ml l^{-1}. The pH of the medium was adjusted to 7.0 at room temperature, boiled and dispensed into Hungate tubes under a N_2 atmosphere, autoclaved and reduced with 10% $Na_2S \cdot 9H_2O$ as required. Growth was monitored using phase-contrast microscopy. Approximately 1 ml of pool water from hot springs in Yellowstone National Park, (WY, USA) was used to inoculate media which was incubated *in situ* in the pool from which the inoculum originated. The media used included the enrichment medium outlined above and a modified enrichment medium, where the cystine was removed and starch was replaced with konjac glucomannan (Shintoa Koeki Kaisha). Tubes were incubated in the pools from 4 to 7 days, following which they were transported to the laboratory under ambient conditions and microbial growth checked by phase-contrast microscopy.

Morphology.

Cells were examined under phase-contrast light microscopy. For scanning electron microscopy (SEM), cells were grown to late exponential phase and filtered through a 0.22 µm filter 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 increasing concentrations of ethanol, 50, 75 and 90%, respectively, then four changes of absolute ethanol. The filter was critically point dried, sputtered with platinum and viewed using a Hitachi S-4100 field emission SEM.

Metabolic studies.

All experiments were undertaken at 90 °C unless otherwise stated, and growth documented using phase-contrast microscopy with a Thoma counting chamber (depth 0.02 mm). Unless otherwise stated, carbon utilization studies involved the addition of substrates at 0.2% (w/v) final concentration to modified enrichment medium. The modified enrichment medium had the starch supplement omitted, reduced concentrations of yeast extract and trypticase peptone (0.05 g l^{-1} and 1.0 g l^{-1}, respectively) and MOPS buffer added to a final concentration of 25 mM and the pH adjusted to approximately 7.0 at room temperature. A positive growth response to a substrate was only recorded when a similar cell density was achieved on three successive transfers in the same medium. For growth response to pH, buffers were added to the medium to a final concentration of 25 mM. The buffers used and the corresponding pH ranges were: MES, pH 4.5-6.5; MOPS, pH 6.5-7.5; Tris, pH 7.5-9.0; CHES, pH 9.0-10.0 and CAPS, pH 10.0-10.5. The pH was adjusted at 75 °C and the corresponding pH value (at 90 °C) was extrapolated using the appropriate d(pkA)/dt coefficient. Different electron acceptors were tested by replacing the cystine of the standard enrichment medium. Alternative electron acceptors were used at final concentrations of both 2.5 mM and 10 mM in medium containing 25 mM MOPS buffer. Elemental sulfur (approximately 20 mg) was added to individual Hungate tubes containing 9 ml of medium prior to autoclaving. Utilization of electron acceptors was recorded as positive when the same cell density was achieved after at least two transfers in the medium. Salt tolerance was determined by adding NaCl directly to the standard enrichment medium. Autotrophic growth was tested on modified enrichment medium (starch omitted and both trypticase peptone and yeast extract at 0.05 g l^{-1}) under H_2:CO_2 atmosphere (80:20 v/v). Autotrophic medium without an electron acceptor was tested and cystine, sulfite, thiosulphate and elemental sulfur were trialled as electron acceptors.

Sensitivity to antibiotics.

To minimize temperature degradation (Peteranderl *et al.*, 1990) antibiotics were added to cells in exponential growth (90 °C) to a final concentration of 100 $\mu g\ ml^{-1}$.

Waste products.

H_2S was determined qualitatively (Huber *et al.*, 1986). The breakdown products of growth on konjac glucomannan were tested by size exclusion chromatography (SEC) through a Biogel P2 (2.6 X 95 cm) size exclusion column. Size standards of mono-saccharide (glucose) and di-saccharide (maltose) were run through the column at 1% w/v in pure water. Konjac glucomannan growth medium with and without an inoculum of AQ1.S1^T cells was incubated at 90 °C. Medium was removed after approximately 3 days incubation, growth was recorded by light microscopy in the inoculated tubes and the control tubes were confirmed as sterile. Medium was passed through a 0.22 µm filter and 10 ml of filtrate was snap frozen in liquid N_2, and freeze-dried. It was then re-suspended in 4-5 ml of 100% methanol and incubated for 30 minutes in an ultrasonic cleaner then centrifuged at 2000 *g* for 7 minutes. The supernatant was transferred to a clean tube and the methanol removed by exposure to N_2 gas flow with the tube being in a 40 °C heat block. The residue was re-suspended in 2 ml of Milli-Q water, sonicated for 5 minutes and passed through a 0.22 µm filter. Finally, 2 ml was loaded onto the column for analysis. In addition, konjac glucomannan suspended in pure water (1% w/v), was processed as described for the growth medium (without the 90 °C incubation) and passed through the column.

Determination of G+C mol%.

Determination of G+C mol% was undertaken in duplicate as described by Gonzalez & Saiz-Jimenez (2002) using a Smart Cycler II (Cepheid). DNA from *E. coli* (strain DH5α) was used as a control.

Genetic studies.

The 16S rRNA gene was amplified by the polymerase chain reaction (PCR) utilizing primers A5F (5′-CCGTTGATCCTGCCGG-3′) and U1522R (5′-AAGGAGGTGATCCARCCGCA-3′). Each PCR consisted of 1 µM of each primer, 200 µM dNTP's, 1X PCR buffer, 1.25 units of *Taq* polymerase and approximately 20 ng template DNA in a final reaction volume of 25 µl. The thermocycling conditions consisted of initial denaturation of 94 °C for 3 minutes and 30 seconds, then 32 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 2 minutes with a final extension of 72 °C for 6 minutes. The 16S rRNA gene was sequenced by the MegaBACE DNA analysis system (Amersham Bioscience) using primers: A5F; A347F (CCAGGCCCTACGGGGCGCA); A915F (AGGAATTGGCGGGGGAGCAC); A907R (CCGTCAATTCCTTTGAGTTT); 519R (GWATTACCGCGGCKGCTG); A1335R (GTGTGCAAGGAGCAGGGAC) and U1522R. The 16S rRNA gene for the coccal members of the Tok10A and Tok1 consortia were obtained by using DNA extracted from a coccal dominated culture for Tok10A.S1 and gene-cloning for Tok1 (Götz, 1998). Both strands of the 16S rRNA gene were sequenced for the Tok37.S1 and AQ1.S1^T isolates, with single strand sequencing undertaken on Tok10A.S1 and Tok1. Sequences were checked for chimeric artifacts using the CHIMERA_CHECK online tool of the Ribosomal Database Project (Maidak *et al.*, 2001). Phylogenetic analysis and alignment of 16S rRNA gene sequences was performed using the ARB software package (Ludwig *et al.*, 2004). The phylogenetic position of the sequences were determined using the PHYLIP package with analysis of sequences undertaken using DNADIST, DNAML, DNAPARS, FITCH, NEIGHBOR and SEQBOOT programs (Felsenstein, 1993).

RESULTS AND DISCUSSION

Enrichments and isolation

From an in-depth study into the microbial ecology of a hot pool (designated AQ1) situated in Kuirau Park, Rotorua, New Zealand, a consortium of rod- and coccoid-shaped organisms was obtained as an enrichment from pool water incubated at 90 °C. Typically, the rod dominated cultures within the first days of incubation with an overgrowth of cocci occurring after 4 to 7 days of incubation. Therefore, serial dilutions (1:10) of a nascent consortium culture was undertaken to obtain a pure culture of the rod. A pure culture of the coccus was eventually achieved by many successive dilutions (1:10) from an older consortium culture where the cocci dominated. Both cultures were maintained by weekly transfer of serial dilutions (1:10) for nearly a year prior to characterization. Sequencing of the 16S rRNA gene identified the rod as a strain of *Pyrobaculum* (results not shown) and the coccus (isolate AQ1.S1^T) matched very closely (16S rRNA gene sequence and morphology) to another previously identified coccus (Tok1) that was isolated from a hot spring in Tokaanu, New Zealand (Götz, 1998). Tok1 was also enriched as a co-culture with a strain of *Pyrobaculum,* however, extensive attempts to obtain a pure culture of Tok1 failed (Götz, 1998). Further geothermal habitats within New Zealand and Yellowstone National Park were then screened by enrichment for the presence of these novel cocci (Table 5.1). Samples from New Zealand hot springs and mud pools were inoculated into anaerobic enrichment medium and incubated at 93 °C. A duplicate enrichment was incubated at 80 °C if the *in situ* pool temperature was below 90 °C (results not shown). No cocci were enriched from springs in Yellowstone National Park. However, for the New Zealand springs, as was the case for the AQ1 consortium, rod-shaped organisms were commonly observed in the medium after 2 to 4 days incubation followed by an overgrowth of cocci (Table 5.1). Conversely, cells of a coccus morphology dominated rod-shaped organisms in the Tok10A enrichment (coccus member designated as Tok10A.S1) and an enrichment containing only cocci was obtained from pool Tok37 (coccus designated Tok37.S1). Comparison of denaturing gradient gel electrophoresis (DGGE) profiles between the enrichments and AQ1.S1^T suggested that all coccal-containing consortia contained AQ1.S1^T-related members (results not shown). All attempts to isolate pure cultures of cocci, from enrichments of pools AQ2, AQ5, Tok10A and Tok13 failed, including attempts at isolation using solid Gelrite medium (Hungate, 1969). The 16S rRNA gene sequences of the coccal isolates from pool Tok10A and Tok37 were obtained and matched closely to that of AQ1.S1^T and Tok1 (results presented below). However, the Tok37 coccal enrichment also contained an atypical 16S rRNA gene PCR product that contained introns and may indicate that it is a mixed culture of at least two different coccal species. Isolate AQ1.S1^T was therefore used for further characterization.

Morphology

Cells of AQ1.S1^T are regular to irregular cocci with a diameter of 1-1.5 μm when grown on starch. Cells of AQ1.S1^T occurred singly, in pairs or in aggregates of many cells. Aggregation of cells was typically common when AQ1.S1^T was grown on mono-, di-, or poly-saccharides as presented in Fig. 5.1. A web-like array of extra-cellular material appeared to hold cells together when cell aggregates were examined under SEM (Fig. 5.2). Attempts to stain this material using alcian blue- and copper sulfate-based capsule/polysaccharide staining methods were unsuccessful. Cells of Tok1 also formed aggregates, but, this was not observed for either the Tok37.S1 or Tok10A.S1 cultures.

Phenotypic characterization

Strain AQ1.S1^T grew at 85-98 °C and pH 5.4-7.0. No growth was observed at 80 or 100 °C, or at pH 4.9 or 7.4. The optimum temperature and pH for growth on starch was between 92 and 95 °C and pH 6.4, with a doubling time of approximately 7.5 h. Cultures of AQ1.S1^T grew to densities of approximately 2.5×10^7 cells ml^{-1}. AQ1.S1^T utilized starch, trypticase peptone, lactose, glucose, konjac glucomannan, mannose, galactose, maltose, glycogen, and β-cyclodextrin. Weak growth was evident on beef extract and sucrose. Growth was not observed on yeast extract, cellobiose, methanol, ethanol, trehalose, pyruvate, acetate, malate, casamino acids (0.1% w/v), carboxymethyl-cellulose, amylopectin (corn), xanthan gum, locust gum (bean), guar gum, dextran, xylan (oat spelts, larch or birch), xylitol, xylose or amylose (corn and potato). It could also grow in the standard growth medium without the addition of starch. The addition of a vitamin solution (Wolin *et al.*, 1963) weakly promoted growth. Cultures grew in the presence of up to 0.5% NaCl (optimally with no added NaCl) but not at 0.75% NaCl. An electron acceptor was not required and the removal of cystine from the growth medium did not affect cell numbers significantly. Other electron acceptors trialled included thioglycolate, nitrate, nitrite, sulfite, thiosulphate and elemental sulfur. The electron acceptors were inhibitory at 10 mM concentration (including cystine) and at 2.5 mM both sulfite and thioglycolate also inhibited growth of AQ1.S1^T. Sulfur inhibited growth of AQ1.S1^T, Tok37.S1, Tok10.S1 and Tok1. No growth was detected in serum bottles under autotrophic conditions. All isolates were strict anaerobes, even trace quantities of oxygen (just sufficient to turn resazurin pink) inhibited growth. Mono- and di-saccharides accumulated in AQ1.S1^T cultures grown on konjac glucomannan and not in sterile medium that had been exposed to the same temperature as the inoculated medium or the stock of konjac glucomannan. This most probably indicates that the konjac glucomannan is being hydrolysed enzymatically by AQ1.S1^T into sugars for metabolism. Hydrogen sulfide was also detected in AQ1.S1^T cultures grown on enrichment medium. Cells of AQ1.S1^T were resistant to novobiocin and streptomycin but sensitive to erythromycin, chloramphenicol and rifampicin.

Analysis of 16S rRNA gene and G+C mol%

A near-full length 16S rRNA gene sequence for AQ1.S1^T (1491 bp) was obtained and had a G+C ratio of 66%. The 16S rRNA gene sequences of the other novel coccal isolates matched very closely to that of AQ1.S1^T: 99 % to Tok37.S1 (1423 bp), 98 % to Tok10A.S1 (1433 bp) and 98 % to Tok1 (1191 bp) and all sequences were free of chimeric artifacts. Sequence similarities between AQ1.S1^T and the members of the *Pyrodictiaceae* ranged from 91.5-94 % and 87-94.5 % for the *Desulfurococcaceae,* with the closest sequence match being *Staphylothermus marinus* (X99560). The G+C content of AQ1.S1^T was 52.9 mol%. Phylogenetic analysis revealed that AQ1.S1^T and the other novel coccal isolates formed an independent group within the *Crenarchaeota* (Fig. 5.3). The topology of the phylogenetic tree did not change using DNADIST (Fitch and Neighbor), DNAML and DNAPARS analysis or with the removal of the shorter 16S rRNA gene sequence of Tok1 (1195 bp). Likewise, the structure of the phylogenetic tree did not change with the inclusion of *Methanopyrus kandleri*, DSM6324^T (M59932), *Archaeoglobus fulgidus*, DSM 4304^T (Y00275) and *Methanocaldococcus jannaschii*, DSM 2661^T (M59126) as additional outgroups.

Discussion and proposal of a novel genus

These novel coccoid micro-organisms are widely distributed in high-temperature near-neutral hot pools and mud pools in New Zealand. An unusual feature was that the pH range for growth for the type strain AQ1.S1^T determined in laboratory culture was between pH 5.4 to 7.0 (at 90 °C) yet isolation was regularly achieved from more alkaline springs. This was not the result of an aberrant pH reading since the pH of the AQ1 pool was measured at regular intervals over at least a year and was consistently above pH 7.0 (range measured was 7.4 to 8.2). Moreover, we failed to isolate or detect molecular signatures of AQ1.S1^T-related organisms by DGGE analysis in pools where the pH was below pH 6.7, yet ostensibly the organism should be able to proliferate under such conditions. These novel coccal-organisms were not enriched from pools with similar characteristics in Yellowstone National Park. Moreover, the use of '*Ignisphaera*-specific' enrichment media, i.e. no electron acceptor and konjac glucomannan as a carbon source did not yield consortia containing coccoid organisms.

Commonly, the novel cocci-shaped organisms grew commensally with species of *Pyrobaculum,* with the exception of Tok37.S1, and although the *Pyrobaculum* sp. could readily be obtained as a pure culture, it proved impossible with the coccal-shaped organisms (with the exception of AQ1.S1^T), and may be indicative of a metabolic dependency of the cocci to *Pyrobaculum*. In fact, the outgrowth of cocci following the development of rods (*Pyrobaculum* sp.) within cultures may be due to the cocci utilising a product excreted by the rod-shaped members of the consortium. Likewise, the presence of a second atypical 16S rRNA gene PCR product for Tok37.S1 may indicate another coccal member that Tok37.S1 may be dependent upon.

AQ1.S1^T is a coccoid-shaped, anaerobic, moderately acidophilic, heterotrophic hyperthermophile, with maximum growth below 100 °C. These morphological and phenotypic attributes fit within the description of the *Desulfurococcaceae* family (Burggraf *et al.*, 1997). The ability of AQ1.S1^T to grow on media with no exogenous electron acceptor indicates its capacity to ferment polysaccharides and complex proteinaceous substrates. The fermentation of polysaccharides and/or peptides has also been noted in other members of this *Desulfurococcaceae* family such as *Desulfurococcus* (Zillig *et al.*, 1982), *Thermosphaera* (Huber *et al.*, 1998), *Staphylothermus* (Fiala *et al.*, 1986), *Sulfophobococcus* (Hensel *et al.*, 1997) and both *Acidilobus* (Prokofeva *et al.*, 2000) and *Caldisphaera* (Itoh *et al.*, 2003) of the 'Acidilobus' group. The formation of cell aggregates which was typical of AQ1.S1^T and Tok1 has also been noted in cultures of *Desulfurococcaceae*, for example, cells of *Caldisphaera lagunensis* clump together (Itoh *et al.*, 2003) and both *Thermosphaera aggregans* and *Staphylothermus marinus* grow as grape-like aggregates (Huber *et al.*, 1998; Fiala *et al.*, 1986). However, the growth inhibition of sulfur on the novel cocci does not accord with the description of the *Desulfurococcaceae*, as they typically use elemental sulfur for reduction or respiration (Burggraff *et al.*, 1997). However, *Aeropyrum camini* (Nakagawa *et al.*, 2004), *Thermosphaera aggregans* (Huber *et al.*, 1998) and *Sulfophobococcus zilligii* (Hensel *et al.*, 1997) are already exceptions within the *Desulfurococcaceae* in this regard.

By themselves, the phenotypic characteristics of the novel cocci may suggest they represent members of the *Desulfurococcaceae*, however, based on 16S rRNA gene analysis the novel coccal isolates form an independent lineage within the *Crenarchaeota* with a 100% bootstrap value and do not group within any other lineage within the *Desulfurococcaceae*. Consequently, the '*Ignisphaera*-group' constitutes a novel deep branching genus of the *Desulfurococcaceae*, branching independently from both the *Desulfurococcaceae* and *Pyrodictiaceae* families. Therefore, inclusion of the '*Ignisphaera*-group' and the proposal of AQ1.S1^T as a new genus within the *Desulfurococcales* may be controversial, as is the case for the 'Acidilobus group', due to its independent grouping based on 16S rRNA gene analysis and acidophilic growth optima (Itoh *et al.*, 2003). Despite the above considerations, with regard to the phylogenetic placement of strain AQ1.S1^T and related isolates, we propose a new genus within the *Desulfurococcales* as the most conservative origin for this lineage. However, as more representatives of the *Crenarchaeota* are cultured and characterized the position of the *Ignisphaera*-type isolates may be resolved.

Description of *Ignisphaera* gen. nov.

Ignisphaera (Ig.ni.sphae'ra. L.n. ignis fire, L. fem. n. sphaera ball, N. L. fem n. *Ignisphaera* fire ball).

Cells are regular to irregular cocci that occur singly, in pairs or in aggregates. Hyperthermophilic anaerobe. Moderate acidophile. Heterotrophic. Electron acceptor is not absolutely necessary. Elemental sulfur and NaCl inhibits growth. The 16S rRNA gene groups within the *Crenarchaeota*. The habitat is terrestrial near-neutral hot springs and mud pots. The type species is *Ignisphaera aggregans*.

Description of *Ignisphaera aggregans* sp. nov.

Ignisphaera aggregans (ag'gre.gans. L part. adj. *aggregans* (aggregate forming), (aggregating clumping)).

Cells are regular to irregular cocci approximately 1–1.5 µm in size when grown on starch. Cells occur singly, in pairs or in aggregates of several cells. Growth occurs in the temperature range 85-98°C with an optimum at 92 to 95°C and in the pH range of 5.4-7.0 with an optimum pH of 6.4. Obligate anaerobe. Heterotrophic, fermentation. Elemental sulfur inhibits growth. Growth occurs at low salinity, < 0.5% NaCl (optimally without addition of NaCl). Resistant to novobiocin and streptomycin and sensitive to erythromycin, chloramphenicol and rifampicin. The G+C content of the DNA is 52.9 mol%. Isolated from a near-neutral, boiling spring in Kuirau Park, Rotorua, New Zealand. The type strain is *Ignisphaera aggregans* AQ1.S1^T (= DSM 17230^T = TG 866^T).

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REFERENCES

Aoshima, M., Nishibe, Y., Hasegawa, M., Yamagishi, A. & Oshima, T. (1996). Cloning and sequencing of a gene encoding 16S ribosomal RNA from a novel hyperthermophilic archaebacterium NC12. *Gene* **180**, 183-187.

Arab, H., Völker, H. & Thomm, M. (2000). *Thermococcus aegaeicus* sp. nov. and *Staphylothermus hellenicus* sp. nov., two novel hyperthermophilic archaea isolated from geothermally heated vents off Palaeochori Bay, Milos, Greece. *Int J Syst Evol Microbiol* **50**, 2101-2108.

Blöchl, E., Rachel, R., Burggraf, S., Hafenbradl, D., Jannasch, H. W. & Stetter, K. O. (1997). *Pyrolobus fumarii*, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113 °C. *Extremophiles* **1**, 14-21.

Burggraf, S., Huber, H. & Stetter, K. O. (1997). Reclassification of the crenarchaeal orders and families in accordance with 16S rRNA sequence data. *Int J Syst Bacteriol* **47**, 657-660.

Felsenstein, J. (1993) PHYLIP – phylogeny inference package (version 3.5c).

Fiala, G., Stetter, K. O., Jannasch, H. W., Langworthy, T. A. & Madon, J. (1986). *Staphylothermus marinus* sp. nov. represents a novel genus of extremely thermophilic submarine heterotrophic Archaebacteria growing up to 98 °C. *Syst Appl Microbiol* **8**, 106-113.

Gonzalez, J. M. & Saiz-Jimenez, C. (2002). A fluorimetric method for the estimation of G+C mol% content in microorganisms by thermal denaturation temperature. *Environ Microbiol* **4**, 770-773.

Götz, D. K. (1998). The characterisation of three hyperthermophilic archaea from New Zealand hot springs. PhD thesis. University of Waikato, Hamilton, New Zealand.

Hensel, R., Matussek, K., Michalke, K., Tacke, L., Tindall, B. J., Kohlhoff, M., Siebers, B. & Dielenschneider, J. (1997). *Sulfophobococcus zilligii* gen. nov. spec. nov. a novel hyperthermophilic archaeum isolated from hot alkaline springs of Iceland. *Syst Appl Microbiol* **20**, 102-110.

Huber, H., Burggraf, S., Mayer, T., Wyschkony, I., Rachel, R. & Stetter, K. O. (2000). *Ignicoccus* gen. nov., a novel genus of hyperthermophilic, chemolithoautotrophic *Archaea*, represented by two new species, *Ignicoccus islandicus* sp. nov. and *Ignicoccus pacificus* sp. nov. *Int J Syst Evol Microbiol* **50**, 2093-2100.

Huber, R., Langworthy, T. A., König, H., Thomm, M., Woese, C. R., Sleytr, U. B. & Stetter, K. O. (1986). *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90 °C. *Arch Microbiol* **144**, 324-333.

Huber, R., Dyba, D., Huber, H., Burggraf, S. & Rachel, R. (1998). Sulfur-inhibited *Thermosphaera aggregans* sp. nov., a new genus of hyperthermophilic archaea isolated after its prediction from environmentally derived 16S rRNA sequences. *Int J Syst Bacteriol* **48**, 31-38.

Hungate, R. E. (1969). A roll-tube method for cultivation of strict anaerobes. Methods Microbiol, **3B**, 117-132.

Itoh, T., Suzuki, K., Sanchez, P. C. & Nakase, T. (2003). Caldisphaera lagunensis gen. nov., sp. nov., a novel thermoacidophilic crenarchaeote isolated from a hot spring at Mt Maquiling, Philippines. Int J Syst Evol Microbiol **53**, 1149-1154.

Jochimsen, B., Peinemann-Simon, S., Völker, H., Stüben, D., Botz, R., Stoffers, P., Dando, P. R. & Thomm, M. (1997). Stetteria hydrogenophila, gen. nov. and sp.

nov., a novel mixotrophic sulfur-dependent crenarchaeote isolated from Milos, Greece. Extremophiles **1**, 67-73.

Ludwig, W., Strunk, O., Westram, R., & 29 other authors (2004). ARB: a software environment for sequence data. Nucleic Acids Res **32**, 1363-1371.

Maidak, B. L., Cole, J. R., Lilburn, T. G. & 7 other authors (2001). The RDP-II (Ribosomal Database Project). Nucleic Acids Res **29**, 173-174.

Nakagawa, S., Takai, K., Horikoshi. K. & Sako, Y. (2004). Aeropyrum camini sp. nov., a strictly aerobic, hyperthermophilic archaeon from a deep-sea hydrothermal vent chimney. Int J Syst Evol Microbiol **54**, 329-335.

Peteranderl, R., Shotts, E. B. Jr. & Wiegel, J. (1990). Stability of antibiotics under growth conditions of thermophilic anaerobes. Appl Environ Microbiol **56**, 1981-1983.

Prokofeva, M. I., Miroshnichenko, M. L., Kostrikina, N. A., Chernyh, N. A., Kuznetsov, B. B., Tourova, T. P. & Bonch-Osmolovskaya, E. A. (2000). Acidilobus aceticus gen. nov., sp. nov., a novel anaerobic thermoacidophilic archaeon from continental hot vents in Kamchatka. Int J Syst Evol Microbiol **50**, 2001-2008.

Sako, Y., Nomura, N., Uchida, A., Ishida, Y., Morii, H., Koga, Y., Hoaki, T. & Maruyama, T. (1996). Aeropyrum pernix gen. nov., sp. nov., a novel aerobic hyperthermophilic archaeon growing at temperatures up to 100 °C. Int J Syst Bacteriol **46**, 1070-1077.

Stetter, K. O., König, H. & Stackebrandt, E. (1983). Pyrodictium gen. nov., a new genus of submarine disc-shaped sulphur reducing archaebacteria growing optimally at 105 °C. Syst Appl Microbiol **4**, 535-551.

Stetter, K. O. (2003) Validation list No. 89. Int J Syst Evol Microbiol 53, 1-2.

Wolin, E. A., Wolin, M. J. & Wolfe R. S. (1963). Formation of methane by bacterial extracts. J Biol Chem **238**, 2882-2886.

Zillig, W., Stetter, K. O., Prangishvilli, D., Schäfer, W., Wunderl, S., Janekovic, D., Holz, I. & Palm, P. (1982) Desulfurococcaceae, the second family of the extremely thermophilic, anaerobic, sulfur-respiring Thermoproteales. Zbl Bakt Hyg I Abt Orig **C3**, 304-317.

Zillig, W., Holz, I. & Wunderl, S. (1991) Hyperthermus butylicus gen. nov. sp. nov., a hyperthermophilic anaerobic, peptide-fermenting, facultatively H_2S-generating archaebacterium. Int J Syst Bacteriol **41**, 169-170.

Table 5.1 Summary of results of hot springs from New Zealand (N.Z.) and Yellowstone National Park (Y.N.P.).

Location	Hot spring	Temp. (°C)	pH	Enrichments	Novel cocci
N.Z. – Kuirau Park, Rotorua	AQ1	91	7.5	Rods and cocci	AQ1.S1^{T}
	AQ2	92	8.0	Rods and cocci	
	AQ3	87	7.5	Rods	
	AQ5	86	8.0	Rods and cocci	
N.Z. – Ohinemutu, Rotorua	OH2	94	9.0	No growth	
N.Z. – Whakarewarewa, Rotorua	WH1	96	8.5-9.0	No growth	
N.Z. – Tokaanu Thermal Park	Tok3	85	6.5	No growth	
	Tok10A	84	7.0	Rods and cocci	Tok10A.S1 (consortium)
	Tok13	97	6.0	Rods and cocci	
	Tok37 (mud pot)	99	7.5	Cocci	Tok37.S1 (probable consortia)
	Tok13	90	Neutral	Rods and cocci	Tok1 (consortium)
Y.N.P. – GOPA*	Obsidian Pool	73.5-76	6.0	Rods and Fervidobacterial morphology	
	South Obsidian Pool	75.5-80	6.0	Fervidobacterial morphology	
	O.H.	75-85	5-6	Rods	
Y.N.P. – Sentinel Meadows	E.M.	81-85	7-8	No growth	
	B.P.	87-92.5	7-8	No growth	
	Boulder Spring	86	8	No growth	
Y.N.P. – Sylvan	Sylvan Spring	75-78	5-6	Few rods	
Y.N.P. – South Sylvan	J.T.P.	89	6-7	No growth	
	F.L.	81	6	Rods	

***Greater Obsidian Pool Area.**

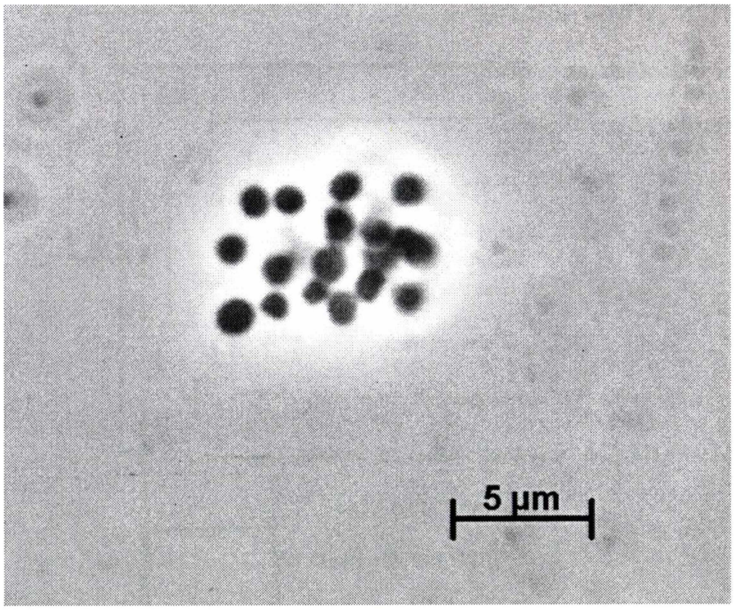

Figure 5.1 Phase-contrast micrograph of an aggregation of AQ1.S1^T cells grown on enrichment media.

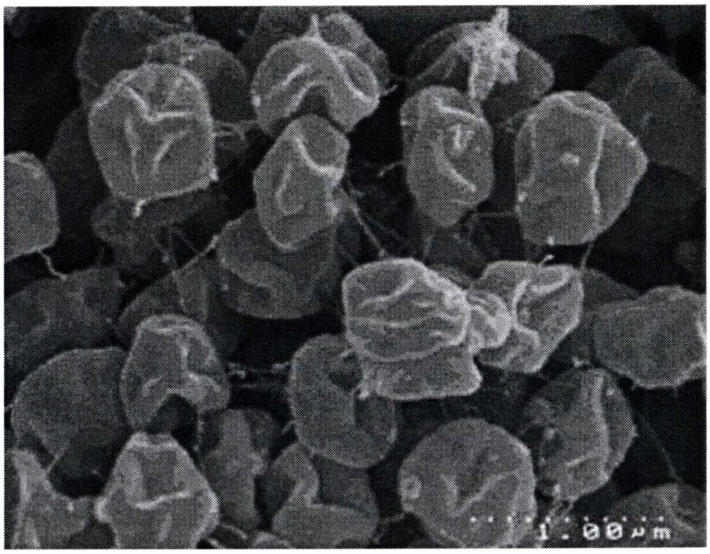

Figure 5.2 SEM micrograph of dehydrated AQ1.S1^T cell aggregate grown on enrichment medium with konjac glucomannan as a carbon source.

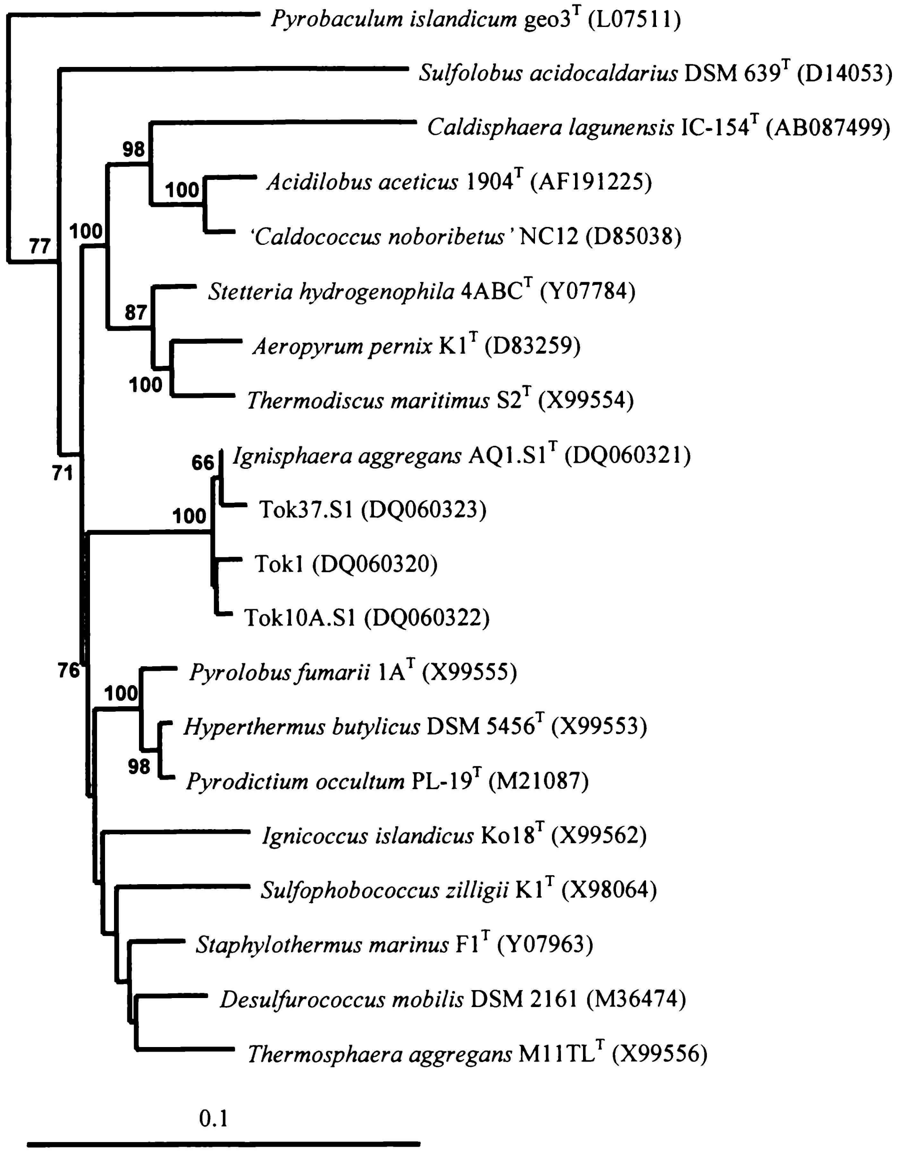

Figure 5.3 Phylogenetic tree based on analysis of the 16S rRNA gene sequences of strain AQ1.S1^{T}, other novel isolates Tok37.S1, Tok10A.S1 and Tok1, and representative species of the Crenarchaeota showing the position of the novel cocci. The dendogram was produced with DNADIST (Neighbor joining) analysis using 1027 base pairs of aligned sequence. The phylogenetic tree was rooted to *Thermococcus celer* DSM 2476^{T} (M21529). Bootstrap values above 65% are shown from 100 replicates. Bar, 10 % sequence divergence.

5.2 Supplementary results

The near-full length 16S rRNA gene sequences of all *Ignisphaera*-related organisms are included in Appendix D. Photographs and GPS coordinates (New Zealand only) of the New Zealand and Yellowstone National Park (WY, USA) hot pools involved in the study are included in Appendix A and B, respectively. The screening of New Zealand and Yellowstone hot pools for *Ignisphaera*-related microorganisms by culture- and molecular-based methods are described in greater detail in the sections below.

5.2.1 Screening the Thermophile Research Unit Culture Collection (TRUCC) for novel *Ignisphaera*-related archaea

Thermophilic archaeal coccal cultures from the TRUCC were screened using 16S rDNA sequencing to determine if any coccal isolates were closely related to AQ1.S1^T. This was deemed likely since many isolates had been purified and deposited mainly on the basis of morphology, and the prospect of mixed cultures of coccal isolates being mistaken for a pure culture. DNA was isolated directly from freeze-dried ampoules or stock glycerol cultures stored at -70°C that contained coccal-shaped microorganisms isolated from high-temperature (>80°C), near-neutral geothermal habitats of New Zealand. For selected cultures, morphologies were checked by inoculation into Db+S enrichment medium (see Appendix E for composition) and incubation at 85°C. Table 5.2 lists the isolates and the tests undertaken.

Table 5.2 TRUCC isolates screened for *Ignisphaera*-related microorganisms.

Isolate	Source	TRUCC#	Temp. (°C)	pH	Db+S inoculation	DNA extraction
Rt59.S1	Rotorua Pool 59	75	88	6.8	Not determined	Ampoule
Tok12.S1	Tokaanu Pool 12	95	85-88	6.0	Not determined	-70°C glycerol
Ket10.S2*	Ketetahi Pool 10	269	85	6.5	No growth	Ampoule
Ket55.S2	Ketetahi Pool 55	318	85-88	Low	Heavy coccal growth	Ampoule
Ket77.S1	Ketetahi Pool 77	321	85-88	Low	Few irregular cocci	Ampoule
Ket55.S1	Ketetahi Pool 55	322	85-88	Low	Regular larger cocci (up to 4 μm)	Ampoule

***Interestingly, Ket10.S2 was also isolated from co-culture with a rod.**

DNA extracted from the TRUCC isolates was amplified by PCR targeting a partial region (1000 bp) of the 16S rRNA gene using primers RR150 (347F) and RR151 (1335R) and the PCR product checked on a 1% agarose gel (Figure 5.4)

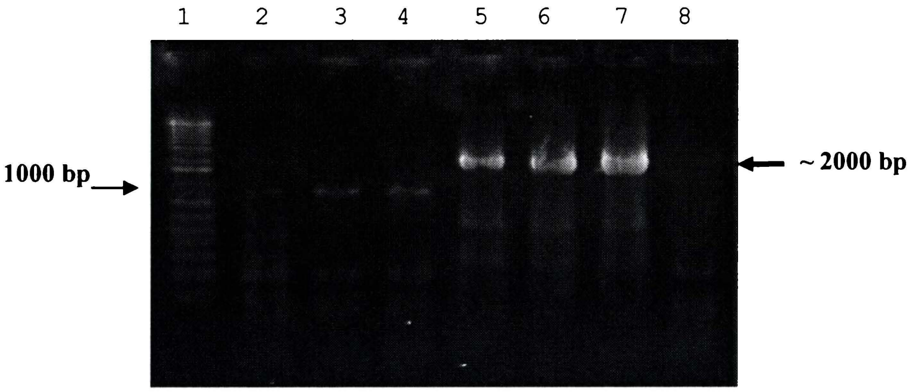

Figure 5.4 Archaeal 16S rDNA amplified by PCR from TRUCC isolates. The track numbers and corresponding samples are: (1) 1 kb ladder; (2) TRUCC 75; (3) TRUCC 95; (4) TRUCC 269; (5) TRUCC 318; (6) TRUCC 320; (7) TRUCC 321; (8) Negative control.

TRUCC isolates, 75, 95 and 269 contained the expected approximate 1000 bp PCR product, however, abnormally large PCR products of approximately 2000 bp were obtained for TRUCC 318, 321 and 322. The DNA bands were extracted and sequenced using primer RR150 (347F) and the closest NCBI BLASTn relatives listed in Table 5.3.

Table 5.3 NCBI BLASTn similarities of the TRUCC isolates.

TRUCC #	Closest NCBI BLASTn	Accession #	Similarity	length (bp)
75	Multiple products	N.A.	N.A.	N.A.
95	*Thermococcus waimanguensis*	AF098975.1	100%	113
	Thermococcus waiotapuensis (DSM12768)	AY099187.1	100%	113
269	*Thermococcus waimanguensis*	AF098975.1	100%	480
	Thermococcus waiotapuensis (DSM12768)	AY099187.1	100%	480
318	Multiple products	N.A.	N.A.	N.A.
321	*Desulfurococcus mobilis*	M36474	97%	130
322	*Desulfurococcus mobilis*	M36474	97%	71

DNA sequences are in Appendix D. N.A. = Not applicable.

From Table 5.3 it seems that the 'normal' (~1000 bp) sized 16S rDNA sequences were closely related to species of *Thermococcus*. However, the abnormally large 16S rDNA sequences were related to *Desulfurococcus*. A DGGE comparison between these two types of sequences (*Thermococcus,* TRUCC 269 and *Desulfurococcus,* TRUCC 321) and against *Ignisphaera* sp. is included in Figure 5.5. The atypical 16S rDNA PCR products are discussed in further detail in Section 5.3.

5.2.2 Screening New Zealand's geothermal habitats for *Ignisphaera*-related microorganisms

Attempts to isolate other *Ignisphaera*-related microorganisms by culturing from New Zealand thermal environments are discussed in the reproduced IJSEM article. The New Zealand pools targeted and the enrichments undertaken (Db+S medium) in the study are presented in greater detail in Table 5.4.

Table 5.4 New Zealand hot pools screened using culture methods for *Ignisphaera* sp.

Hot pool	*In situ* temp. (°C)	*In situ* pH	Lab pH	Incubation temp. (°C)	Enrichment
AQ2	92	8.0	8.26	93	Rods and cocci
AQ3	87	7.5	7.43	93	Rods and cocci
				80	*'Fervidobacterial'* morphologies
AQ5	86	8.0	8.38	93	Rods and cocci
				80	*'Fervidobacterial'* morphologies
TK3	85	6.5	6.91	93	*'Thermococcus'* morphologies
				80	No growth
Tok10A	84	7.0	7.15	93	Rods and cocci
				80	*'Fervidobacterial'* and *'Thermococcus'* morphologies
Tok13	97	6.0	6.72	93	Rods and cocci
WH1	96	8.5-9.0	8.72	93	No growth
OH2	94	9.0	9.13	93	No growth
Tok37	99	Not tested	7.58	93	Cocci

Extensive dilution series were undertaken on Tok10A, AQ2, AQ5 and Tok13 enrichments in an attempt to obtain pure cultures of cocci, but without success. The mixed cultures from Tok13, AQ5, AQ2 and Tok10A were stored at -70°C in glycerol cryoprotectant as listed in Appendix F. DNA was extracted from all cultures that contained coccal members and archaeal DGGE (45-65%) was undertaken as a comparison between cultures (Figure 5.5). Unfortunately, DNA could not be PCR amplified from AQ2, so it was not included in the DGGE profile.

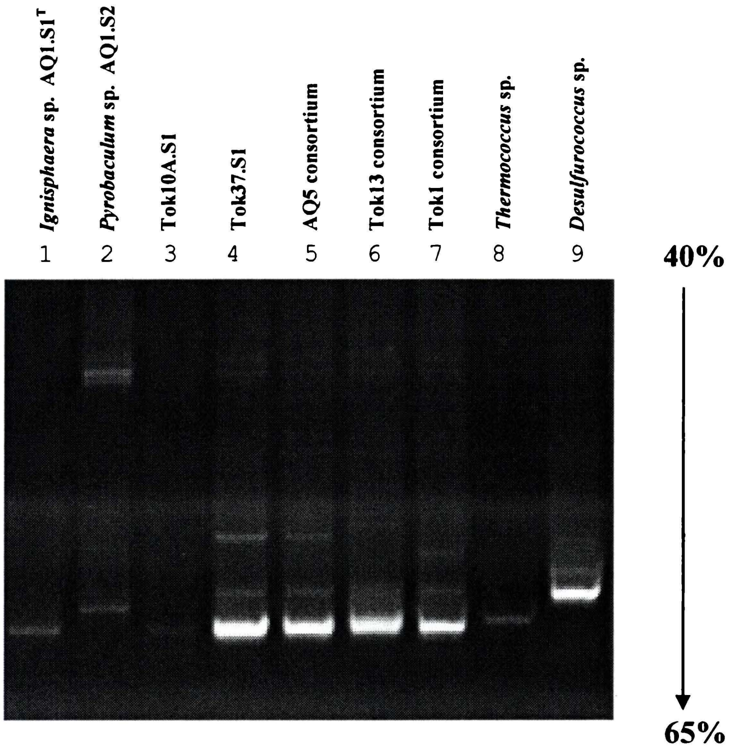

Figure 5.5 Archaeal DGGE comparison between suspected *Ignisphaera* isolates. The track numbers and corresponding samples are: (1) *Ignisphaera* strain AQ1.S1^T; (2) *Pyrobaculum* sp., AQ1.S2; (3) Tok10A.S1; (4) Tok37.S1; (5) AQ5 consortium; (6) Tok13 consortium; (7) Tok1 consortium; (8) *Thermococcus* sp., TRUCC 269; (9) *Desulfurococcus* sp., TRUCC 321.

DGGE comparison (Figure 5.5) shows that the enrichments containing coccal members have a dominant DGGE band at the same position as the *Ignisphaera* type strain AQ1.S1^T and Götz's (1998) coccal consortium member: Tok1 (lane 7). Moreover, the two other coccal DNAs: *Thermococcus* sp. (TRUCC 269) and *Desulfurococcus* sp. (TRUCC 321) produced DGGE bands in differing positions, reinforcing that the enrichments may contain *Ignisphaera*-like coccal isolates. Tok37.S1 was used for further analysis as discussed in the following section.

5.2.2.1 *Ignisphaera* sp. Tok37.S1

Tok37.S1 was assumed to be a pure culture. It was freeze-dried and is stored in the TRUCC as TG 879 (Table F.1, Appendix F). An SEM image of Tok37.S1 cells is presented in Figure 5.6 (comparative to the SEM micrograph (Figure 5.2) of *Ignisphaera* strain AQ1.S1^T in the IJSEM manuscript) and the 16S rRNA gene of this isolate was sequenced and identified it as a close relative to *Ignisphaera* strain AQ1.S1^T (as discussed in the IJSEM manuscript). Therefore, it was characterised with the aim of including its description in the IJSEM paper. Its characteristics are presented in Table 5.5 with comparison against the *Ignisphaera* type strain.

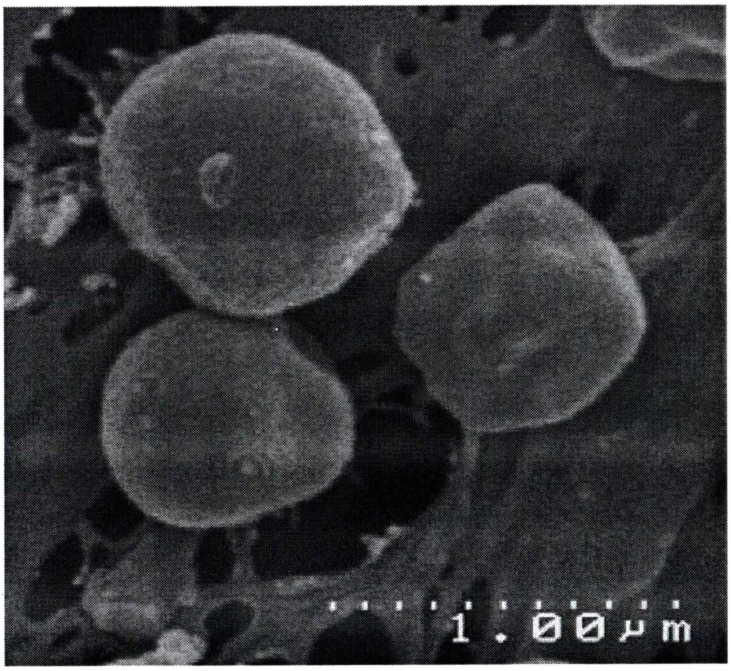

Figure 5.6 SEM micrograph of Tok37.S1 cells

Table 5.5 Comparison of phenotypic characteristics of *Ignisphaera* strains AQ1.S1^T and Tok37.S1.

	AQ1.S1^T	Tok37.S1
Morphology	1 to 1.5 µm diameter	1-4 µm
	Cell aggregation	No aggregation
Temperature (°C)	Optimum: 92 to 95	Optimum: 92
	Range: 85 to 98	Range: 75 to 100
	No growth at 80 or 100	No growth at 70 or 103
pH	Optimum: 6.4	Optimum: ~6.0
	Range: 5.4 to 7.0	Range: 5.4 to 7.4
	No growth at 4.9 or 7.4	No growth at 4.9 or 7.4
NaCl	Optimum: None added	Optimum: None added
	Growth up to 0.5%	Growth up to 0.75%
	No growth at 0.75%	No growth at 1.0%
Carbon substrates	Starch	Starch
	Trypticase peptone	Trypticase peptone
	Lactose	Lactose
	Glucose	Sucrose (weakly)
	Konjac glucomannan	Beef Extract (weakly)
	Mannose	
	Galactose	
	Maltose	
	Glycogen	
	B-cyclodextrin	
	Sucrose (weakly)	
	Beef Extract (weakly)	
Antibiotics	Resistant to novobiocin and streptomycin. Sensitive to erythromycin, chloramphenicol and rifampicin	Same as for AQ1.S1^T
Electron acceptors	Not absolutely necessary	Best growth with addition of electron acceptor, i.e. 2.5 mM cystine.
Autotrophic growth	No	Not determined
Notes		H_2S was detected in culture

Following the characterization of Tok 37.S1, its 16S rDNA was PCR amplified again (primer pair RR150 (347F) and RR151 (1335R)) and a dominant, abnormally large (2000 bp) 16S rDNA band was obtained rather than the expected 1000 bp product. Therefore, all stock Tok37.S1 glycerol cultures that were stored at -70°C were screened for this atypical band (Figure 5.7).

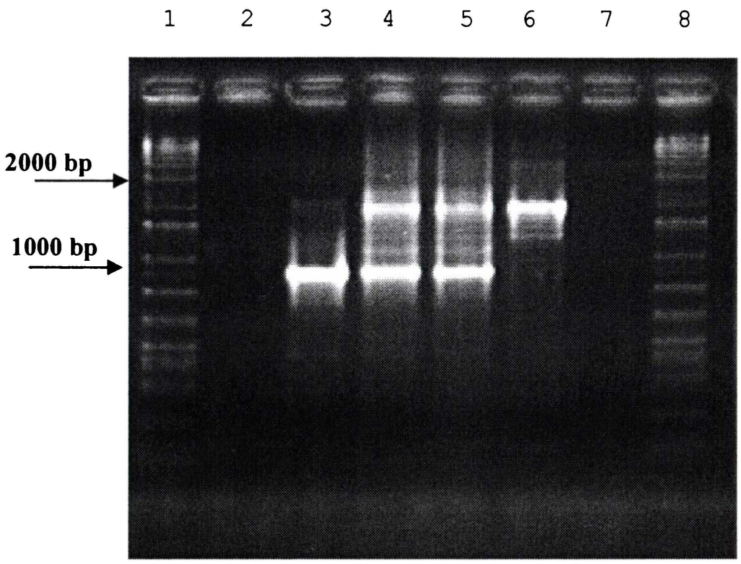

Figure 5.7 Archaeal 16S rDNA PCR from Tok37.S1 stored -70°C glycerol cultures. The track numbers and corresponding samples are: (1) 1 kb ladder; (2) 22 Jan 2003 #1; (3) 22 Jan 2003 #2; (4) 19 Jan 2003 #1, (5) 19 Jan 2003 #2; (6) 17 April 2004; (7) Negative control; (8) 1 kb ladder.

From the agarose gel (Figure 5.7) the initial Tok37.S1 culture; sample 22 Jan 2003 #2 (unfortunately 22 Jan 2003 #1 did not amplify) contained a dominant 'normal' sized (1000 bp) 16S rDNA band and a small atypical band at 2000 bp, however, as the culture aged the 'normal' sized PCR product lost its dominance and the large atypical product became dominant and it was the only band present in the profile of the 17 April 2004 sample (lane 6).

Consequently, the full 16S rRNA gene was PCR amplified from Tok37.S1 (sample 22 Jan 2003 #3) with primers RR149 (~ 2F) and RR77 (1522R) and two bands were obtained; an expected 1500 bp product and an atypical 2500 bp product. The resulting amplicons were sequenced using primer RR149 (~ 2F). The expected, 'normal' sized band proved to be the previously identified *Ignisphaera*-type sequence; however, the larger atypical product proved to be most closely related (95%) to *Desulfurococcus mobilis*, (M36474) by NCBI BLASTn. When aligned to the *Desulfurococcus mobilis* 16S rDNA sequence an intron of 36 bp (indicate by bold font below in the alignment) was evident and might suggest that other introns exist in the Tok37.S1 16S rRNA gene that can account for its large size (alignment below).

```
Desulfurococcus                      CCGCCGCTGCGGGGCGTGGCGG-ACGGCTG-AGTAACACGTGGC
Tok37.S1 large band                  CCGCCGCCGCGGGGCGTGGCNGGACGGCTGTATTAACACGTGGC
                                     ******* ************ * ******* * ***********

Desulfurococcus      TAACCTACCCTCGGGAGGGGGATAACACCGGGAAACTGGTGCTAATCCCCCATAGGGGAG
Tok37.S1 large band  TAACCTACCCTCGGGAGGGGGATAACACCGGGAAACTGGTGCTAATCCCCCATAGGGGAG
                     ************************************************************

Desulfurococcus      GAGGCCTGGAAGGGTTCCTCCCCGAAAGGGTGTGGCAGGGGTTAACGCTGCTACACCGCC
Tok37.S1 large band  GAGGCCTGGAAGGGTTCCTCCCCGAAAGGGTGT---ACAGGCCAACGCTGCTACACCGCC
                     *********************************   *  **  *****************

Desulfurococcus      CGAGGATGGGGCTACGGCCCATTAGGTTGTTGGCGGGGTAACGGCCCGCCAAGCCGATAA
Tok37.S1 large band  -GAGGATGGGGCTACGGCCCATTAGGTAGTTGGCGGGGTAACGGCCCGCCAAGCCGATAA
                      ************************* *********************************

Desulfurococcus      TGGGTAGGGGCCGTGAGAGCGGGAGCCCCCAGATGGGCACTGAGACAAGGGCCCAGGCCC
Tok37.S1 large band  TGGGTAGGGGCCGTGAGAGCGGGAGCCCCCAGATGGGCACTGAGACAAGGGCCCAGGCCC
                     ************************************************************

Desulfurococcus      TACGGGGCGCACCAG-GCG------------------------------------CGAAA
Tok37.S1 large band  TACGGGGCGCACCAGCGCGAAACTCCGCAAATGGGGTTCTCGGGGCACACTACCCCGAAG
                     *************** ***                                    ****

Desulfurococcus      CCTCCGC--AATGCGGGAAACCGTGACGGGGCCACCCCGAGTGCCCCCTTACGGGGGCTT
Tok37.S1 large band  CCCCAGCGGAATGCGGGAAACCGTGACGGGGCCACCCCGAGTGCCCCCTCACGGGGGCTT
                     ** * **  **************************************** **********

Desulfurococcus      TTCCCCGCTGTAGGAAGGCGGGGGAATAA-GCGGGGGGCAAGTCTGGTGTCAGCCGCCGC
Tok37.S1 large band  TTCCCCGCTGTAGGAATGCGGGGGAATAACGCGGGGGGCAAGTCTGGTGTCAGCCGCCGC
                     **************** ************ ******************************

Desulfurococcus      GGTAATACCAGCCCCG
Tok37.S1 large band  GGTAATACCAGCCCCG
                     ****************
```

The intron sequence was used for a BLASTn search against the NCBI database, however, no matches were found amongst any other 16S rDNA sequences. The sequence was also aligned against other crenarchaeota that contain 16S rDNA introns, including, *Pyrobaculum aerophilum, Thermoproteus sp.* and *Caldivirga maquilingensis*, but no homologous intron in any other 16S rDNA was found.

The above results suggest that the population of the Tok37.S1 culture was a mixed culture of at least two species of cocci, where the population structure changed from an *Ignisphaera* sp. dominant culture to one dominated by a *Desulfurococcus*-related organism. An unlikely alternative scenario is that the Tok37.S1 organism contains two 16S rRNA genes but this cannot explain the differential amplification of each with transfer, and it might be expected that the phylogenetic affiliation of each would be similar. It is interesting to note the closest relative (*D. mobilis*, M36474) for the longer amplicon from Tok37.S1 is the same as that obtained for the two TRUCC *Desulfurococcus*-related isolates 321 and 322 that also contain large 16S rRNA sequences (Section 5.2.1). The 16S rRNA sequences of the long amplicons from Tok37.S1, TRUCC 321 and 322 are discussed further in Section 5.3.

5.2.3 Screening of Yellowstone National Park hot pools for *Ignisphaera*-related archaea

Attempts to isolate *Ignisphaera*-like archaea from hot pools of superficially similar chemistry in Yellowstone National Park are discussed in the IJSEM paper. However, Table 5.6 includes information about the enrichments in greater detail, including cell morphologies and lengths of incubation in the hot pools.

The enrichments were removed from the pools and transported to the laboratory and 1 ml taken to inoculate fresh medium. Dilutions series (1:10) were undertaken in an attempt to isolate pure cultures from enrichments. In addition, media from Jan's Tide Pool and Frog Lips that were inoculated in the field and transported back to the laboratory under ambient conditions were incubated at 85°C and isolation attempts undertaken using dilution series. All resulting cultures are listed in Table 5.7.

Table 5.6 Yellowstone National Park hot pools screened by culture methods for *Ignisphaera*-related microorganisms.

Location	Hot Pool	*In situ* temp. (°C)	*In situ* pH	Medium	Incubation time (days)	Enrichment morphologies
Greater Obsidian Pool Area (GOPA)	OB1 Heim	75-85	5-6	Enrichment media (Db+S)	7	*Fervidobacterial*, long, thin rods
				Db+Konjac glucomannan	7	*Fervidobacterial*
	South Obsidian Pool	75.5-80	6	Enrichment media (Db+S)	7	*Fervidobacterial*
				Db+Konjac glucomannan	7	*Fervidobacterial*, long, thin rods
	Obsidian Pool	73.5-76	6	Enrichment media (Db+S)	7	*Fervidobacterial*, long, thin rods
				Db+Konjac glucomannan	7	*Fervidobacterial*, long, thin rods
Sentinel Meadows	Bison Pool	87-92.5	7-7.4	Enrichment media (Db+S)	8	No Growth
				Db+Konjac glucomannan	8	Few *Fervidobacteria*
	Evil Mouth	81-85	7-8	Enrichment media (Db+S)	8	No growth
				Db+Konjac glucomannan	8	No growth
Boulder Spring	Boulder Spring	86.4	8	Enrichment media (Db+S)	5	No growth
				Db+Konjac glucomannan	5	No growth
Sylvan	Sylvan Spring	75-78	5-6	Enrichment media (Db+S)	8	Long, thin rods
				Db+Konjac glucomannan	8	Long, thin rods
South Sylvan	Jan's Tide Pool	89.2	6.6	Enrichment media (Db+S)	transported to laboratory	Not applicable
				Db+Konjac glucomannan	transported to laboratory	Not applicable
	Frog Lips	80.7	6	Enrichment media (Db+S)	transported to laboratory	Not applicable
				Db+Konjac glucomannan	transported to laboratory	Not applicable

Table 5.7 Cultures obtained from Yellowstone National Park enrichments.

Hot Pool	Incubation temp. (°C)	Medium	Culture morphologies
OB1 Heim	80	Db+S	No growth
	80	Db+Konjac glucomannan	No growth
	85	Db+S #1	Golf club *Pyrobaculum*-like rods
	85	Db+S #2	Thin rods and *Pyrobaculum*-like rods
South Obsidian Pool	80	Db+S	No growth
	80	Db+Konjac glucomannan	No growth
	85	Db+Konjac glucomannan	Thin rods
Obsidian Pool	80	Db+S	*Fervidobacterial*
	80	Db+Konjac glucomannan	*Fervidobacterial*
	85	Db+S	Thin rods and *Pyrobaculum*-like rods
	85	Db+Konjac glucomannan	Thin rods
Sylvan Spring	85	Db+S	Very few *Pyrobaculum*-like rods
Jan's Tide Pool	85	Db+S	No growth
	85	Db+Konjac glucomannan	No growth
Frog Lips	85	Db+S	Thin rods and *Pyrobaculum*-like rods
	85	Db+Konjac glucomannan	No growth

In conclusion, cocci were not enriched from hot pools in Yellowstone National Park, with the dominant microbial morphologies in the media being rod-shaped.

5.3 Analysis of 16S rDNA introns in some New Zealand coccal-shaped archaea

Large atypical 16S rDNA amplicons were obtained for TRUCC 318, 321 and 322 (Section 5.2.1) and Tok37.S1 (Section 5.2.2.1). All atypical sequences (with the exception of TRUCC 318) had a single sequencing reaction already undertaken on them and all proved to be most closely related to *Desulfurococcus mobilis* (M36474).

An undergraduate student James Connell was assigned a project to investigate these atypical 16S rDNA sequences under my supervision. Initially, partial 16S rDNA PCR products (Tok37.S1, and TRUCC 318, 321 and 322) were obtained using primers RR150 (347F) and RR151 (1335R) and were digested with *Hin*d III and *Rsa* I restriction endonucleases to compare resulting restriction fragment length polymorphisms (RFLP) patterns to provide a comparison of similarity between sequences (Figure 5.8).

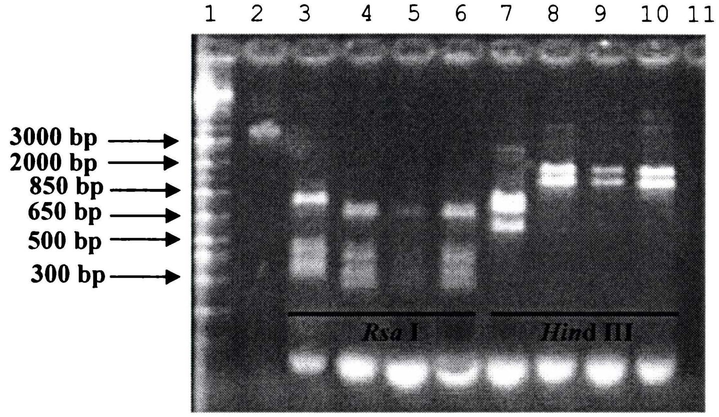

Figure 5.8 Restriction endonuclease patterns from atypical 16S rDNA PCR products. The track numbers and the corresponding samples are: (1) 1 kb ladder; (2) Uncut Ket55.S2 control; Lanes 3 to 6 were digested with *Rsa* I; (3) Tok37.S1; (4) TRUCC 318; (5) TRUCC 320; (6) TRUCC 321; Lanes 7 to 10 were digested with *Hind* III; (7) Tok37.S1; (8) TRUCC 318; (9) TRUCC 320; (10) TRUCC 321; (11) Negative control.

From the restriction endonuclease digests it is evident that the three TRUCC isolates contain similar RFLP patterns, with Tok37.S1 containing the same number of cut sites but giving different fragment sizes. Therefore, it was decided to sequence 16S rDNA from one of the TRUCC isolates (i.e. Ket55.S1) which contained a RFLP pattern typical of all three, and also Tok37.S1.

A PCR product was obtained from Tok37.S1 using primer pair RR150 (347F) and RR151 (1335R) and the resulting sequence obtained utilising primer RR151 (1335R) is aligned to *Desulfurococcus mobilis* (M36474) below (alignment taken from James Connell's report). An intron is indicated in bold font for the Tok37.S1 sequence. The intron sequence was used in a BLASTn search against the NCBI database, however, no matches were found amongst any other 16S rDNA sequences.

```
Tok37.S1                ----------ACAAGGGGTTGGAGCT-CGGTTCAATTGGAGTCAACGCCCGGGAATCTCA 50
Desulfurococcus         GGGAGCACCACAAGGGGTGGAGCCTGCGGTTCAATTGGAGTCAACGCC--GGGAATCTCA 1018
                                  ********* *   ** **********************  **********

Tok37.S1                CCGGGGGAGACAGCAGGATGACGGCCAGGTTAAAGGCCTTGCCTGACGCGCTGAGAGGAG 110
Desulfurococcus         CCGGGGGAGACAGCAGGATGACGGCCAGGTTAAAGGCCTTGCCTGACGCGCTGAGAGGAG 1078
                        ************************************************************

Tok37.S1                GTGCATGGCCGTCGCCAGCTCGTGCTGTGAAGTGTCCGGTTAAGTCCGGAAAATCCCCGA 170
Desulfurococcus         GTGCATGGCCGTCGCCAGCTCGTGCTGTGAAGTGTCCGGTTAAGTCCGGAAA-------- 1130
                        ****************************************************
```

```
Tok37.S1            GTGGGGTAGTACATGGTTTACTTCTCCACCGGGGATCCGAACGAGCGAGACCCCCACCCC 230
Desulfurococcus     -----------------------------------------CGAGCGAGACCCCCACCCC 1149
                                                             *******************

Tok37.S1            TAGTTGCTACCCGGGGCTATGGCTCCGGGGCACA-TAGGGGGAC-GCCGCCGTTTAAG-C 287
Desulfurococcus     TAGTTGCTACCCGGGGCTACGGCTCCGGGGCACACTAGGGGGACTGCCGCCGTTTAAGGC 1209
                    ******************* ************** ********* ************* *

Tok37.S1            GGAGGAAGGAGGGGGCCACGGCAGGTCAGCATGCCCCGAACCCCCCGGGCTACACGCGGG 347
Desulfurococcus     GGAGGAAGGAGGGGGCCACGGCAGGTCAGCATGCCCCGAACCCCCCGGGCTACACGCGGG 1269
                    ************************************************************

Tok37.S1            CTACAATGGCGGGGACAGCGGGATCCGACCCCGAAAGGGGGAGGTAATCCCTCAAACCCC 407
Desulfurococcus     CTACAATGGCGGGGACAGCGGGATCCGACCCCGAAAGGGGGAGGCAATCCCTCAAACCCC 1329
                    ******************************************** ***************

Tok37.S1            GCCGTGGTTGGAATCGAGGGCGGCAACTCGCCCTCGTGAACGTAAAAATCCCCTNGAACC 467
Desulfurococcus     GCCGTGGTTGGGATCGAGGGCTGCAACTCGCCCTCGTGAACG-AGGAATCCCTAGTAACC 1388
                    *********** ********* ******************** *  ******    ****

Tok37.S1            GCGCGTCAACATCGCGCGAAACC------------------------------------- 490
Desulfurococcus     GCGCGTCAACATCGCGCGGTGAATACGTCCCTGCTCCTTGCACACACCGCCCGTCGCTCC 1448
                    ******************
```

Similarly, a sequence using primer RR151 (1335R) was obtained from the PCR product of Ket55.S1 (primer pair RR150 (347F) and RR151 (1335R)). It was aligned to *Desulfurococcus mobilis* (M36474) as presented below (alignment taken from James Connell's report), however, introns were not detected in the sequence.

```
Ket55.S1            ---------------------------------------------------------GAC 3
Desulfurococcus     GCATGGCCGTCGCCAGCTCGTGCTGTGAAGTGTCCGGTTAAGTCCGGAAACGAGCGAGAC 1140
                                                                             ***

Ket55.S1            CACCACCCCTAG--GCTACCCGGGGCTANGGCCCCGGGGCACACTAGGGGGAC-GCCGCC 60
Desulfurococcus     CCCCACCCCTAGTTGCTACCCGGGGCTACGGCTCCGGGGCACACTAGGGGGACTGCCGCC 1200
                    * **********  ************** *** ******************** ******

Ket55.S1            GTT-AAGGCGGAGGAAGGAGGGGGCCACGGCAGGTCAGCATGCCCCAAACCCCCCGGGCT 119
Desulfurococcus     GTTTAAGGCGGAGGAAGGAGGGGGCCACGGCAGGTCAGCATGCCCCGAACCCCCCGGGCT 1260
                    *** ****************************************** *************

Ket55.S1            ACACGCGGGCTACAATGGCGGGGACAGCGGGATCCGACCCCGAAAGGGGGAGGTAATCCC 179
Desulfurococcus     ACACGCGGGCTACAATGGCGGGGACAGCGGGATCCGACCCCGAAAGGGGGAGGCAATCCC 1320
                    ***************************************************** ******

Ket55.S1            TCAAACCCCGCCGTGGTTGGAA-CGAGGGCTGCAACTCGCCCATCGTGAACGTTAAATCC 238
Desulfurococcus     TCAAACCCCGCCGTGGTTGGGATCGAGGGCTGCAACTCGCCC-TCGTGAACGAGGAATCC 1379
                    ******************** * ******************* *********   *****

Ket55.S1            TNGTAAACCGCGCGTCA-CATCGCGCGT--GATNC------------------------- 270
Desulfurococcus     CTAGTAACCGCGCGTCAACATCGCGCGGTGAATACGTCCCTGCTCCTTGCACACACCGCC 1439
                         ************ *********    ** *
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Therefore, in conclusion, two introns were found in the Tok37.S1 16S rDNA sequence (the first is presented in Section 5.2.2.1) and introns were not found in the Ket55.S1 sequence. Therefore, if time had permitted, a sequencing effort to obtain the near-full length 16S rDNA sequence of these isolates would have provided the data needed to account for their atypical large size.

5.4 Discussion and conclusion

The IJSEM manuscript and supplementary data suggests that there are novel-archaeal cocci residing in New Zealand's hot springs, including the characterised *Ignisphaera*-type archaea and *Desulfurococcus*-related microbes with large, intron containing 16S rDNAs. In fact, the Tok37.S1 coccal culture may have started as a consortium with a dominant species of *Ignisphaera,* through which, serial transfers lead to dominance of the culture changing to a large 16S rRNA containing *Desulfurococcus*-related organism.

Introns within the 16S rRNA genes of archaea species have already been documented including those in *Aeropyrum pernix* (Nomura *et al.*, 1998, 2002), *Pyrobaculum aerophilum* (Burggraf *et al.*, 1993), *Thermoproteus neutrophilus* (Itoh *et al.*, 1998a) and uncultured archaeal species (Takai and Horikoshi, 1999). In addition, the 23S rDNA of archaea can also harbour introns, for example, in *Desulfurococcus mobilis* (Kjems and Garrett, 1985) and *Aeropyrum pernix* (Nomura *et al.*, 1998).

The introns residing in archaeal rRNA genes are normally mobile elements which encode for homing endonuclease-like open reading frames (ORF) containing sequences with a LAGLIDADG motif (Nomura *et al.*, 2002). Homing endonucleases are grouped into three families according to their structural similarity, where the majority are LAGLIDADG-types (Nakayama *et al.*, 2003). However, increasing numbers of introns found in crenarchaeaotal 16S rDNAs are short in sequence (~30-200 bp) and do not contain ORFs (Nomura *et al.*, 2002), examples include *Staphylothermus marinus* (Kjems and Garrett, 1991), *Thermoproteus neutrophilus* (Itoh *et al.*, 1998a), *Caldivirga maquilingensis* (Itoh *et al.*, 1999) and uncultured species (Takai and Horikoshi, 1999). There is debate as to whether these introns are related to the ORF-containing introns and whether they have the capacity to be mobile. However, a link between these two types of introns has been shown by Nakayama *et al.*, (2003), whereby, a homing endonuclease which is encoded for within the 16S rRNA gene of *Pyrobaculum oguniense* cleaves at the insertion site of a neighbouring intron that does not contain an ORF. From the introns of Tok37.S1 16S rDNA it seems that they are also short ORF-less sequences, however, other introns must reside in the 16S rRNA gene to account for the abnormally large 16S rDNA PCR product. Therefore, a full length 16S rDNA sequencing effort of the atypical 16S rRNA genes of the New Zealand isolates may reveal the presence of other introns and perhaps homing endonuclease signatures.

Chapter 6 : Studies undertaken on samples from hydrothermal vents at the East Pacific Rise (EPR)

6.1 Introduction

Black smokers are hydrothermal vents discharging superheated water in excess of 350°C from the sea floor. Metal sulfides dissolved in the superheated water are precipitated when mixed with the cold sea water leading to the production of 'black smoke' (Haymon, 1983), and as a consequence, chimney-like structures (mainly metal sulfide precipitates) form around these vents. The vents provide the chemical energy for chemosynthetic metabolism and support a plethora of microorganisms (Alain *et al.*, 2004; Harmsen *et al.*, 1997; Slobodkin *et al.*, 2001). In fact, hydrothermal vents have been stated to be one of most productive eco-systems on Earth (Alain *et al.*, 2004). The primary microbial consumers allow the development of higher organisms which use these microorganisms for direct consumption or for symbiotic grazing or filter feeding. For example, vents along the East Pacific Rise (EPR) are commonly colonised by the polychete worm *Alvinella pompejana* that contains symbiotic bacteria (Cary *et al.*, 1997) and *Riftia pachyptila* which contain bacterial symbionts that produce energy by the oxidation of reduced sulfur compounds, and also fix inorganic carbon which is utilized by its host (Distel *et al.*, 1988).

DNA-based microbial ecology studies have shown that novel uncultured microbes are associated with hydrothermal chimney structures (Reysenbach *et al.*, 2000b; Schrenk *et al.*, 2003; Takai *et al.*, 2001; Hoek *et al.*, 2003). However, a recent study has shown that the pioneer colonisers of high temperature hydrothermal chimneys include novel nanoarchaeal signatures (McCliment *et al.*, in press), i.e. if a chimney was removed and allowed to re-form, these were some of the first microorganisms detected by 16S rDNA cloning within hours of chimney formation.

Nanoarchaea were first discovered in a culture of *Ignicoccus* sp. (Huber *et al.*, 2002). Currently, the *Ignicoccus* genus consists of two coccal-shaped hyperthermophilic chemolithoautotrophic species (*Ignicoccus islandicus* and *pacificus*) isolated from submarine hydrothermal systems (Huber *et al.*, 2000a). The nanoarchaeal cells were very small in size (400 nm in diameter) and grew attached to the 'host' *Ignicoccus* cells. It was characterised and its 16S rRNA gene sequenced and was named *Nanoarchaeum equitans* and placed within a new phylum (or may in fact constitute a novel Kingdom). The existing archaeal specific primers used in microbial ecology did not amplify nanoarchaeal 16S rDNA. Therefore, nanoarchaeal specific PCR primers were developed to target and subsequently amplify by PCR the 16S rDNA from nanoarchaea (Huber *et al.*, 2002). These new primers were utilised by Hohn *et al.*, (2002) in a number of 'hot' environments and nanoarchaeal signatures were discovered in Yellowstone National Park, WY, USA, the Uzon Caldera, Kamchatka, Russia and an abyssal vent system (EPR). These three environments include both terrestrial and marine high-temperature (70-98°C) hydrothermal systems, and therefore, it was theorized that nanoarchaea are present in other high-temperature habitats including terrestrial settings. Since these first ecological studies the genome of *Nanoarchaeum equitans* has been sequenced (Waters *et al.*, 2003) and its lipids characterised (Jahn *et al.*, 2004). From these studies it was revealed that *Nanoarchaeum equitans* has the smallest sequenced genome of any organism apart from viruses (~ 480,000 bp) and lacks the genes encoding for enzymes involved in lipid, cofactor, nucleic or amino acid bio-synthesis. Therefore, it was concluded that *Nanoarchaeum equitans* is completely dependent on its host and may in fact be parasitic towards its *Ignicoccus* host.

6.2 Objective and approach

Due to the discovery of nanoarchaea in New Zealand environments (see Section 4.5) and experience in anaerobic, high-temperature culturing, an invitation from Prof. S. C. Cary (University of Delaware, USA and University of Waikato, NZ) was made to join the Extreme 2004 expedition to sample hydrothermal vents (http://www.ocean.udel.edu/extreme2004/home.html) at the EPR (9°40" North, 104°16" West) to continue on from the study undertaken by McCliment *et al.*, (in press) with the aim of obtaining a culture of the nanoarchaeal colonisers that were detected by this work. Chimney structures from hydrothermal vents were displaced by the *Alvin* submersible manipulator arms and 'frying pans' (see Figure 6.1) were placed over the vent holes. Frying pans were constructed from titanium and were sterilised by exposure to a gas blow-torch. Frying pans contained a titanium mesh which gave a support structure to allow chimneys to grow onto (rendering them less fragile) and a titanium handle and rope to allow for easy deployment and recovery of the frying pan and subsequent transportation to the laboratory in a nondestructive manner. Following incubation of a frying pan over the vent (approximately 2 days) the frying pan was collected by *Alvin*, stored in a sample box (containing 0.22 µm filtered sea water) attached to the basket on the front of the *Alvin* submersible and returned to the surface. Sulfide from the newly-formed chimney was then used for inoculation of enrichment media. The enrichment media chosen to attempt the isolation of colonisers included:

1. Two media used to cultivate nanoarchaea and its *Ignicoccus* host (medium 897 and SME, Appendix E for composition).
2. A standard *Thermococcus* cultivation medium (medium 760, Appendix E) because species of *Thermococcus* were also detected by McCliment *et al.*, (in press).
3. Modified Db+S (prepared in half concentration sea water), which is a universal medium used to culture terrestrial heterotrophic hyperthermophiles (composition in Appendix E).

Other minor projects included culturing attempts from sulfide chimneys "of opportunity", which were collected by *Alvin* and transported back to the laboratory and used to inoculate the growth media. Also, *Alvinella pompejana* and the tubes that it inhabits were collected by *Alvin* and used to inoculate anaerobic *Spirochete*-based media and aerobic tryptic soy agar with starch (TSA/S) medium (media compositions in Appendix E).

The materials and methods used to obtain the results for this Chapter are included in the general Materials and Methods, Chapter 2.

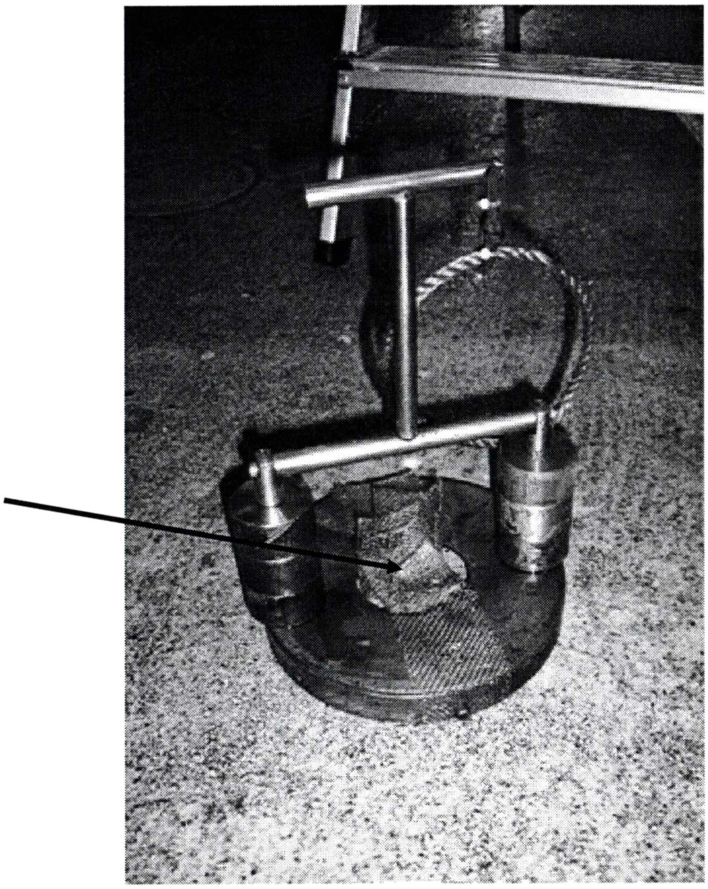


Figure 6.1 A titanium 'frying pan'. The arrow indicates the hole which is placed over a hydrothermal vent.

6.3 Results

The results of the culturing from *Alvinella pompejana* samples are discussed initially in Section 6.3.1 below, followed by chimney (sulfide)-based culturing in Section 6.3.2.

6.3.1 *Alvinella pompejana*-associated samples

Samples were taken from *Alvinella pompejana* and used to inoculate modified *Spirochete* (medium 590) and TSA/S media. All enrichments and the resulting cultures obtained are presented in Table 6.1 below.

Table 6.1 Cultures obtained from *Alvinella*-associated samples.

Date	Dive #	Sample used to inoculate medium	Incubation temp. (°C)	Medium/Cultures	Morphologies of cultures
3/12/4	4062	Fleece removed from *Alvinella* (Biovent), re-suspended in sterile sea water.	37	*Spirochete* medium - 4X (1:10) dilution series from enrichment.	Short rods and shorter motile rods.
As above	As above	As above	45	*Spirochete* medium - 2X (1:10) dilution series from enrichment.	Bent, short motile rods.
As above	As above	As above	Room-temp.	*Spirochete* medium - 2X (1:10) dilution series from enrichment.	Motile, short thick rods.
5/12/4	4065	Liquid used to wash *Alvinella*.	55	*Spirochete* medium - 3X (1:10) dilution series from enrichment.	Cocci, short rods and small motile cells.
As above	As above	Tubes *Alvinella* live in.	55	*Spirochete* medium - 2X (1:10) dilution series from enrichment.	Cocci, short rods and small motile cells.
As above	As above	Liquid used to wash *Alvinella*.	45 and 55	Modified TSA/S- 250 µl spread onto plates.	No growth (10 day incubation)
As above	As above	Tubes *Alvinella* live in.	45 and 55	Modified TSA/S- 250 µl spread onto plates.	No growth (10 day incubation)

All enrichment cultures were transported to New Zealand under ambient conditions and eventually stored at 4°C. An undergraduate student, Lisa Everett was assigned to attempt to isolate the two organisms (rod and a motile rod) into pure culture from the 37°C consortia (Table 6.1). The non-motile rod was isolated into pure culture by its ability to grow aerobically on agar plates. However, the motile rod was dominated by the non-motile rod and could not be isolated into pure culture (Everett, 2005). The full length 16S rDNA was amplified by PCR from the rod isolate using the universal bacterial primer pair RR69 (27F) and RR77 (1522R), however, attempts at sequencing this amplicon were not successful and only a short sequence length was obtained. This was repeated but again the sequence result was poor. Only 75 bp of 16S rDNA could be used for a subsequent NCBI BLASTn search (sequence is contained in Appendix D), the resulting closest relatives (approximately 50 hits) were 98% similar in sequence to the query sequence and the NCBI taxonomy report placed the isolate within the Alphaproteobacteria class within the Sphingomonadaceae family, which is in accordance with the rod staining Gram negative (Everett, 2005).

A bacterial DGGE was undertaken to compare the pure rod culture and the original consortium culture in the hope of sequencing a DGGE band that would be considered to be derived from the motile rod, i.e. a band that was present in the consortium profile and distinct from that of the short rod in pure culture. Thus, sequencing the band would prove the identity of the motile rod. The DGGE is presented below in Figure 6.2.

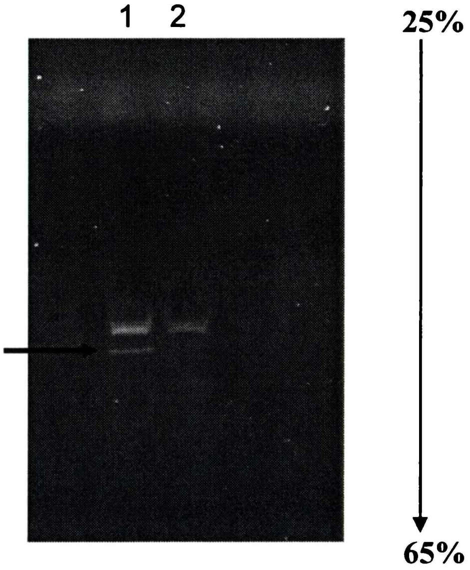


Figure 6.2 Bacterial DGGE of *Alvinella pompejana* associated (37°C) rod and consortium cultures. The track numbers and corresponding samples are: (1) consortium; (2) rod culture. The band indicated by an arrow was extracted and sequenced.

The dominant band indicated on the DGGE (Figure 6.2) was not present in the DGGE profile of the pure culture of rods and was assumed to represent the motile rod of the original consortium. Therefore, the band was sequenced and a BLASTn search undertaken against the NCBI database with the closest relatives listed in Table 6.2 below (the original sequence is contained in Appendix D).

Table 6.2 NCBI BLASTn results of the DGGE band from the *Alvinella pompejana* derived consortium.

Accession #	Organism	Sequence length (bp)	Similarity
AY264938	*Vibrio alginolyticus*	148	98%
AY264936	*Vibrio vulnificus*	148	98%
AF493804	*Vibrio fischeri*	148	98%
X76333 and X74726	*Vibrio vulnificus* (ATCC 27562T) and (ATCC 27562T)	148	98%

The closest relatives to the sequence of the DGGE band from the consortium (Table 6.2) indicate a close relationship to species of *Vibrio*. This is in accordance with its morphological characteristics, i.e. a motile curved rod. In fact, a species of *Vibrio*, namely; *Desulfovibrio hydrothermalis* has been isolated from a deep-sea hydrothermal chimney sample collected at 13°N on the EPR (Alazard *et al.*, 2003). Therefore, attempts were undertaken by a visiting scientist (Associate Prof. Hong Chen) to isolate the motile rod using *Desulfovibrio hydrothermalis* medium (see Appendix E for composition), however, attempts were unsuccessful.

6.3.2 Hydrothermal chimney-associated samples

6.3.2.1 Isolation attempts from chimney sulfide

Chimney samples collected by *Alvin* and nascent chimney samples recovered from the frying pans were used to inoculate enrichment media, described below. Sulfide from each sample was also preserved by a variety of methods for long-term storage and future work, namely: frozen at -20°C, in 100% ethanol at room temperature and fixation by paraformaldehyde and subsequent dehydration in ethanol. Samples for the inoculation of media was re-suspended in sterile sea water and the supernatant used to inoculate enrichment media (in duplicate) including: *Thermococcus* marine medium

(medium 760), Db+S medium in half concentration sea water, *Ignicoccus* medium (medium 897) and SME medium (general marine medium used to grow *Ignicoccus* and nanoarchaeal consortia). The compositions of all the media used are presented in Appendix E. Table 6.3 lists and describes the frying pan and chimney samples of 'opportunity'.

Table 6.3 Frying pan and chimney samples of 'opportunity' from the EPR.

Frying pan #	**Vent**	**Dive deployed**	**Dive collected**	***In situ* incubation time**	**Temp. (°C)**
1	Tica	4066	4068	2 days	188-330
2	Ty	4067	4069	2 days	250-300
3	Ty	4069	4071	2 days	166-319
4	Q	4070	4072	2 days	149-325
N.A.	Biovent	N.A.	4062	N.A.	N.D.
N.A.	Alvinella Stump	N.A.	4064	N.A.	~ 250
N.A.	Near Ty & Io	N.A.	4065	N.A.	~ 200
N.A.	Tika	N.A.	4066	N.A.	~ 308
N.A.	Un-named	N.A.	4067	N.A.	N.D.

N.A. = Not applicable. N.D. = Not determined.

The enrichments obtained from the samples listed in Table 6.3 are described in Table 6.4. Enrichments with positive signs of growth were diluted (1:10) into fresh medium. However, cells could only be transferred twice at most, as another dilution did not yield a culture containing an outgrowth of microorganisms. Moreover, cell lysis was commonly observed amongst the enrichments.

Table 6.4 Enrichments obtained from chimney and frying pan sulfide samples.

Sample	Incubation temp. (°C)	Inoculated media	Enrichments and cell morphologies
Sulfide 4062	85 & 95	All 4*	No growth.
Sulfide 4064	85	All 4*	Medium 897 = regular cocci.
	95	All 4*	Medium 897 = regular cocci.
			Medium 760 = regular cocci.
			Medium SME = lysed cells.
Sulfide 4065	85	All 4*	All media (exception of Db+S) = regular cocci.
	95	All 4*	Medium SME = cocci.
			Medium 897 = cocci.
Sulfide 4066	85 & 95	All 4*	Medium SME (95°C) = very few cocci
Sulfide 4067	85 & 95	SME & 897	Medium SME (85°C) = regular, motile cocci, lysis observed.
			Medium 897 (95°C) = regular, motile cocci, lysis observed.
Frying Pan 1	85	All 4*	Medium 760 = regular cocci.
			Medium 897 = lysed cocci.
	95	All 4*	Medium SME = lysed cocci.
			Medium 897 = lysed cocci.
			Medium 760 = regular cocci.
Frying Pan 2	85 & 95	All 4*	Medium 897 (95°C) = regular cocci.
Frying Pan 3	85	All 4*	Medium 897 = regular cocci.
			Medium 760 = regular cocci.
	95	All 4*	Medium 897 = regular cocci.
			Medium SME = regular cocci.
Frying Pan 4	85	All 4*	Medium 897 = regular, motile cocci.
			Medium SME = regular cocci.
	95	All 4*	Medium 897 = regular cocci.
			Medium SME = regular cocci.

***SME medium, medium 897, medium 760 and Db+S in half concentration seawater.**

In conclusion, growth was not observed in any of the Db+S media and the only morphology observed in all other media were regular coccal-shaped cells. Two types of cocci were documented, one being approximately 2 µm or greater in diameter and the other which was approximately 1 µm in diameter and motile. The cultures were transported to the laboratory under ambient conditions.

6.3.2.2 DNA analysis of enrichments

Due to the cultures not being able to be transferred into fresh media, and therefore not able to be cultivated within the laboratory, it was decided to analyse cultures by DNA-based approaches. All enrichments and serially diluted cultures were checked microscopically on return to the laboratory and media containing cells (under phase-contrast microscopy) were used for subsequent DNA isolation and analysis. DNA was extracted from the cultures by the CTAB method, with an archaeal 16S rDNA PCR undertaken using primer pair RR150 (347F) and RR151 (1335R). The cultures used for DNA isolation and the result of their archaeal PCR amplifications are listed in Table 6.5.

Table 6.5 Enrichments used for DNA extraction from chimney enrichments.

Sample #	Dive #	Medium	Temp. (°C)	Culture type	Positive archaeal 16S rDNA PCR?
1	4067	SME	85	10^{-1} dilution	Yes
2	4067	SME	85	10^{-1} dilution	Yes
3	4067	897	95	10^{-2} dilution	Yes
4	4067	SME	85	10^{-1} dilution	Yes
5	4067	SME	85	Enrichment	Yes
6	4064	897	85	Enrichment	No
7	4065	897	95	Enrichment	No
8	4065	897	85	Enrichment	No
9	4065	SME	85	10^{-1} dilution	Yes
10	4065	760	85	Enrichment	Yes
11	4065	760	85	Enrichment	Yes
12	4066	SME	95	Enrichment	No
13	4071	SME	95	Enrichment	No
14	4072	897	85	Enrichment	No
15	4068	SME	95	10^{-1} dilution	Yes
16	4068	760	95	Enrichment	Yes
17	4068	760	85	Enrichment	No
18	4068	897	85	Enrichment	Yes
19	4068	SME	95	Enrichment	Yes
20	4068	897	95	Enrichment	Yes
21	4069	897	95	10^{-1} dilution	No

An archaeal DGGE was then undertaken on any samples with positive archaeal amplifications (see Table 6.5) so that the DGGE profiles of all cultures could be compared as presented in Figure 6.3, below. From the archaeal DGGE it appeared that all cultures gave a band at the same position on the gel, this was repeated with increasing denaturant (50-70%) and the same result obtained.

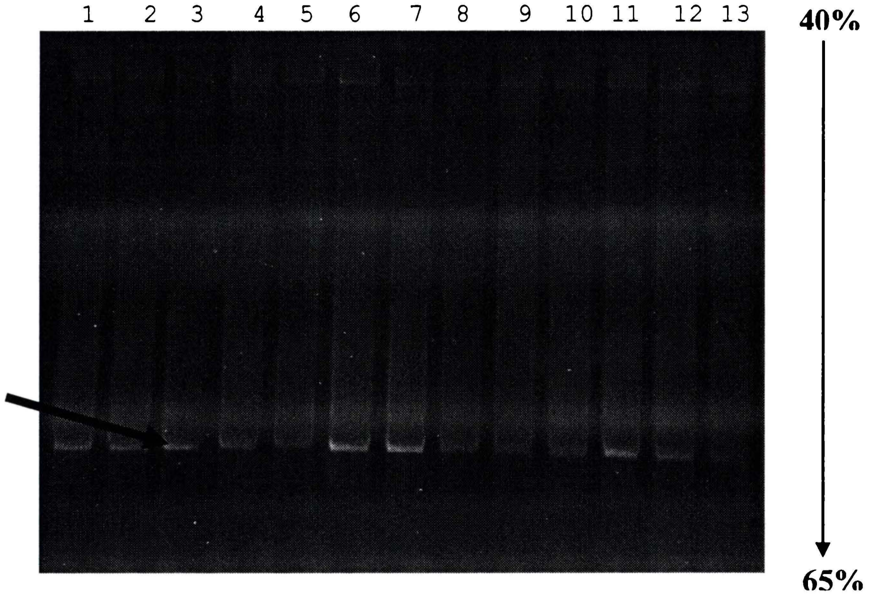


Figure 6.3 Archaeal DGGE of sulfide enrichments. The track numbers and corresponding samples (See Table 6.5) are: (1) Sample 1; (2) Sample 2; (3) Sample 3; (4) Sample 4; (5) Sample 5; (6) Sample 9; (7) Sample 10; (8) Sample 11; (9) Sample 15; (10) Sample 16; (11) Sample 18; (12) Sample 19; (13) Sample 20. The arrow indicates the band that was extracted and sequenced.

It was decided to sequence the DGGE band from one of the bands (sample 3, lane 3) as indicated by the arrow on Figure 6.3. The sequence was used in a BLASTn search against the NCBI database and it was established to be closely related to species of *Thermococcus*, whereby, the eight closest relatives (446 of 448 bp, 99% similar) included seven *Thermococcus* species from hydrothermal vents (Lepage *et al.*, 2004) and an uncultured clone from an *in-situ* sampler deployed at a 13°N hydrothermal vent (Nercessian *et al.*, 2003). In addition, a PCR assay targeting nanoarchaeal 16S rDNA (McCliment *et al.*, in press) was undertaken on all 21 extracted DNA samples (Table 6.5); however, no cultures yielded a positive PCR product.

6.4 Discussion and conclusion

In conclusion, the major aim of obtaining a culture of the pioneer nanoarchaeal hydrothermal vent colonisers was not achieved, with the cultures being dominated by species of *Thermococcus*. Moreover, nanoarchaea were not detected by PCR assay within the enrichments. The two major coccal morphologies (larger non-motile cocci and smaller motile cocci) observed in the enrichments from chimney inocula do not contradict the 16S rDNA signatures of *Thermococcus* that were obtained, as some species of *Thermococcus* are motile. It seems most probable that if nanoarchaea were to be cultivated, they would most probably be associated with host cells. If the *Thermococcus* cells within the enrichments were nanoarchaeal hosts, the nanoarchaea might be below the level of detection because of the lack of growth of the coccal host in the medium used. The inability to cultivate the cocci can be due to several possible factors: lack of required substrate, suitable ionic strength or composition and a tendency of the cells to lyse in the medium used. Since the microbial 'host' for the nanoarchaeal colonisers is unknown (only assumed) this increases the difficulty of attempting enrichments and further sampling efforts and culturing work is required to obtain the pioneering microbial colonisers of the hydrothermal vents. The development of media that closely mimics the chemistry of the hydrothermal vents at the site of colonisation would possibly aid these isolation attempts.

In contrast, the lower-temperature cultures obtained from the *Alvinella pompejana*-associated samples grew to high cell densities and contained various cell morphologies. Further work on the 37°C consortia led to the isolation of a rod identified to be a member of the Sphingomonadaceae family from co-culture with a species of *Vibrio*. The *Alvinella pompejana*-associated cultures are stored and further work on these samples may produce pure cultures of novel microorganisms and further the understanding of the microbes inhabiting hydrothermal vents and their association with higher organisms that are dependent on microbial-based metabolic processes at these extreme environments.

Chapter 7 : General Conclusion

This final chapter is intended to give a brief conclusion to the preceding chapters and a discussion of future considerations.

Methodologies were developed and successfully used to document biofilm formation on surfaces incubated in three New Zealand hot pools (Chapter 3) which were subsequently applied to a number of hot pools in both New Zealand (NZ) and Yellowstone National Park (YNP) as described in Chapter 4. DGGE analysis provided a quick method to gauge the microbial community diversity of extracted DNA including that from colonised slides, pool water and culture. As was predicted, the lower temperature pool, KP1, had a high diversity of microbes and was dominated by bacteria, with the hotter pool, AQ1, being dominated by a low diversity of archaea. Unexpectedly, the highest temperature pool, AQ1, had the faster rate of colonisation and attained the highest level of biomass on incubated slides of all three pools. For a number of reasons, work on the pools of KP1 and Champagne Pool was suspended, however, further work is currently being undertaken by a Ph.D. student into the microbial ecology of Champagne Pool.

The aim of the thesis was to target the active members of the microbial community by documenting colonisation, thus, avoiding dead or dormant cells or those adapted to rapid growth in artificial culture media. This aim seems to have been met for the lower temperature (~ 75°C) pool KP1, as DGGE profiles of the DNA from colonised slides differed from the DGGE profiles of DNA extracted from pool water and cultures isolated from the pool. Those species developing on the slide represent the more active and physiologically more significant members of the community, while organisms obtained only by enrichment may not be the most active or numerous species within the pool habitat. In contrast, a colonisation approach may not have been required to document the dominant microbial members of the high-temperature pools (>80°C) of NZ as DGGE profiles of DNA extracted from pool water provided similar profiles to DNA extracted from a colonised slide and organisms within the pools were typically recalcitrant to enrichment. Therefore, the similarity of colonisation and pool water DGGE profiles may also indicate that dead or dormant cells within these high-temperatures environments do not influence molecular-based community analysis. However, in the study, it was assumed that the DNA isolated and the consequent DGGE-PCR amplicons used for analysis was exclusively obtained from the dominant microorganisms colonising the surfaces incubated in the pools. As discussed in the Introduction (Section 1.1.2.2) due to the various shortcomings of PCR-based analysis, i.e. DNA extraction and PCR bias, this may not hold true. Therefore, if time and money had permitted, fluorescent in situ hybridisation (FISH) analysis using probes specific to the microorganisms identified by DGGE would have conclusively proved that these organisms were in fact the dominant colonisers of surfaces in the environments. Also, as discussed in the Introduction, the minor microbial members of a habitat may not be detected by DGGE. The use of DGGE in microbial ecological studies has recently been reviewed by Ercolini (2004) and has defined the detection limits of DGGE-PCR as approximately 10^4-10^8 cfu/ml (dependent on the environment under investigation, DNA extraction and PCR methods used). This project was aimed at targeting the dominant members of the hot pool environment; therefore the detection limits of DGGE may not have posed a problem for this study. However, further work could include investigating the minor microbial members of the community. This could involve the production of 16S rDNA clone libraries which would provide a greater and more detailed perspective of these communities.

The microbial colonisers of slides incubated in NZ hot pools were closely related to cultured and uncultured (hyper)thermophiles reported with other overseas thermal habitats, with the exception of superficially similar hot pools in YNP, which possessed different colonising organisms. As other studies have established, bacterial signatures dominated the microbial communities of colonised slides in the high-temperature pools of YNP; whereas the NZ pools were more typical, with archaea being dominant and bacteria absent. To the best of our knowledge this is the first reported molecular-based study of New Zealand's high-temperature hot pools and the first to provide the phylogenetic identity of the microbes within these pools. A worldwide perspective of colonisation in high-temperature (>80°C) hot pools cannot be made, as colonisation of surfaces in NZ hot pools can only be compared to the analogous YNP study. However, further work is currently being conducted by Prof. Hugh Morgan involving the application of the same methods used in this study to follow colonisation of slides incubated in hot pools of Iceland, Fiji and Japan and should provide a greater international perspective of colonisation of surfaces in hot springs.

Standard media and enrichment protocols were used to attempt the isolation of type strains whose molecular signatures had been detected on colonised slides incubated in the NZ pools, but generally did not yield the cultures expected. While some of the interesting colonising organisms remain to be cultured, unexpectedly the enrichment methods resulted in the isolation of a pure culture of a novel clade of crenarchaeaota, *Ignisphaeara* sp., (Chapter 5) which on characterisation were only distantly related to other hyperthermophiles. Although *Ignisphaera* sp. was widely dispersed in NZ hot pools, it was only detected by culturing and not by molecular methods from either pool water or colonised slides. Therefore, *Ignisphaera* sp. may exist at population densities below those detected by molecular methodology. Although this might diminish its importance in the overall functioning of the ecosystem it nevertheless demonstrates the power of culture-based approaches to enrich for the minority members of a community. Intriguingly, *Ignisphaera* sp. could not be enriched from YNP hot pools and therefore, may be endemic to New Zealand's hot pools, although, further investigation of other overseas thermal habitats is required to substantiate this. Also, of interest is the *Aeropyrum*-related coloniser of slides incubated in NZ pools with a pH >7.0. The presence of this organism was totally unexpected as it is most closely related to marine-associated microorganisms and it seems to be a novel organism only distantly related to organisms of other terrestrial hot pools. Therefore, further in-depth enrichment efforts may be justified to isolate this organism.

A further point of difference between pools in NZ and YNP was the apparently ubiquitous presence of nanoarchaea in the former and absence in the latter. An apparently pure culture of *Pyrobaculum* (strain AQ1.S2) isolated from pool AQ1 also gave a positive indication of nanoarchaeal presence. At this time some care needs to be exercised in the interpretation of such results. Although nanoarchaea are implicated there is no further supporting evidence in the form of microscopic observation or isolation of putative nanoarcheal cells. If time had permitted, the application of a fluorescently labelled nanoarchaea-specific oligonucleotide probe would have conclusively proven the presence of nanoarchaea within the *Pyrobaculum* culture and justified further work into the characterisation of this probable consortium. The high likelihood that *Pyrobaculum* sp. are a host of nanoarchaea and the ubiquitous distribution of *Pyrobaculum* (as detected by DGGE and culturing) within the pools (83-100°C, pH 6-9) may explain the presence of nanoarchaea within the NZ hot pools and the apparent lack of nanoarchaea within the YNP hot pools. Therefore, the distribution of nanoarchaea might simply reflect the distribution of infected *Pyrobaculum* cells. The direction of future work could be to confirm the association between these two microorganisms by two different approaches. Firstly, by screening NZ hot pools that are outside the temperature and pH growth requirements for *Pyrobaculum* for the presence of nanoarchaea. Secondly the ratio of nanoarchaeal 16S genes to the host 16S genes of *Pyrobaculum* can be obtained by quantitative PCR to establish a "multiplicity of infection" which might be expected to be constant in *Pyrobaculum* isolates from different pools.

Finally, as outlined in Chapter 6, the goal of obtaining cultures of nanoarchaea from nascent hydrothermal vent samples was not achieved. However, the inability to isolate nanoarchaea using media previously used to isolate nanoarchaea from Icelandic hydrothermal vents may indicate the presence of nanoarchaea with differing types of metabolism. Together with the terrestrial NZ and YNP nanoarchaeal data, these results provide future directions for further work on the ecology of terrestrial and marine nanoarchaea. All enrichments from the East Pacific Rise have been retained and therefore, further work into the isolation and characterisation of organisms from these samples is possible.

Appendices

Appendix A – New Zealand hot pools.

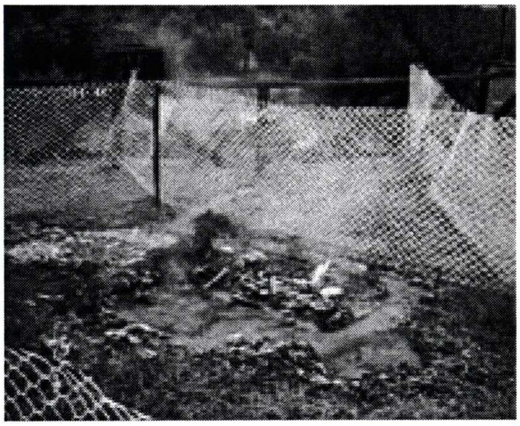
Table A. 1 Global positions of New Zealand hot pools.

Geographical area	Pool	GPS (NZMG*)	Latitude/Longitude
Rotorua, Kuirau Park - (near the Aquatic Centre).	AQ1 (close to wood platform)	E2794311	38° 07' 54" S
		N6336135	176° 14' 31" E
	AQ1 (left of wood platform)	E2794312	38° 07' 53" S
		N6336142	176° 14' 31" E
	AQ2	E2794308	38° 07' 54" S
		N6336127	176° 14' 31" E
	AQ3	E2794307	38° 07' 54" S
		N6336111	176° 14' 31" E
	AQ4	E2794311	38° 07' 54" S
		N6336112	176° 14' 31" E
	AQ5	E2794272	38° 07' 52" S
		N6336187	176° 14' 29" E
Rotorua, Kuirau Park.	KP1	E2794295	38° 07' 40" S
		N6336569	176° 14' 30" E
	KP2	E2794536	38° 08' 00" S
		N6335916	176° 14' 40" E
	KP3	E2794560	38° 07' 52" S
		N6336163	176° 14' 41" E
Rotorua, Sulfur Point.	SP1	E2795629	38° 09' 41" S
		N6332760	176° 15' 30" E
	SP2	E2796123	38° 08' 08" S
		N6335610	176° 15' 46" E
	SP3	E2796082	38° 08' 10" S
		N6335572	176° 15' 44" E
Rotorua, Ohinemutu.	OH1	E2794831	38° 07' 41" S
		N6336519	176° 14' 52" E
	OH2	E2794839	38° 07' 40" S
		N6336529	176° 14' 52" E
Rotorua, Whakarewarewa.	WH1	E2795678	38° 09' 43" S
		N6332712	176° 15' 32" E
	WH2	E2795705	38° 09' 39" S
		N6332838	176° 15' 33" E
Tokaanu Thermal Park.	TK2 (Tok2)	E2749591	38° 58' 04" S
		N6244781	175° 45' 52" E
	TK3 (Tok3)	E2749569	38° 58' 04" S
		N6244771	175° 45' 51" E
	Tok10A	E2749406	38° 58' 08" S
		N6244672	175° 45' 44" E
	Tok37 (mud pool)	E2749396	38° 58' 07" S
		N6244698	175° 45' 44" E
	Tok12	E2749582	38° 58' 02" S
		N6244834	175° 45' 52" E
	Tok13	E2749662	38° 58' 03" S
		N6244796	175° 45' 55" E

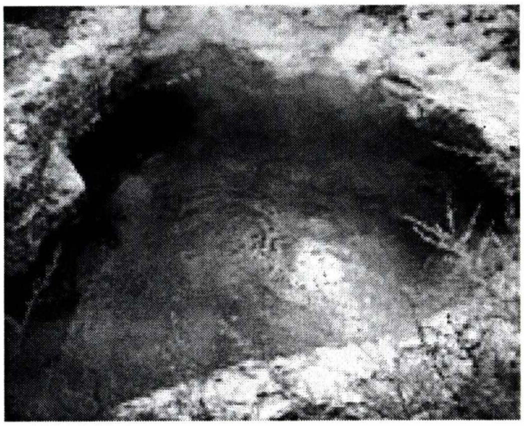
*New Zealand Map Grid, i.e. GPS output (Multinavigator, Silva Sweden, AB) used to convert to the New Zealand Geodetic Datum 2000 at www.linz.govt.nz (the final four digits were removed due to spurious results that were outside the accuracy of the GPS output).

AQ1

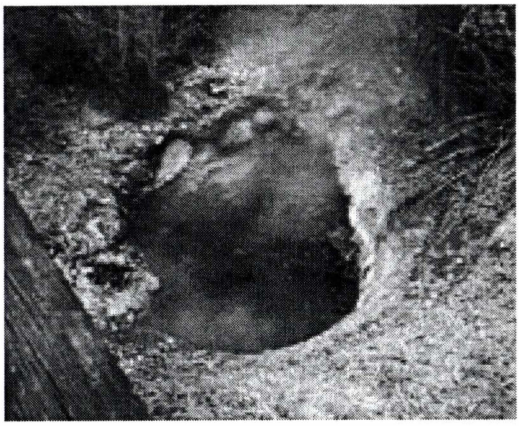
AQ2



AQ3



AQ4



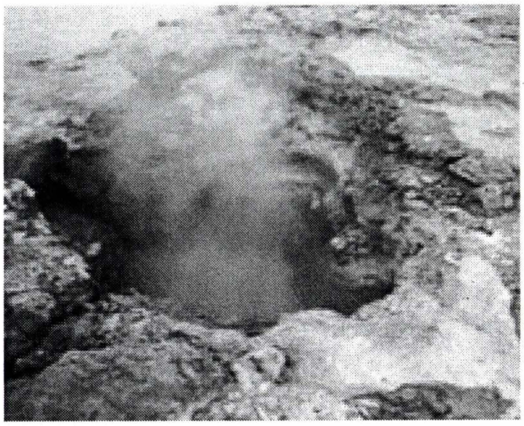
AQ5

KP1

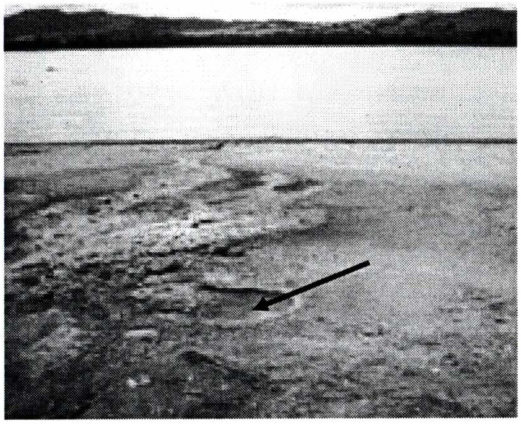
KP2

KP3

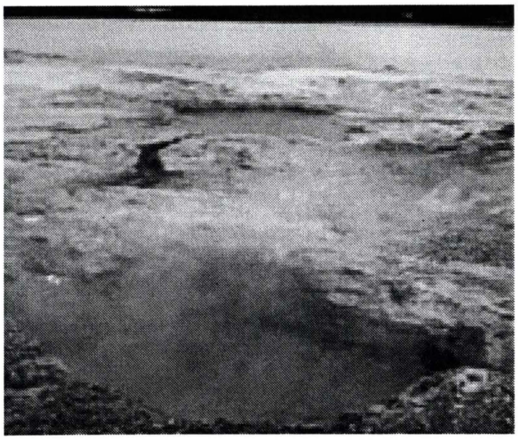
SP1



SP2

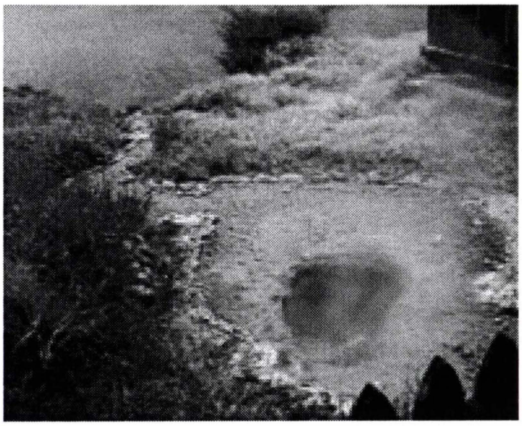


SP3 **(Pool in foreground)**



OH1 **(Drain out-flow, direction of arrow)**

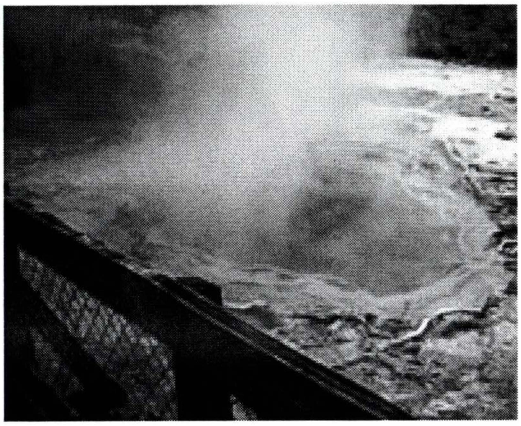
OH2



WH1

WH2

TK2 (Tok2)

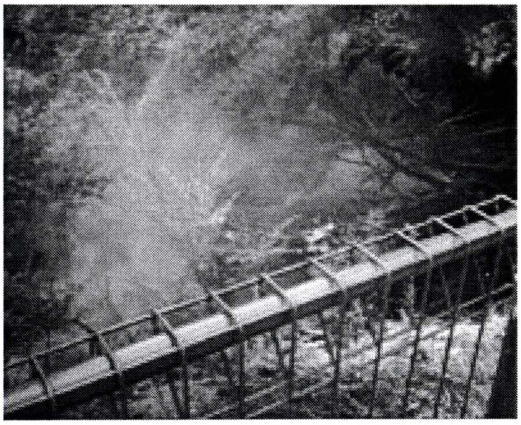


TK3 (Tok3)

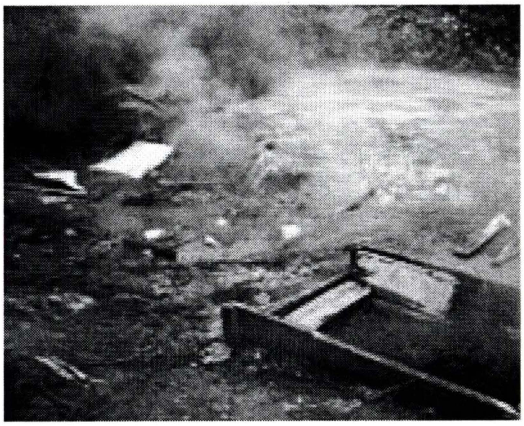
Tok10A

Tok37 (mud pool on right, indicated by arrow)

Tok12



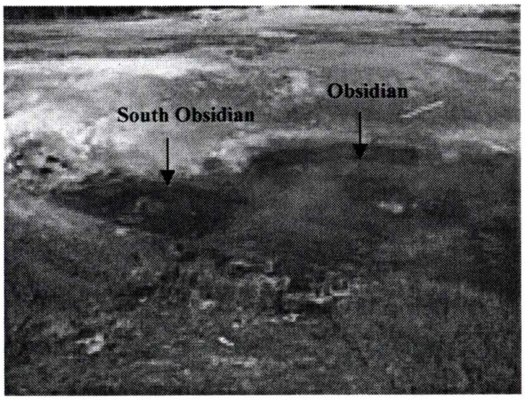
Tok13 (Hangi pit runoff)



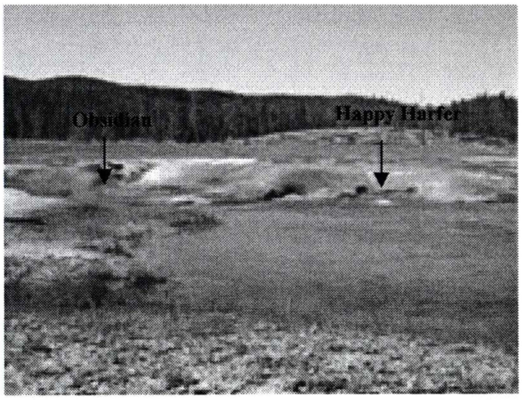
Champagne Pool

Appendix B – Yellowstone National Park hot pools.

South Obsidian Pool and Obsidian Pool



Happy Harfer



OB1 Heim

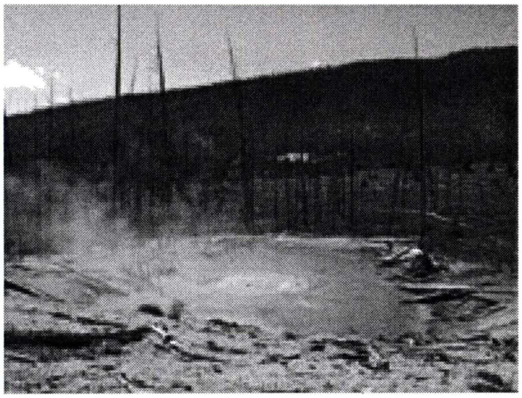
Steep Cone

Bison Pool

Evil Mouth

Boulder Spring

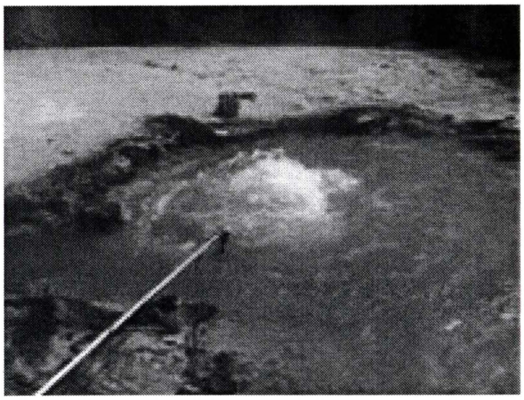
Sylvan Spring



Evening Primrose

Gunter's Fancy

Avocado Pool



Jan's Tide Pool

Frog Lips

Appendix C – DNA size standard marker

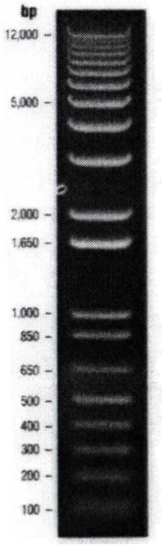


Figure C. 1 1 kb plus DNA ladder (Invitrogen, CA, USA).

Appendix D – 16S rRNA gene sequences

Chapter 3: DNA sequences

```
> ALA isolate from KP1
GGGGCTTGCCTTGATTCGGTCAGCGGCGGACGGGTGAGTAACACGTTGGCAACCTGCCCGCAAGACCGGGATAACTCCGGGAAACC
GGAGCTAATACCGGATAACACCGAAGACCGCATGGTCTTGGGTTGAAAGGCGGCTNTTGGCTGTCACTTGCGGATGGGCCCGCGGC
GCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGGCCTGAGAGGGTGACACGGCCACA
```

```
> Rod (Pyrobaculum) colonisation band
GGAAACCTCACCCGGGGCGACAGCAGGATGAAGGCCAGGCTAACGACCTTGCCGGACGAGCTGAGAGGAGGTGCATGGCCGTCGTC
AGCTCGTGCCGTGAGGTGTCCGGTTAAGTCCGGCAACGAGCGAGACCCCCGCCCCTAGTTGCTACCCCTCCCTACGGGGAGGGGGG
CACACTAGGGGGACTGCCGGCGTAAGCCGGAGGAAGGAGGGGGCCACGGCAGGTCAGTATGCCCCGAAACCCCGGGGCTGCACGCG
AGCTGCAATGGCGGGGACAGCGGGATCCGACCCCGAAAGGGGGAGGCAATCCCGTAAACCCCGCCCCAGTAGGGATCGAGGGCTGC
AACTCGCCCTCGTGAACGTGGAATCCCTAGTAACCG
```

```
> Coccal colonisation band
CGCCGGGAATCTCACCGGGGGCGACAGCAGGATGACGGCCAGGTTAAAGGCCTTGCCCGACGCGCTGAGGGGAGGTGCATGGCCGT
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GGGCACACTAGGGGGACTGCCGCCGCTAAGGCGGAGGAAGGAGGGGGCCACGGCAGGTCAGCATGCCCCTAATCCCCCGGGCTGCA
CGCGGGCTACAATGGCGGGGACAGCGGGTGCCGACCCCGAAAGGGGGAGGCAATCCCTCAAACCCCGCCGCAGTTGGGATCGAGGG
CTGCAACTCGCCCTCGTGAACGCGGAATCCCTAGTAACCGCGCGTCAACATCGCGCGGTGAATACGTCCCTGCTCC
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```
> OTU 1 (AQ1 pool water clone)
CCGGTTGATCCTGCCGGACCCGACCGCTATCGGGGTAGGGCTAAGCCATGCGAGTCGTGCGCCCGGGGGCGCCCGGGAGCGGCGCA
CGGCTCAGTAACACGTGCCTAACCTAACCTCGGGAGGGGGATACCCCCGGGAAACTGGGGTCAATCCCCCATAGGGGAAGGGCGCT
GGAAGGCCCCTTCCCCGAAAGGGCTGGCGGCCGATCCGCCGCCAGCCGCCCGAGGGTGGGGGCACGGCCCATCAGGTAGTTGGCGG
GTTAAAGGCCCGCCAAGCCGAAGACGGGTAGGGGCGGTGAGAGCCGTGAGCCCCGAGATGGGCACTGAGACAAGGGCCCAGGCCCT
ACGGGGCGCAGCAGGCGCGAATACTCCGCAATGCGGGCAACCGCGACGGGGCTACCCCGAGTGCCGGGCGAAGAGCCCGGCTTTTG
CCCGGTCTAAAAAGCCGGGAGAATAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGGTAATACCAGCTCCGCGAGTGGTCGGGGT
GTTTACTGGGCTTAAAGCGCCCGTAGCCGGCCCGGCAAGTCGCTCCTGAAATCCCCGGGCTCAACCCGGGGG
```

```
> OTU 2A (AQ1 pool water clone)
AAGGAGGTGATCCAGCCGCAGGTTCCCCTACGGCTACCTTGTTACGACTTCACCCCCCTTGGGAGCCCCCTGCTCGTCCCCCCACC
TTGCCGGGCAA
```

```
> OTU 2B (AQ1 pool water clone)
CCCGACTGCTATCGGGGTGAGGCTAAGCCATGGGAGTCGTACGCCCCGCCGCCGCGGGGCGTGGCGCACGGCTGAGTAACACGTGG
CTAACCTACCCTCGGGAGGGGGATAACACCGGGAAACTGGTGCTAATCCCCCATAGGGGAGGGCGCCTGGAAGGGTCCCTCCCCGA
AAGGGTACGGCGGGGGTTATCGCCGCCGTGCCGCCCGGGGATGGGGCTGCGGCCCATCAGGTAGTTGGCGGGGTAACGGCCCGCCA
AGCCGAAGACGGGTAGGGGCCGTGAGAGCGGGAGCCCCCAGATGGGCACTGAGACAAGGGCCCAGGCCCTACGGGGCGCACCAGGC
GCGAAACCTCCGCAATGCGGGAAACCGTGACGGGGTCACCCCGAGTGCCCCCGATAAGGGGGCTTTTCCCCGCTGTAAGAAGGCGG
GGAAATAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGGTAATACCAGCCCCGCGAG
```

> OTU 3 (AQ1 pool water clone)

GAGGTGATCCAGCCGCAGGTTCCCCTACGGCTACCTTGTTACGACTTCACCCCCCTTGGGAGCCCCCTGCTCGTCCCCCCACCTCG
GCGGCAAGGGGCCTCGCAGAGGGCT

> OTU 4 (AQ1 pool water clone)

GCCGCCCGAGGGTGGGGCTGCGGCCCATCATGGTAGTTGGCGGGGTAACGGCCCGCCAAGCCGACGACGGGTAGGGGCCGTGAGAG
CGGGAGCCCCCAGATGGGCCCTGAGACAAGGGCCCAGGCCCTACGGGGCGCACCAGGCGCGAAACCTCCGCAATGCGGGAAACCGT
GACGGGGTCACCCCGAGTGCCCCGAAGAGGGGCTGTTCCCCGCTGTAAAACGGCGGGGGAGTAAGCGGGGGGCAAGTCTGGTGTCA
GCCGCCGCGGTAATACCGAGCCCCGCGAGTGGTCGGGACGATTATTGGGCCTAAAGCGCCCGTAGCCGGCCCGACAAGTCCCCGCT
GAAATCCCCGGGCCCAACCCGGGGGCAGGCGGGGATACTGTCGGGCTAGGGGGCGGGAGAGGCCGAGGGTACTCCCGGGGTAGGGG

> OTU 6 (AQ1 pool water clone)

AAGGAGGTGATCCAGCCGCAGGTTCCCCTACGGCTACCTTGTTACGACTTCACCCCCCTTGGGAGCCCCCTGCTCG
CGAAATCCGATAATCCCGGGAGGACCACCAGTGGCG

> Nanoarchaeal sequence from AQ1 pool water (see Chapter 4 DNA sequences)

> AQ1.S1^{T} (*Ignisphaera aggregans*) [Accession no. DQ060321]

GGTTGATCCTGCCGGACCCGACCGCTATCGGGGTGGGGCTAAGCCATGGAAGTCGTACGCCCACCAAGTGGTGGGCGTGGCGGACG
GCTGAGTAACACGTGGCTAACCTACCCTCGGGACGGGGATAGCCCCGGGAAACTGGGGCTAATCCCCGATAGGTGGAGGGGCCTGG
AATGGTCCTCCACCGAAAGGGTCAGACCGCGATGAAGGTCTGACCGCCCGAGGATGGGGCTGCGGCCCATCATGGTTGTTGGCGGG
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GGGGTGCACCAGGCGCGAAACCTCCGCAATGCGGGAAACCTGTGACGGGGTCACCCCGAGTGCCCCGAAGAGGGGCTTTTCCCCGC
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GCTAGGGGGCGGGAGAGGCCGAGGGTACTCCCGGGGTAGGGGCGAAATCCGATAATCCCGGGAGGACCACCAGTGGCGAAGGCGCT
CGGCTGGAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGCGAACCGGATTAGATACCCGGGTAGTCCCGGCTGTAAACGATG
CGGGCTAGGTGTTGGACGGGCTTTGAGCCCGTCCAGTGCCGCAGGGAAGCCGTTAAGCCCGCCGCCTGGGGAGTACGGCCGCAAGG
CTGAAACTTAAAGGAATTGGCGGGGGAGCACCACAAGGGGTGGAGCCTGCGGCTTAATTGGAGTCAACGCCGGGAATCTTACCGGG
GGCGACAGCAGGATGACGGCCAGGCTAACGACCTTGCCCGACACGCTGAGAGGAGGTGCATGGCCGTCGCCAGCTCGTGCCGTGAG
GCGTCCGGTTAAGTCCGGCAACGAGCGAGATCCCCGCCCCCAGTTGCTACCCCGACCTCCGGGTCGGGGCACACTGGGGGGACTG
CCGCCGATAAGGCGGAGGAAGGAGGGGGCCACGGCAGGTCAGCATGCCCCGAATCCCCCGGGCTGCACGCGGGCTACAATGGCGGG
GACAGCGGGTACCGACCCCGAAAGGGGGAGGTAATCCCTGAAACCCCGCCGTAGTTGGGATCGAGGGTTGCAACTCACCCTCGTGA
ACGTGGAATCCCTAGTAACCGCGCGTCAACATCGCGCGGTGAATACGTCCCTGCTCCTTGCACACACCGCCCGTCGCTCCACCCGA
GCGGGGGAGAGGTGAGGCCTGCTCCATCCGGGGTTATCCCGGGTGGGGTGGGTCGAACCTCTCCTCCGCAAGGGGGGAGAAGTCGT
AACAAGGTAGCCGTACCGGAAGGTGCGGC

> AQ1.S2 (*Pyrobaculum* sp.)

CCGACCGCTATCGGGGTAGGGCTAAGCCATGCGAGTCGCGCGCCCGGGGGCGCCCGGGAGCGGCGCACGGCTCAGTAACACGTGCC
TAACCTAACCTCGGGAGGGGACACCACCGGGAAACTGGGGTCAATCCCCCATAGNGGAAGGGCGCTGGAAGGCCCTTCCCCGAAAG
GGCTGGCGACCGATCCNCCGCCAGCCGNCCGAGGGTGGGGGCACGGCCCATCAGGTAGTTGGCGGGTTAAAGGCCCGCCAAGCCGA
ANACGGGTAGGGGCGGTGAGAGCCGTGAGCCCCGAGATGGGCACTGAGACAAGGGCCCAGGCCCTACGGGGTGCAGCAGGCGCGAA
TACTCCGCAATGCGGGCAACCGCGACGGGGTCTACCCGAGTGCGGGCGAAGAGCCCGGCTTTTGCCCGGTCTAAAAAGCCGGGAGA
ATAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGGTAATACCAGCTCCGCGAGTGGTCGGGGTGTTTACTGGGCTTAAAGCGCCC
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GCCGCCGGTACTCCGGGGGTAGGGGCGAAATCCGTTAATCCCCGGAGGACCACCAGTGGCGAAAGCGGGCGGCCAGAACGCGCCCG
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CCCCTCGCCGCAAGGTGGGGGGACGAGCAGG
```

Chapter 4: DNA sequences

```
> OH1 glass slide bacterial DGGE band
ACTGANNTGGCATGGGCGAACCTGACCAGCGACGCTGCGTGCAGGACGAAGCCTTCGGGGTGTAACNCCTTTTGTCGAGGACGAAC
TCCTACACGTGTACTCCCCGCAATTACCCCCGGCTTACTCTCTTCCCACCACCCCCGTTAT

> AQ3 glass slide bacterial DGGE band
GATGGGCATGGGCGAAAGCTGACCAGCGACGCCGCGTGGGGGAAGAAGGCTTCGGGTCGTAACCCTGTTCTGGGGGAAGAACAGGC
CTGGGTTATAGCCCGGGTCTGCTGACGGTACCCCAGGAGAAAGCCACGGCTAACTGCTGCCAGCAGCCGCGG

> TK3 glass slide bacterial DGGE band
ACCGTACCGCGACGCTTCGTCGGGAAGAAGCCTTCGGGGTGTAACCGCTTTTGTGGGGGACGAAGTCCTGACGGTACCCCACGAAT
AAGCCACGGCTAACCTCGTGCCAGCAGCCGCGTAATA

> WH2 pool water bacterial DGGE band
CCCGCGACGCCGCGTATGGGAAGAAGCCCTGCGGTGGTTAAACCCCTGTCGTGTGGGGGACGAAGCGGCTATTGGTTAATAGCCCA
TAGTCGTGACGGTACCCCCAGATGAATGGACGTGCTAACTACGTAGCCAGCAGCCGCGGTAATACG

> TK2 pool water bacterial DGGE band
CCCGCGACGCTGCGTGCGGGAAGAAGCCTTCGGGGTGTAAACC

> Steep Cone slide bacterial DGGE band
TGGGGATGAAGCCCNTCGGGGTGTAAACCCCNTCGGGGGGGACGNTGTGTCCTGGGGTGNATAATCCCAGGGCATGACGGTACCCC
CAGAGG

> Sylvan Spring slide bacterial DGGE band
CGAAAGGCTGACCAGCGANTCCGCGTGGTGGATGAAGCCCTTCGGGGTGTAAACACCTTTTCTGGGGGAAGATAATGACGGTACCC
CAGGAATAAGGGACGGCTAACTACGTGCCAGCCGCCGCGGTAATACG

> Obsidian Pool slide bacterial DGGE band
TCCGCGTGGNGGANGAAGCCCNTCGGGGTGTAAACTCCTGTTCCAGTGGGGAAGCATCGTGACAGGGCACT

> SP1 slide archaeal DGGE band
CTTGCGGTTTATTGGAGTCACGCCGGAAACTTACCGGGGGCGACAGCAGGATGAAGGCCAGGCTGACGACCTTGCCAGACGACCTG
AGAGGAGGTGCATGGCCGTCGCCGGCTCGTGCCGTGAGGTGTCCTGTTAAGTTCAGGAACGAGCGAGACCCCCACCCCTAGTTGCT
ACCCGGCCCTTCGGGGCCGGGGCACTCTAGGGGGACTGCCGGCGATAAGCCGGAGGAAGGTGGGGGCTACGGCAGGTCAGTATGCC
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TCCCTGCTCCTTGCA
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> KP3 slide archaeal DGGE band
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ACCCCTAGTTGGTACCCCGGTCTCCGGACCGGGGCCACACTAGGGGGACTGCCGCCGTAAGGCGGAGGAAGGAGGGGGCCACGGCA
GGTCAGCATGCCCCGAAACCCCCGGGCTGCACGCGGGTTACAATGGCAGGGACAGCGGGAGGCTGACCCCGAAAGGGGGAGCCAAT
CCCCAAACCCTGCCGCAGTTGGGATCGAGGGCTGCAACTCGCCCTCGTGAACGAGGAATCCCTAGTAACCGCGGGTCAACAACCCG
CGGTGAATACGTC

> AQ1 slide archaeal DGGE band
CGGCTTATTGGAGTCAACGTCCGGAAACTCACCCGGGGCGACAGCAGGATGAAGGCCAGGCTAACGACCTTGCCGGACGAGCTGAG
AGGAGGTGCATGGCCGTCGTCAGCTCGTGCCGTGAGGTGTCCGGTTAAGTCCGGCAACGAGCGAGACCCCCGCCCCTAGTTGCTAC
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GAAACCCCGGGGCTGCACGCGAGCTGCAATGGCGGGGACAGCGGGATCCGACCCCGAAAGGGGGAGGCAATCCCGTAAACCCCGCC
CCAGTAGGGATCGAGGGCTGCAACTCGCCCTCGTGAACGTGGAATCCCTAGTAACCGCGTGTCACCAACGCGCGGTGAATACGTCC
CTGCT

> TK3 slide archaeal DGGE band
CTCACCCGGGGCGACAGCAGGATAAGGCCAGGCTAACGACCTTGCCGGACGAGCTGAGAGGAGGTGCATGGCCGTCGTCAGCTCGT
GCCGTGAGGTGTCCGGTTAAGTCCGGCAACGAGCGAGACCCCCACCCCTAGTTGCTACCCCTCCCTACGGGGAGGGGGGCACACTA
GGGGGACTGCCGGCGTAAGCCGGAGGAAGGAGGGGGCCACGGCAGGTCAGTATGCCCCGAAACCCCGGGGCTGCACGCGAGCTGCA
ATGGCGGGGACAGCGGGATCCGACCCCGAAAGGGGGAGGCAATCCCGTAAACCCCGCCCCAGTAGGGATCGAGGGCTGCAACTCGC
CCTCGTGAACGTGGAATCCCTAGTAACCGCGT

> TK2 slide archaeal DGGE band
AAGGCCTTGCCCGACGCGCTGAGGGGAGGTGCATGCCGTCGCCAGCTCGTGCCGTGAGGTGTCCTGTTAAGTCAGGCAACGAGCGA
GACCCCCGCCCCTAGTTGCTACCCCAGGGCGACCCTGGGGGCACACTAGGGGGACTGCCGCCGCTAAGGCGGAGGAAGGAGGGGGC
CACGGCAGGTCAGCATGCCCCTAATCCCCCGGGCTGCACGCGGGCTACAATGGCGGGGACAGCGGGTNCCGACCCCGAAAGGGGGA
GGCAATCCCTCAAACCCCGCCGCAGTTGGGATCGAGGGCTGCAACTCGCCCTCGTGAACGCGGAATCCCTAGTACCGCGCGTCAAC
ATCGCGCGGTGAATACGTCCCTGCTCCTTGCACACA

> OH2 slide archaeal DGGE band
CTCACCCGGGGCGACAGCAGGATAAGGCCAGGCTAACGACCTTGCCGGACGAGCTGAGAGGAGGTGCATGCCGTCGTCAGCTCGTG
CCGTGAGGTGTCCGGTTAAGTCCGGCAACGAGCGAGACCCCCGCCCCTAGTTGCTACCCCTCCCTACGGGGAGGGGGGCACACTAG
GGGGACTGCCGGCGTAAGCCGGAGGAAGGAGGGGGCCACGGCAGGTCAGTATGCCCCGAAACCCCGGGGCTGCACGCGAGCTGCAA
TGGCGGGACAGCGGGATCCGACCCCGAAAGGGGGAGGCAATCCCGTAAACCCCGCCCCAGTAGGGATCGAGGGCTGCAACTCGCCC
TCGTGAACGTGGAATCCCTAGTAACCGCGTGTCACCAACGCGCGGTGAATACGTCCCTGCTCCTTGCCGCAAAA

> AQ4 water archaeal DGGE band
ATTGGATTCATCGCCGGGAACTCACGGGGGCGACGGCAGGATAAGGCCAGGCTGAAGGTCTTGCCGGACACGCCTGAGAGGAGGTG
CATTGCCGCCGTCAGCTCGTACCGTGAGGCGTCACTTAAGTGTGGTAACGAGCGAGACCCGCGCCCCTAGTTGCCAGTCCTCCCTG
TTGGGGAGGAGGCACTCTGGGGGGACCGCCGGCGATAAGCCGGAGGAAGGAGCGGGCGACGGTAGGTCAGTATGCCCCGAAACCCC
CGGGNTACACGCGCGCTACAATGGGCGGGACAATGGGATCCGACCCCGAAAGGGGAAGGAAATCCCCTAAACCCGCCC

> AQ2 slide archaeal DGGE band
AGGGGAGGTGCATTGCCGTCGCCAGCTCGTGCCGTGAGGTGTCCTGTTAAGTCAGGCAACGAGCGAGACCCCCGCCCCTAGTTGCT
ACCCCAGGTGCGACCCTTGGGGCACACTAGGGGGACTGCCGCCGCTAAGGCGGAGGAAGGAGGGGGCCACGGCAGGTCAGCATGCC
CCTAATCCCCCGGGCTGCACGCGGGCTACAATGGCGGGGACAGCGGGTGCCGACCCCGAAAGGGGGAGGCAATCCCTCAAACCCCG
CCGCAGTTGGGATCGAGGGCTGCAACTCGCCCTCGTGAACGCGGAATCCCTAGTAACCGCGCGT
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> WH1 slide archaeal DGGE band
TAAGNCGGAGGAAGGAGGGGGCCACGGCAGGTCAGTATGCCCCTAANCCCCCGGGCTGCACGCGAGCTACAATGGCGGGGACAGCG
GGTTCCGACCCCGAAAGGGGGAGGCAATCCCTTAAACCCCGCCCCAGTTGGGATCGAGGGCTGCAACTCGCCCTCGTGAACGTGGA
ATCCCTAGTAACCGCGTGTCANCATCGCGCGGTGAATACGTCCCTGCTCCTTGCA

> SP2 water archaeal DGGE band
CGAGCGAGACCCGCGCCCCCAGTTGCCAGTCCTCCCCGTTGGGGAGGAGGCACTCTGGGGGGACCGCCGGCGATAAGCCGGAGGAA
GGAGCGGGCGACGGTAGGTCAGTATGCCCCGAAACCCCCGGGCTACACGCGCGCTACAATGGGCGGGACAATGGGATCCGACCCCG
AAAGGGGAAGGAAATCCCCTAAACCCGCCCCCAGTTCGGATTGCGGGCTGCAACTCGCCCGCATGAAGCTGGAATCCCTAGTACCC
GCGTGTCATCATCGCGCGGCGAATAC

> Obsidian Pool slide archaeal DGGE band
GAAGCTTACGGGGGAGACGCAGGATTGACGGCCAGGCTAATGACCTTGCCTGACTCGCGGAGAGGAGTGTGCATGGCCGTCGCCAG
TTCGTGTTGTGAAATGTCCGGTTAAGTCCGGCAACGAGCGAGACCCCCACCCCTAGTTGGTACTCTGGCCTCCGGGCCAGTGGCCA
CACTAGAGGGACTGCCGCCGTAAGGCGGAGGAAGGAGGGGGCCACGGCAGGTCAGCATGCCCCGAAACCCCCGGGCCGCACGCGGG
TTACAATGGCAGGACAGCGGGATCCTGCCCCGAGAGGGGTGGGCAATCCCTAAACCCTGCCTCAGTTGGGATCGAGGGCTGCAACT
CGCCCTCGTGAACGAGGAATCCCTAGTAACCGCAGGTCACCAACCTGCG

> South Obsidian Pool water slide archaeal DGGE band
GGCTAATGACCTTGCCTGACTCGCGGAGAGGAGGTGCATGGCCGTCGCCAGTTCGTGTTGTGAAATGTCCGGTTAAGTCCGGCAAC
GAGCGAGACCCCCACCCCTAGTTGGTACTCTGGCCTCCGGGACCAGGCCACACTAGAGGGACTGCCGCCGTAAGGCGGAGGAAGGA
GGGGGCCACGGCAGGTCAGCATGCCCCGAAACCCCCGGGCCGCACGCGGGTTACAATGGCAGGGACAGCGGGATCCTGCCCCGAGA
GGGGTGGGCAATCCCTAAACCCTGCCTCAGTTGGGATCGAGGGCTGCAACTCGCCCTCGTGAACGAGGAATCCCTAGTAACCGCAG
GTCACC

> Sylvan Spring slide archaeal DGGE band
CTCACCGGGGGCGACAGCAGGATTACGGCCAGGCTAATGACCTTGCCCGACGCGCTGAGAGGAGGTGCATGGCCGTCGCCAGCTCG
TGCTGTGAAGTGTCCTGGTTAAGTCCGGAAACGAGCGAGACCCCTGCCCCTAGTTGCCACCCGGGGCTCCGGCCTCGGGGCACACT
AGGGGGACTGCCGCCGTTTAAGGCGGAGGAAGGAGGGGGCCACGGCAGGTCAGCATAGGGGCTTCTCTTATGAGAGCCCCGCTGAG
GCCCCGAAACCCCCGGGCTACACGCGGGCTACAATGGCGGGGACAGCGGGATCCGAACCCGAAAGGGGGAGGTAATCCCTCAAACC
CCGCCGTAGTTGGGATCGAGGGCTGCAAATCGCCCTCGTGAACGCGGAATCCC

> OB1 Heim pool water archaeal DGGE band
CCATATGGTGAGCTGCGGCTTATTGCAGTCACTNCAGGAACTTACCGGGGGCGACAGCAGGATTACGGCCAGGCTAATGACCTTGC
CTGACGCGCTGAGAGGAGTGTGCACGGCCGTCGCCAGCTCGTGCCGTGAGGTGTCTGGTTAAGTCCAGCAACGAGCGAGACCCCTG
CCCCTAATTGCTACCTGCTCCTTCGGGAGTAGGGCACATTAGGGGGACTGCCGGCGTCTAAGCCGGAGGAAGGTGGGGGCCACGGC
AGGTCAGCATGCCCCGAAACCCCCGGGCTGCACGCGGGCTACAATGGCTGGGACAGCGGGAACCGACCTCGAAAGAGGAAGGTAAT
CCCTCAAACCCAGCCGCAGTTGGGATCGAGGACTGAAACCCGTCCTCGTGAACGAGGAATCCCTAGTAACCGCGCGTCATCATCGC
GCGGTGAATACGTCCCTGC

> Frog Lips pool water archaeal DGGE band
ACGAGCTGAGAGGAGGTGCATGGCCGTCGCCAGCTCGTGCCGTGAGGTGTCCTGTTAAGTCA

> Nanoarchaeal sequence from AQ1 pool water
GAGTAACACGTCGGCAACTACCCTCGGGAGGGGGATAACCCCGGGAAACTGGGGATAAACCCCCATAGGAGATGGGTGGTGGAATT
CCCCCATCTCCGAAAGGGAGTGGGGATAATCCNTCCCCACTCCGCCCGAGGATGGGCCGGCGGCCTATCATGGTAGTTGGCGGGGT
AACGGCCCGCCAAGCCGAAGACGGGTAGGGGCCGTGAGAGCGGGAGCCCCCAGATGGGCACTGAGACAAGGGCCCAGGCCCTACGG
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GGCGCACCAGGGGGCGAAACCTCCGCAATGGGTATATGCCTGACGGGGGACGGGAGTGCCCAGGGGTTTCCCCTGGGTTTTCGGG
AATTTTAAAAATCC

> Bacterial DGGE from White Island mixed culture
CCGCGTGAGCGAAGAAGGCCTTCGGGTTGTAAAGCTCTGTTGCCCGGGGCGAGCGGCATGGGGAGTGGAAAGCCCCATGCGAGACG
GTACCGGGTGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAA

> Archaeal DGGE band from White Island slide 1
CCAGGCTAACGACCTTGCCTGACTCGCGGAGAGGAGGTGCATGGCCGTCGCCAGCTCGTGTTGTGAAATGTCCTGTTAAGTCAGGC
AACGAGCGAGACCCCCACCCCTAGTTGGTACCCCGGTCTCCGGACCGGGGCCACACTAGGGGGACTGCCGCCGTAAGGCGGAGGAA
GGAGGGGGCCACGGCAGGTCAGCATGCCCCGAAACCCCCGGGCTGCACGCGGGTTACAATGGCAGGGACAGCGGGAGGCGACCCCG
AAAGGGGGAGCCAATCCCTAAACCCTGCCGCAGTTGGGATCGAGGGCTGCAACTCGCCCTCGTGAACGAGGAATCCCTAGTAACCG
CGGGTCAACAACCCGCGGTGAATACGTCC

> Archaeal DGGE band from White Island slide 3
TGCCAGGCTAACGACCTTGCCTGACTCGCGGAGAGGAGGTGCATGGCCGTCGCCAGCTCGTGTTGTGAAATGTCCTGTTAAGTCAG
GCAACGAGCGAGACCCCCACTCCTAGTTGGTATCACGGTCTCCGGACCGTGACCACACTAGGAGGACTGCCGCCGTAAGGCGGAGG
AAGGAGGGGGCCACGGCAGGTCAGCATGCCCCGAAACCCCCGGGCCGCACGCGGGTTACAATGGCAGGGACAGCGGGATGCTACCC
CGAAAGGGGGAGCCAATCCCTAAACCCTGCCGCAGTTGGGATCGAGGGCTGAAACCCGCCCTCGTGAACGAGGAATCCCTAGTAAC
CGCGGGTCAACAACCCGCGGTGAATACGTCCCTGCT

Chapter 5: DNA sequences

> AQ1.S1^{T} (*Ignisphaera aggregans*) [Accession no. DQ060321]
(See the Chapter 3 DNA sequences)

> Tok10A.S1 (*Ignisphaera* sp.) [Accession no. DQ060322]
CCACCAAGTGGTGGGCGTGGCTGACGGCTGAGTAACACGTGGCTAACCTACCCTCGGGACGGGGATAGCCCCGGGAAACTGGGGCT
AATCCCCGATAGGTGGAGGGGCCTGGAACGGTCCTCCACCGAAAGGGTCAGACCGCGATGAAGGTCTGATCGCCCGAGGATGGGGC
TGCGGCCCATCATGGTAGTTGGCGGGGTAACGGCCCGCCAAGCCGATAACGGGTAGGGGCCGTGAGAGCGGGAGCCCCCAGATGGG
CACTGAGACAAGGGCCCAGGCCCTACGGGGTGCACCAGGCGCGAAACCTCCGCAATGCGGGAAACCGTGACGGGGTCACCCCGAGT
GCCCCGAAGAGGGGCTTTTCCCCGCTGTAAGTAGGCGGGGGAATAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGGTAATACCA
GCCCCGCGAGTGGTCGGGACGTTTACTGGGCCTAAAGCGCCCGTAGCCGGCCCAGTAAGTCCCCACAGAAATCCTCGGGCTCAACC
CGAGGGCCTGTGGGGATACTGCTGGGCTAGGGGGCGGGAGAGGCCGAGGGTACTCCCGGGGTAGGGGCGAAATCCGATAATCCCGG
GAGGACCACCAGTGGCGAAGGCGCTCGGCTGGAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGCGAACCGGATTAGATACC
CGGGTAGTCCCGGCTGTAAACGATGCGGGCTAGGTGTTGGACGGGCTTTGAGCCCGTCCAGTGCCGCAGGGAAGCCGTTAAGCCCG
CCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCGGGGGAGCACCACAAGGGGTGGAGCCTGCGGCTTAATTGG
AGTCAACGCCGGGAATCTTACCGGGGGCGACAGCAGGATGACGGCCAGGTTAACGACCTTGCCCGACACGCTGAGAGGAGGTGCAT
GGCCGTCGCCAGCTCGTGCCGTGAGGCGTCCGGTTAAGTCCGGCAACGAGCGAGATCCCCGCCCCCAGTTGCTACCCCGACCTCCG
GGTCGGGGGCACACTGGGGGGACTGCCGCCGANAAGGCGGAGGAAGGAGGGGGCCACGGCAGGTCAGCATGCCCCGAATCCCCCGG
GCTGCACGCGGGCTACAATGGCGGGGACAGCGGGTACCGACCCCGAAAGGGGGAGGCAATCCCTGAAACCCCGCCGTGGTTGGGAT
CGAGGGTTGCAACTCACCCTCGTGAACGTGGAATCCCTAGTAACCGCGCGTCAACATCGCGCGGTGAATACGTCCCTGCTCCTTGC
ACACACCGCCCGTCGCTCCACCCGAGCGGGGGAGAGGTGAGGCCTGCTCTACCCGGGGGTTAACCCCGGGCGGGGTGGGTCGAACC
TCTCCTCCGCAAGGGGGGAGAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGC

> Tok37.S1 (*Ignisphaera* sp.) [Accession no. DQ060323]

GGTGGGCGTGGCGGACGGCTTAATAACACGTGGCTAACCTACCCTCGGGACGGGGATAGCCCCGGGAAACTGGGGCTAATCCCCGA
TAGGTGGAGGGGCCTGGAATGGTCCTCCACCGAAAGGGTCAGACCGCGATGAAGGTCTGACCGCCCGAGGATGGGGCTGCGGCCCA
TCATGGTATGTTGGCGGGGTAACGGCCCGCCAAGCCGATAACGGGTAGGGGCCGTGAGAGCGGGAGCCCCCAGATGGGCACTGAGA
CAAGGGCCCACGCCCTACGGGGTGCACCAGGCGCGAAACCTCCGCAATGCGGGAAACCTGTGACGGGGTCACCCCGAGTGCCCCGA
AGAGGGGCTTTTCCCCGCTGTAAGTAGGCGGGGGAATAAGCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGGTAATACCAGCCCCGC
GAGTGGTCGGGACGTTTACTGGGCCTAAAGCGCCCGTAGCCGGCCCAGTAAGTCCCCACAGAAATCCTCGGGCTCAACCCGAGGGC
CTGTGGGGATACTGCTGGGCTAGGGGGCGGGAGAGGCCGAGGGTACTCCCGGGGTAGGGGCGAAATCCGATAATCCCGGGAGGACC
ACCAGTGGCGAAGGCGCTCGGCTGGAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAGCGAACCGGATTAGATACCCGGGTAG
TCCCGGCTGTAAACGATGCGGGCTAGGTGTTGGACGGGCTTTGAGCCCGTCCAGTGCCGCAGGGAAGCCGTTAAGCCCGCCGCCTG
GGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCGGGGGAGCACCACAAGGGGTGGAGCCTGCGGCTTAATTGGAGTCAAC
GCCGGGAATCTACCGGGGGCGACAGCAGGATGACGGCCAGGCTAACGACCTTGCCCGACACGCTGAGAGGAGGTGCATGGCCGTCG
CCAGTCGTGCCGTGAGGCGTCCGGTTAAGTCCGGCAACGAGCGAGATCCCCGCCCCCAGTTGCTACCCCGACCTCCGGGTCGGGGG
CACACTGGGGGGACTGCCGCCGATAAGGCGGAGGAAGGAGGGGGCCACGGCAGGTCAGCATGCCCCGAATCCCCCGGGCTGCACGC
GGGCTACAATGGCGGGGACAGCGGGTACCGACCCCGAAAGGGGGAGGTAATCCCTGAAACCCCGCCGTAGTTGGGATCGAGGGTTG
CAACTCACCCTCGTGAACGTGGAATCCCTAGTAACCGCGCGTCAACATCGCGCGGTGAATACGTCCCTGCTCCTTGCACACACCGC
CCGTCGCTCCACCCGAGCGGGGGAGAGGTGAGGCCTGCTCCATCCGGGGTTACCCCGGGTGGGGTGGGTCGAACCTCTCCTCCGCA
AGGGGGGAGAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGC

> Tok1 (*Ignisphaera* sp.) [Accession no. DQ060320]

CCATGGAGGTCGTACGCCCACCAAGTGGTGGGCGTGGCGGACGGCTGAGTAACACGTGGCTAACCTACCCTCGGGACGGGGATAGC
CCCGGGAAACTGGGGCTAATCCCCGATAGGTGGAGGGGCCTGGAACGGTCCTCCACCGAAAGGGTCAGACCGCGATGAAGGTCTGA
CCGCCCGAGGATGGGGCTGCGGCCCATCATGGTAGTTGGCGGGGTAACGGCCCGCCAAGCCGATAACGGGTAGGGGCCGTGAGAGC
GGGAGCCCCCAGATGGGCACTGAGACAAGGGCCCAGGCCCTACGGGGTGCACCAGGCGCGAAACCTCCGCAATGCGGGAAACCGTG
ACGGGGTCACCCCGAGTGCCCCGAAGAGGGGCTTTTCCCCGCTGTAAGTAGGCGGGGGAATAAGCGGGGGGCAAGTCTGGTGTCAG
CCGCCGCGGTAATACCAGCCCCGCGAGTGGTCGGGACGTTTACTGGGCCTAAAGCGCCCGTAGCCCGGCCCAGTAAGTCCCCACAG
AAATCCTCGGGCTCAACCCGAGGGCCTGTGGGGATACTGCTGGGCTAGGGGGCGGGAGAGGCCGAGGGTACTCCCGGGGTAGGGGC
GAAATCCGATAATCCCGGGAGGACCACCAGTGGCGAAGGCGCTCGGCTGGAACGCGCCCGACGGTGAGGGGCGAAAGCCGGGGGAG
CGAACCGGATTAGATACCCGGGTAGTCCCGGCTGTAAACGATGCGGGCTAGGTGTTGGACGGGCTTTGAGCCCGTCCAGTGCCGCA
GGGAAGCCGTTAAGCCCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCGGGGGAGCACCACAAGGGGTGG
AGCCTGCGGCTTAATTGGAGTCAACGCCGGGAATCTTACCGGGGGCGACAGCAGGATGATGGCCAGGCTAACGACCTTGCCCGACA
CGCTGAGAGGAGGTGCATGGCCGTCGCCAGCTCGTGCCGTGAGGCGTCCGGTTAAGTCCGGCAACGAGCGAGATCCCTGCCCCCAG
TTGCTACCCCGACCTTCGGGTCGGGGGCACACTGGGGGGACTGCCCGCCGATAAGGCGGANGAAGGAGGGGGCCACGGCANGTCAG
CATGCCCCGAATCCCCCGGGCT GCACGCGGGCTNCAATNGCGGGGACAAGNGGNTCCGACCCCGAAAAGGGGGGAG

> TRUCC 95

CCGAGTGGTGGCCACTATTATTGGGCCTAAAGCGTCCGTAGCCGGGCCCGTAAGTCCCTGGCGAAATCTCACGGCTCAACCGTGGG
GCTCGCTGGGGATACTGCGGGCCTTGG

> TRUCC 269

CGGCGGCCCGAGTGGTGGCCACTATTATTGGGCCTAAAGCGTCCGTAGCCGGGCCCGTAAGTCCCTGGCGAAATCTCACGGCTCAA
CCGTGGGGCTCGCTGGGGATACTGCGGGCCTTGGGACCGGGAGAGGCGGAGGGTACCCCTGGGGTAGGGGTGAAATCCTATAATCC
CAGGGGGACCGCCAGTGGCGAAGGCGCTCCGCTGGAACGGGTCCGACGGTGAGGGACGAAGGCCAGGGGAGCAAACCGGATTAGAT
ACCCGGGTAGTCCTGGCTGTAAAGGATGCGGGCTAGGTGTCGGGTGAGCTTCGGGCTCGCCCGGTGCCGGAGGGAAGCCGTTAAGC
CCGCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTGGCGGGGGAGCACCACAAGGGGTGGAGCGTGCGGTTTAAT
TGGATTCAACGCCGGGAACCTCACCGGGGGCGACGGCAGGATGAAGGCCA

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> TRUCC 321
AATGCGGGAAACGTGACGGGGCCACCCCGAGTGCCCCCTCACGGGGGCTTTTCCCCGCTGTAGGAATGCGGGGGAATAAGCGGGGG
GCAAGTCTGGTGTCAGCCGCCGCGGTAATACCAGCCCCGCGAG

> TRUCC 322
ACGGGGCCACCCCGAGTGCCCCCTCACGGGGGCTTTTCCCCGCTGTAGGAATGCGGGGGAATAAGCGGGGG

> Tok37.S1 Top band
CCGCCGCCGCGGGGCGTGGCNGGACGGCTGTATTAACACGTGGCTAACCTACCCTCGGGAGGGGGATAACACCGGGAAACTGGTGC
TAATCCCCCATAGGGGAGGAGGCCTGGAAGGGTTCCTCCCCGAAAGGGTGTACAGGCCAACGCTGCTACACCGCCGAGGATGGGGC
TACGGCCCATTAGGTAGTTGGCGGGGTAACGGCCCGCCAAGCCGATAATGGGTAGGGGCCGTGAGAGCGGGAGCCCCCAGATGGGC
ACTGAGACAAGGGCCCAGGCCCTACGGGGCGCACCAGCGCGAAACTCCGCAAATGGGGTTCTCGGGGCACACTACCCCGAAGCCCC
AGCGGAATGCGGGAAACCGTGACGGGGCCACCCCGAGTGCCCCCTCACGGGGGCTTTTCCCCGCTGTAGGAATGCGGGGGAATAAC
GCGGGGGGCAAGTCTGGTGTCAGCCGCCGCGGTAATACCAGCCCCG

> Tok37.S1 (obtained by James Connell)
ACAAGGGGTTGGAGCTCGGTTCAATTGGAGTCAACGCCCGGGGAATCTCACCGGGGGAGACAGCAGGATGACGGCCAGGTTAAAGG
CCTTGCCTGACGCGCTGAGAGGAGGTGCATGGCCGTCGCCAGCTCGTGCTGTGAAGTGTCCGGTTAAGTCCGGAAAATCCCCGAGT
GGGGTAGTACATGGTTTACTTCTCCACCGGGGATCCGAACGAGCGAGACCCCCACCCCTAGTTGCTACCCGGGGCTATGGCTCCGG
GGCACATAGGGGGACGCCGCCGTTTAAGCGGAGGAAGGAGGGGGCCACGGCAGGTCAGCATGCCCCGAACCCCCCGGGCTACACGC
GGGCTACAATGGCGGGGACAGCGGGATCCGACCCCGAAAGGGGGAGGTAATCCCTCAAACCCCGCCGTGGTTGGAATCGAGGGCGG
CAACTCGCCCTCGTGAACGTAAAAATCCCCTNGAACCGCGCGTCAACATCGCGCGAAACC

> Ket55.S1
GACCACCACCCCTAGGCTACCCGGGGCTANGGCCCCGGGGCACACTAGGGGGACGCCGCCGTTAAGGCGGAGGAAGGAGGGGGCCA
CGGCAGGTCAGCATGCCCCAAACCCCCCGGGCTACACGCGGGCTACAATGGCGGGGACAGCGGGATCCGACCCCGAAAGGGGGAGG
TAATCCCTCAAACCCCGCCGTGGTTGGAACGAGGGCTGCAACTCGCCCATCGTGAACGTTAAATCCTNGTAAACCGCGCGTCACAT
CGCGCGTGATNC
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Chapter 6: DNA sequences

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> Rod isolate (obtained by Lisa Everett)
TGGTGGGGTAAAGGCCTACCAAGCCGACGAATCCATAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAG

> Bacterial DGGE band from Alvinella pompejana-associated consortium
CAGCCATNCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTGGTANTGTTAATAGCACTATCA
TTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA

> Archaeal DGGE band from chimney enrichment
GCGGTTTAATTGGATTCAACGCCGGGAACCTCACCGGGGGCGACGGCAGGATGAAGGCCAGGCTGAAGGTCTTGCCGGACGCGCCG
AGAGGAGGTGCATGGCCGCCGTCAGCTCGTACCGTGAGGCGTCCACTTAAGTGTGGTAACGAGCGAGACCCGCGCCCCCAGTTGCC
AGTCCTCCCCGCTGGGGAGGAGGCACTCTGGGGGGACCGCCNGGCGATNAAGCCGGAGGAAGGAGCGGGCGACGGTAGGTCAGTAT
GCCCCGAAACCCCCGGGCTACACGCGCGCTACAATGGGCGGGACAATGGGAACCGACCCCGAAAGGGGAAGGGAATCCCCTAAACC
CGCCCTCAGTTCGGATCGCGGGCTGCAACTCGCCCGCGTGAAGCTGGAATCCCTAGTACCCGCGTGTCATCATCGCGCGGCGAATA
CGTCCCTGCTCCTTGCAC
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Appendix E – Growth media

10% $Na_2S \cdot 9H_2O$

A 10% $Na_2S \cdot 9H_2O$ (w/v) solution was prepared, and the pH adjusted to ~ 7.0 with concentrated H_2SO_4 acid. This was undertaken inside a fume-hood due to the production of H_2S gas. The pH was checked using pH strips (Merck KGaA, Darmstadt, Germany) and the mixture dispensed into a serum bottle under N_2 gas and autoclaved. The stock solution was used to reduce anaerobic medium as required.

2/1 + C medium

	g/L
NaCl	0.9
$MgCl_2 \cdot 6H_2O$	0.2
K_2HPO_4	1.5
KH_2PO_4	0.75
NH_4Cl	0.9
Cysteine	0.75
Yeast extract	1.0
Tryptone	2.0
Cellobiose	1.2
Selenite/Tungstate	1.0 ml
SL 10 trace elements	1.0 ml
$FeCl_3$ (0.28 mg/ml)	1.0 ml
Wolin's vitamins*	0.1 ml
Resazurin (0.1%)	1.0 ml

The pH was adjusted as required and the medium was boiled and dispensed under a N_2 atmosphere, then autoclaved. *Added after autoclaving. Following autoclaving, the medium was reduced with 10% $Na_2S \cdot 9H_2O$ as required.

***Aeropyrum* medium (modified)**

	g/L
Trypticase peptone	5.0
Yeast extract	1.0
$Fe_{(III)}$ citrate	0.1
NaCl	0.25
$MgCl_2 \cdot 6H_2O$	0.59
$NaSO_4$	0.324
$CaCl_2 \cdot 2H_2O$	0.18
KCl	0.55
Na_2CO_3	0.16
KBr	0.08
$SrCl_2 \cdot 6H_2O$	34.0 mg
H_3BO_3	22.0 mg
Na-silicate	4.0 mg
NaF	2.4 mg
$(NH_4)_2NO_3$	1.6 mg
Na_2HPO_4	80.0 mg
Resazurin (0.1%)	1.0 ml

This medium was based on medium 820 (obtained from DSMZ) and was originally used to culture *Aeropyrum camini* and *Aeropyrum pernix* (Sako *et al.*, 1996). The medium was adjusted to pH 8.0, boiled, and dispensed under N_2 or N_2:CO_2 (80:20) gas. Following autoclaving, $Na_2S_2O_3 \cdot 5H_2O$ was added from a filter sterilised stock solution to a final concentration of 1.0 g/L. Medium was reduced with 10% $Na_2S \cdot 9H_2O$ as required.

AN1 medium

	g/L
KH_2PO_4	1.5
$MgCl_2 \cdot 6H_20$	0.3
NaCl	2.5

Wolin's vitamins*	1.0 ml
Zeikus trace elements	1.0 ml
Trypticase peptone	8.0
Na-thioglycollate	0.5
Sulfur	~ 2.0 (powder added to each individual vessel)

AN1 medium was orginially used to cultivate *Thermococcus zilligii*, strain AN1. Medium was adjusted to the required pH, boiled and dispensed under N_2 gas atmosphere. *Added following autoclaving to individual tubes.

Arsenic-based medium

	g/L
KCl	0.017
$MgCl_2 \cdot 6H_2O$	1.215
NH_4Cl	0.012
$CaCl_2 \cdot 2H_2O$	0.007
KH_2PO_4	0.007
NaCl	0.9
$NaHCO_3$	0.05
$CaCO_3$	0.05
$Fe(NH_4)(SO_4)_2 \cdot 6H_2O$	0.1
Zeikus trace elements	1.0 ml
Yeast extract	0.1
Trypticase peptone	0.1
$Na_2HAsO_4 \cdot 7H_2O$	10 mM
Resazurin (0.1%)	1.0 ml

The medium was adjusted to pH 6.5, boiled and dispensed under N_2. Following autoclaving, the medium was reduced with sterile 10% $Na_2S \cdot 9H_2O$ as required. This medium was based on the medium used for the isolation of *Pyrobaculum arsenaticum* (Huber *et al.*, 2000b).

Arsenic-based solid medium

	g/L
Yeast extract	2.0
Starch	2.0
$(NH_4)_2SO_4$	0.8
KH_2PO_4	0.4
$MgSO_4 \cdot 7H_2O$	0.18
NaCl	1.75
As_2O_3	0.075
Bacteriological agar	15.0

This growth medium was based on the medium used to test arsenic oxidation by *Thermus* species (Gihring *et al.*, 2001).

Arsenic medium

	g/L
NaCl	1.2
KCl	3.0
NH_4Cl	0.3
KH_2PO_4	0.2
Na_2SO_4	0.3
$MgCl_2 \cdot 6H_2O$	0.4
$CaCl_2 \cdot 2H_2O$	0.15
$NaHCO_3$	0.6
Zeikus trace elements	1.0 ml
Sodium arsenate	10 mM
Sodium acetate	10 mM
Yeast extract	0.1
Resazurin (0.1%)	1.0 ml

Medium was based on the medium used by Macy *et al.*, (2000).

Castenholtz medium (CMD)

	g/L
Nitrilotriacetic acid	0.1
$CaSO_4 \cdot 2H_2O$	0.06
$MgSO_4 \cdot 7H_2O$	0.1
NaCl	0.008
KNO_3	0.103
$NaNO_3$	0.689
$Na_2HPO_4 \cdot 2H_2O$	0.140
Nitsch's trace elements	10.0 ml
Trypticase peptone	1.0
Yeast extract	1.0

Medium was adjusted to the required pH and heated to dissolve. For solid medium, 15.0 g/L bacteriological agar was added.

Cryoprotectant

	g/L
Trypticase Peptone	10.0
NaCl	5.0
Beef extract	3.0
Yeast extract	5.0
Cysteine HCl	1.0
Glucose	1.0
Na_2HPO_4	4.0
Glycerol	150 ml

The pH was adjusted to 7.0 and the medium dispensed under N_2 and sterilised by autoclaving.

Db basal[1]

	g/L
$(NH_4)_2SO_4$	1.3
$CaCl_2 \cdot 2H_2O$	0.074
$MgSO_4 \cdot 7H_2O$	0.28
KH_2PO_4	0.28
Yeast extract	0.1
Trypticase peptone	0.2
Nitsch's trace elements	1.0 ml
$FeCl_3$ (0.28 g/L)	1.0 ml
Resazurin (0.1%)	1.0 ml
Bacteriological agar	15.0

Medium was adjusted to pH 7.0, boiled, and dispensed under N_2 gas. Medium was supplemented with compounds (0.5%) as stated in Chapter 3. Following autoclaving the medium was reduced with 10% $Na_2S \cdot 9H_2O$ as required.

Db basal[2]

	g/L
$(NH_4)_2SO_4$	1.3
$CaCl_2 \cdot 2H_2O$	0.074
$MgSO_4 \cdot 7H_2O$	0.28
KH_2PO_4	0.28
K_2HPO_4	1.22
Yeast extract	0.1
Trypticase peptone	0.1
Resazurin (0.1%)	1.0 ml
Nitsch's trace elements	1.0 ml
$FeCl_3$ (0.28 g/L)	1.0 ml

Medium was supplemented with 1% of carbon sources as described in Chapter 3. Gelrite medium was prepared as described in Section 2.3.3. Following autoclaving, the medium was reduced with 10% $Na_2S \cdot 9H_2O$ as required.

Db+S (Db medium with starch)

	g/L
$(NH_4)_2SO_4$	1.3
$CaCl_2 \cdot 2H_2O$	0.074
$MgSO_4 \cdot 7H_2O$	0.28
KH_2PO_4	0.28
Yeast extract	0.1
Trypticase peptone	2.0
L-cystine	0.6
Nitsch's trace elements	1.0 ml
$FeCl_3$ (0.28 g/L)	1.0 ml
Resazurin (0.1%)	1.0 ml
Soluble starch	2.0

The pH of the medium was adjusted to pH 7.0, boiled and dispensed under N_2 gas. Following autoclaving the medium was reduced with 10% $Na_2S \cdot 9H_2O$ as required. For salt water medium (Chapter 6), 500 ml of sea water was used for 1.0 L of medium and the pH adjusted to 6.0.

Db+Fe^{3+} citrate

Db+S medium was prepared with the soluble starch replaced with Fe^{3+} citrate at a 25 mM concentration.

Desulfovibrio hydrothermalis

	g/L
Solution A:	
Na_2SO_4	3.0

KH_2PO_4	0.2
NH_4Cl	0.3
NaCl	21.0
$MgCl_2 \cdot 6H_2O$	3.0
KCl	0.5
$CaCl_2 \cdot 2H_2O$	0.15
Resazurin (0.1%)	1.0 ml
Distilled water	930.0 ml

Solution B:

Trace elements SL-10	1.0 ml

Solution C:

$NaHCO_3$	2.5
Distilled water	50.0 ml

Solution D (a):

Na-L-lactate	10.0
Distilled water	25.0 ml

Solution D (b):

Na-acetate	0.5
Distilled water	20.0 ml

Solution E:

Wolin's vitamins	10.0 ml

Solution F:

$Na_2SeO_3 \cdot 5H_2O$ (3 mg in 1 L of 0.01 M NaOH)	1.0 ml

Solution G:

$Na_2S \cdot 9H_2O$	0.4
Distilled water	10.0 ml

Solution A was prepared anaerobically under $N_2:CO_2$ (80:20) and autoclaved. Solution C was filter-sterilised and gassed for 20 minutes with $N_2:CO_2$. Solutions B, D, E and F were filter-sterilised and gassed with N_2. Solution G was autoclaved under N_2. Solutions B to G were then added to the sterile, cooled solution A. The final pH of the medium was approximately 7.0.

Dictyoglomus medium

	g/L
KH_2PO_4	1.5
$Na_2HPO_4 \cdot 12H_2O$	4.2
NH_4Cl	0.5
$MgCl_2 \cdot 6H_2O$	0.38
$CaCl_2 \cdot 2H_2O$	0.05
$Fe(NH_4)(SO_4)_2 \cdot 6H_2O$	0.039
Na_2CO_3	1.0
Yeast extract	2.0
Trypticase peptone	2.0
Soluble starch	5.0
Cysteine	1.0
Resazurin (0.1%)	1.0 ml
Wolin's vitamins*	1.0 ml
Dictyoglomus trace elements	1.0 ml

The pH of the medium was adjusted, and the medium boiled and dispensed under N_2 atmosphere then autoclaved. *Added after autoclaving. Following autoclaving, the medium was reduced with 10% $Na_2S \cdot 9H_2O$ as required.

***Dictyoglomus* trace elements**

	mg/L
$CoCl_2·6H_2O$	2.9
$NaMoO_4·2H_2O$	2.4
Na_2SeO_3	0.17
$MnCl_2·4H_2O$	2.0
$ZnSO_4$	2.8

LB medium

	g/L
Trypticase peptone	10.0
Yeast extract	5.0
NaCl	5.0

For solid medium, 15 g/L bacteriological agar was added. The pH was adjusted to 7.0 with NaOH and the medium autoclaved. If required, ampicillin was added (final concentration 100 μg/ml) to the cooled medium (approximately 55°C) and 100 μl of 100 mM IPTG and 20 μl of 50 mg/ml X-gal asceptically spread over the surface of LB plates and the plates dried at 37°C for up to one hour.

Medium 63

	g/L
Solution A:	
KH_2PO_4	0.5
NH_4Cl	1.0
Na_2SO_4	1.0
$CaCl_2·2H_2O$	0.1
$MgSO_4·7H_2O$	2.0
Na-lactate	2.0
Yeast extract	1.0
Resazurin (0.1%)	1.0 ml

Nitsch's trace elements	1.0 ml
Water	980.0 ml

Solution B:

$FeSO_4 \cdot 7H_2O$	0.5
Water	10.0 ml

Solution C:

Na-thioglycolate	0.1
Ascorbic acid	0.1
Water	10.0 ml

Medium obtained from DSMZ and used to grow *Thermodesulfobacterium hveragerdense*. The ingredients were dissolved in the appropriate amount of water for each solution. Solution A was boiled and cooled to room temperature under a stream of N_2 gas. Solutions B and C were added and the pH adjusted to 7.0 and the medium distributed into tubes under N_2 gas and autoclaved.

Medium 88

	g/L
$(NH_4)_2SO_4$	1.30
KH_2PO_4	0.28
$MgSO_4 \cdot 7H_2O$	0.25
$CaCl_2 \cdot 2H_2O$	0.07
Nitsch's trace elements	1.0 ml
Yeast extract	1.0

Medium obtained from DSMZ. Growth medium was utilised for the growth of species of *Sulfolobus* and *Sulfurisphaera*. Medium was prepared to pH 5.0 and also prepared anaerobically whereby; 1 ml/L (0.1%) resazurin was added and medium dispensed under N_2 gas and reduced with 10% $Na_2S \cdot 9H_2O$ following autoclaving.

Medium 182

	g/L
Yeast extract	1.0
Casamino acids	1.0
KH_2PO_4	3.1
$(NH_4)_2SO_4$	2.5
$MgSO_4{\cdot}7H_2O$	0.2
$CaCl_2{\cdot}2H_2O$	0.25
Nitsch's trace elements	1.0 ml
Resazurin (0.1%)	1.0 ml

Medium obtained from DSMZ. Medium was adjusted to pH 5.5. If required, powdered sulfur was added to tubes containing growth medium prior to autoclaving. Medium was prepared for anaerobic growth by boiling medium and dispensing under N_2 gas prior to autoclaving and reduced with 10% $Na_2S{\cdot}9H_2O$ as required.

Medium 206

	g/L
Na_2SO_4	3.0
NH_4Cl	1.0
$MgCl_2{\cdot}6H_2O$	0.2
KH_2PO_4	0.3
$Na_2HPO_4{\cdot}12H_2O$	2.0
Zeikus trace elements	10 ml
$FeSO_4{\cdot}7H_2O$	1.5 mg
Wolin's vitamins	5.0 ml
Resazurin (0.1%)	1.0 ml
Yeast extract*	1.0
Na-lactate*	4.0

Medium was obtained from DSMZ and used to culture *Thermodesulfobacterium commune*. Medium was adjusted to pH 7.0 and prepared anaerobically by boiling and dispensing under N_2 gas prior to autoclaving. *Added following autoclaving from anaerobic sterile stock solutions. Following autoclaving, the medium was reduced with 10% $Na_2S{\cdot}9H_2O$ as required.

Medium 760

	g/L
Peptone	5.0
Yeast extract	1.0
Fe^{3+} citrate	0.1
NaCl	19.45
$MgCl_2{\cdot}6H_2O$	5.9
Na_2SO_4	3.24
$CaCl_2{\cdot}2H_2O$	1.8
KCl	0.55
Na_2CO_3	0.16
KBr	0.08
$SrCl_2{\cdot}6H_2O$	34.0 mg
H_3BO_3	22.0 mg
Na-silicate	4.0 mg
NaF	2.4 mg
$(NH_4)NO_3$	1.6 mg
Na_2HPO_4	8.0 mg
Resazurin (0.1%)	1.0 ml

Medium obtained from DSMZ and used for isolation of *Thermococcus* species. Final pH was adjusted to 6.0 at room temperature and filtered to remove iron deposits. The medium was then boiled and dispensed under N_2 atmosphere into tubes containing 0.5% w/v sulfur. The medium was sterilised by exposure to steam at 98°C for 15 minutes on 3 consecutive days. Medium was reduced with 10% $Na_2S{\cdot}9H_2O$ as required. If a black precipitate was produced the iron sediment was not adequately removed.

Medium 897

	g/L
NaCl	13.65
$MgSO_4 \cdot 7H_2O$	3.5
$MgCl_2 \cdot 6H_2O$	2.75
KH_2PO_4	0.5
$(NH4)_2SO_4$	0.25
$CaCl_2 \cdot 2H_2O$	0.38
KCl	0.33
NaBr	0.05
H_3BO_3	15.0 mg
$SrCl_2 \cdot 6H_2O$	7.5 mg
KI	0.05 mg
Beef extract	1.0
$Na_2S \cdot 9H_2O^*$	0.2
Resazurin (0.1%)	1 ml
Sulfur	5.0 (added to individual tubes)

Medium was obtained from DSMZ and used for the isolation of *Ignicoccus* sp. Medium was prepared and adjusted to pH 5.5, boiled and sparged with N_2 gas. The medium was dispensed into tubes under N_2 gas. $^*Na_2S \cdot 9H_2O$ was added from a 10% stock solution to the required concentration and the medium stored at room temperature. Prior to inoculation medium was checked for purity by phase-contrast microscopy then the medium incubated at 90°C for one hour, allowed to cool and inoculated. Then the tubes were flushed and over-pressurised with H_2:CO_2 (80:20) gas.

Nitsch's trace elements

	g/L
$MnSO_4 \cdot 4H_2O$	0.0022
$ZnSO_4 \cdot 7H_2O$	0.0005
H_3BO_4	0.0005

$CuSO_4$	0.000016
$Na_2MoO_4 \cdot 2H_2O$	0.000025
$CoCl_2 \cdot 6H_2O$	0.000046

Consituents were dissolved at 1000X concentration in acidified water (0.5 ml of concentrated H_2SO_4).

Selenite/Tungstate

	g/L
NaOH	0.5
$Na_2SeO \cdot 5H_2O$	0.003
$Na_2WO_4 \cdot 2H_2O$	0.004

SL 10 trace elements

	g/L
$FeCl_2 \cdot 4H_2O$	1.5
5M HCl	15.0 ml
$ZnCl_2$	0.07
$MnCl_2 \cdot 4H_2O$	0.1
H_3BO_4	0.006
$CoCl_2 \cdot 6H_2O$	0.130
$CuCl_2 \cdot 2H_2O$	0.002
$NiCl_2 \cdot 6H_2O$	0.024
$Na_2MoO_4 \cdot 2H_2O$	0.036

SME medium

	g/L
NaCl	13.85
$MgSO_4 \cdot 7H_2O$	3.5
$MgCl_2 \cdot 6H_2O$	2.75
Tri-sodium citrate$\cdot 2H_2O$	1.0

$CaCl_2 \cdot 2H_2O$	0.75
KH_2PO_4	0.5
KCl	0.325
NaBr	50.0 mg
H_3BO_3	15.0 mg
$SrCl_2 \cdot 6H_2O$	7.5 mg
$(NH_4)_2Ni(SO_4)_2$	2.0 mg
KI	0.05 mg
Wolin's vitamins*	10.0 ml
Yeast extract	0.2
Resazurin (0.1%)	1.0 ml
Sulfur	25.0 (added to individual tubes)

The pH was adjusted 5.5, boiled and dispensed into tubes under N_2 gas. Medium was sterilised by exposure to steam at 98°C for 15 minutes on 3 consecutive days. Medium was reduced with 10% $Na_2S \cdot 9H_2O$ as required. On board the *Atlantis* (WHOI), medium was flushed and over-pressurized with H_2:CO2 (80:20) gas prior to inoculation.

SME medium (modified)

	g/L
NaCl	0.25
$MgSO_4 \cdot 7H_2O$	0.35
$MgCl_2 \cdot 6H_2O$	0.275
Tri-sodium citrate·$2H_2O$	0.275
$CaCl_2 \cdot 2H_2O$	0.75
KH_2PO_4	0.5
KCl	0.325
NaBr	50.0 mg
$SrCl_2 \cdot 6H_2O$	7.5 mg
$(NH_4)_2Ni(SO_4)_2$	2.0 mg

KI	0.05 mg
Wolin's vitamins*	10.0 ml
Yeast extract	0.2
Resazurin (0.1%)	1.0 ml
Sulfur	25.0 (added to individual tubes)

The pH was adjusted to 7.5, boiled and dispensed into tubes under N_2:CO_2 (80:20) gas. Medium was sterilised by exposure to steam at 98°C for 15 minutes on 3 consecutive days. Medium was reduced with 10% $Na_2S \cdot 9H_2O$ as required.

SOC medium

	g/L
Trypticase peptone	2.0
Yeast extract	0.5
NaCl (1.0 M)	1.0 ml
KCl (1.0 M)	0.25 ml
Mg^{2+} stock (composition below)*	1.0 ml
Glucose (2.0 M)	1.0 ml

*Added following autoclaving when medium had cooled to room temperature.

Mg^{2+} stock	g/L
$MgCl_2 \cdot 6H_2O$	20.33
$MgSO_4 \cdot 7H_2O$	24.65

Dissolved and passed through a 0.22 μm pore filter to sterilise.

Spirochete medium*

	g/L
NaCl	15.0
$MgCl_2 \cdot 6H_2O$	2.3

KCl	0.5
NH_4Cl	0.3
KH_2PO_4	0.2
$CaCl_2{\cdot}2H_2O$	0.03
Resazurin (0.1%)	1.0 ml
Yeast extract	1.0
SL10 trace elements	1.0 ml
Selenite/Tungstate	1.0 ml

*Modified DSMZ medium 590. The medium was prepared, boiled to dissolve constituents and then cooled to room temperature. The pH was then equilibrated to below 6.0 with N_2:CO_2 gas (80:20) and distributed into tubes (containing approximately 5.0 g/L chitin) under N_2:CO_2 gas (80:20) and autoclaved. Subsequent to autoclaving, the following were added to 10 ml medium: 0.5 ml Na_2CO_3 (5% w/v); 0.2 ml of Wolin's vitamins; 0.1 ml of soluble starch (10% w/v); 0.45 ml of N-acetyl glucosamine (100 mM), and finally reduced with 10% $Na_2S{\cdot}9H_2O$ as required.

***Thermofilum librum* medium**

Medium 88 was prepared without yeast extract and the following added (g/L): soluble starch 2.0: yeast extract 0.2, and 1.0 ml of resazurin (0.1%). Medium was adjusted to pH 6.0, boiled to dissolve and added to tubes containing powdered sulfur (approximately 1 g/L) under N_2 gas flow. Medium was sterilised by incubating at 85°C on each of two successive days. Medium was reduced with 10% $Na_2S{\cdot}9H_2O$ as required.

***Thermoproteus* medium**

	g/L
$(NH_4)_2SO_4$	0.264
$FeSO_4{\cdot}7H_2O$	0.556
$MgSO_4{\cdot}7H_2O$	0.492
$CaSO_4{\cdot}7H_2O$	0.344

KH_2PO_4	0.014
Yeast extract	0.2
Soluble starch	5.0
Sulfur	10.0
Resazurin (0.1%)	1.0 ml

Trace elements (from 0.1% (w/v) solutions):

NaF	0.84 ml
$MnCl_2 \cdot 4H_2O$	0.18 ml
$Na_2B_4O_7 \cdot 10H_2O$	0.45 ml
$ZnSO_4 \cdot H_2O$	0.022 ml
$CuCl_2 \cdot 2H_2O$	0.005 ml
$Na_2MoO_4 \cdot 2H_2O$	0.003 ml
$CoSO_4 \cdot 7H_2O$	0.001 ml

The pH was adjusted to 7.0 and the medium boiled and dispensed under N_2 gas. Following autoclaving medium was reduced with 10% $Na_2S \cdot 9H_2O$ as required.

***Thermoproteus uzoniensis* medium**

	g/L
$(NH_4)_2Cl$	0.33
$CaCl_2 \cdot 2H_2O$	0.33
$MgCl_2 \cdot 6H_2O$	0.33
KCl	0.33
KH_2PO_4	0.33
SL10 trace elements	1.0 ml
Wolin's vitamins*	10.0 ml
Yeast extract	0.1
Resazurin (0.1%)	1.0 ml

The pH was adjusted to pH 7.5, boiled and dispensed under N_2:CO_2 (80:20) into tubes containing powdered sulfur (approximately 10 g/L). The medium was autoclaved, and when cooled, $NaHCO_3$ was added from an anaerobic stock to a final concentration of 2.0 g/L and the medium reduced with sterile 10% $Na_2S \cdot 9H_2O$ as required. *Added following autoclaving.

***Thermotoga* MSM* (modified)**

	g/L
$(NH_4)_2SO_4$	0.65
$CaCl_2 \cdot 2H_2O$	0.074
$MgSO_4 \cdot 7H_2O$	0.28
KH_2PO_4	0.28
NaCl	0.5
Yeast extract	2.0
Casamino acids	2.0
$FeCl_3$ (0.28 g/L)	1.0 ml
Nitsch's trace elements	1.0 ml
Resazurin (0.1%)	1.0 ml
Maltose	2.0

*Mineral salts medium. Medium was adjusted to pH 7.5, boiled and dispensed under N_2. Medium was autoclaved, reduced with 10% $Na_2S \cdot 9H_2O$ if required and caps loosened for the cultivation of isolate Oh.A3.

TSA/S (modified)

	g/L
Tryptic soy broth	30.0
Bacteriological agar	15.0
Soluble starch	2.0
Sea water	500 ml

The pH was adjusted to 6.0 at room temperature, autoclaved and Petri plates poured aseptically under laminar flow hood.

Wolin's vitamins

	mg/100mL
Biotin	2.0
Folic acid	2.0
Pyridoxin HCl	10.0
Riboflavin	5.0
Thiamine	5.0
Nicotinic acid	5.0
Pantothenic acid	5.0
Vitamin B12	0.1
p-amino benzoic acid	5.0
Thioctic acid (lipoil)	5.0

Consituents were dissolved and filter sterilised. The solution was stored away from light and at 4°C.

Zeikus trace elements

	g/L
Nitrilotriacetic acid	12.5

The pH was adjusted to approximately 6.5 with KOH until all the nitrilotriacetic acid had dissolved and the following added:

$FeCl_3·6H_2O$	0.2
$MnCl_2·4H_2O$	0.1
$CoCl_2·6H_2O$	0.017
$CaCl_2·2H_2O$	0.1
$ZnCl_2$	0.1

$CuCl_2$	0.02
H_3BO_4	0.01
$NaMoO_4 \cdot 2H_2O$	0.01
NaCl	1.0
Na_2SeO_3	0.02

Appendix F – Freeze-dried and cryoprotected microorganisms

Table F. 1 Freeze-dried and cryoprotected microorganisms.

Hot pool/Culture	Probable species/ Morphology	Pure or Consortia	Storage type[A]	TRUCC #	Medium	Temp. (°C)	pH
AQ1.S1^{T}	*Ignisphaera aggregans*	Pure	F.D. & G.	TG 866	Db+S	65	7.0
AQ1.S2	*Pyrobaculum sp.*	Pure	F.D. & G.	TG 867	Db+ S	65	7.0
KP1.A1	‘*Thermonena*’	Pure	F.D.	TG 857	CMD agar	65	7.0
KP1.A2	‘*Thermus*’	Pure	F.D.	TG 858	CMD agar	65	7.0
KP1.A3	‘*Bacillus*’	Pure	F.D.	TG 859	CMD agar	70	7.0
KP1.A4	‘*Bacillus?*’	Pure	F.D.	TG 860	CMD agar	70	7.0
KP1.B1	‘*Fervidobacteria*’	Pure	F.D.	TG 869	2/1+C and *Dictyoglomus*	65	6.5
KP1.B2	Long rod	Pure	F.D.	TG 870	AN1 medium	65	7.0
KP1.B3	Long rod	Pure	F.D.	TG 871	Arsenic medium	65	7.0
Tok13	Rods and cocci	Consortia	G.	N.A.	Db+ S	93	7.0
AQ5	Rods and cocci	Consortia	G.	N.A.	Db+ S	93	7.0
AQ2	Rods and cocci	Consortia	G.	N.A.	Db+ S	93	7.0
Tok37.S1	Cocci & *Ignisphaera* sp.	Consortia	F.D. & G.	TG 879	Db+ S	90	6.5
Tok10A	Rods & *Ignisphaera* sp.	Consortia	G.	N.A.	Db+ S	93	7.0
OH1	‘*Fervidobacteria*’	Pure	G.	N.A.	Db+ S	80	7.5
WH2 (sediment MPN)	Cocci and ‘*Fervidobacteria*’	Consortia	G.	N.A.	AN1	80	7.0
WH2 (sediment MPN)	‘*Dictyoglomus*’	Pure	G.	N.A.	Db+ S	80	7.0
SP1 (water MPN)	See Section 4.3.1	Consortia	G.	N.A.	Db+ S	85	6.0
AQ4 (water MPN)	See Section 4.3.1	Consortia	G.	N.A.	Db+ S	85	6.0
AQ3 (water MPN)	See Section 4.3.1	Consortia	G.	N.A.	Db+ S	85	8.0
WH1 (water MPN)	See Section 4.3.1	Consortia	G.	N.A.	Db+ S	80	6.0
SP2 (water MPN)	See Section 4.3.1	Consortia	G.	N.A.	*Thermofilum*	85	6.0
Oh.A3	‘*Thermus*’	Pure	F.D.	TG 834	*Thermotoga* MSM	75	7.5
Orakei Korako[B]	Long thin rods	Consortia	G.	N.A.	Db+ S	93	7.0
AQ1	‘*Thermococcus*’	Pure	G.	N.A.	*Aeropyrum* (N_2:CO_2)	85	8.0
AQ3	‘*Pyrobaculum*’	Pure	G.	N.A.	*Aeropyrum* (O_2)	85	8.0

[A] F.D. = Freeze-dried; G. = glycerol.

[B] Cultures sent to Dr. Hiroshi Xavier Chiura (International Christian University, Osawa, Mitaka, Tokyo, Japan).

References

Alain, K., Zbinden, M., Le Bris, N., Lesonguer, F., Quérellou, J., Gaill, F. and Cambon-Bonavita, M. **2004**. Early steps in microbial colonization processes at deep-sea hydrothermal vents. *Environmental Microbiology* 6:227-241.

Alazard, D., Dukan, S., Urios, A., Verhe, F., Bouabida, N., Morel, F., Thomas, P., Garcia, J. and Ollivier, B. **2003**. *Desulfovibrio hydrothermalis* sp. nov., a novel sulfate-reducing bacterium isolated from hydrothermal vents. *International Journal of Systematic and Evolutionary Microbiology* 53:173-178.

Altschul, S., Gish, W., Miller, W., Myers, E. and Lipman, D. **1990**. Basic local alignment search tool. *Journal of Molecular Biology* 215:403-410.

Amann, R., Ludwig, W. and Scheifer, K.-H. **1995**. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiology Reviews* 59:143-169.

Amend, J. and Shock, E. **2001**. Energetics of overall metabolic reactions of thermophilic and hyperthermophilic Archaea and Bacteria. *FEMS Microbiology Reviews* 25:175-243.

Amo, T., Paje, M., Inagaki, A., Ezaki, S., Atomi, H. and Imanaka, T. **2002**. *Pyrobaculum caldifontis* sp. nov., a novel hyperthermophilic archaeon that grows in atmospheric air. *Archaea* 1:113-121.

Aoshima, M., Nishibe, Y., Hasegawa, M., Yamagishi, A. and Oshima, T. **1996.** Cloning and sequencing of a novel gene encoding 16S ribosomal RNA from a novel hyperthermophilic archaebacterium NC12. *Gene* 180:183-187.

Atkinson, T., Cairns, S., Cowan, D., Danson, M., Hough, D., Johnson, B., Norris, P., Raven, N., Robinson, C., Robson, R. and Sharp, R. **2000**. A microbiological survey of Montserrat Island hydrothermal biotopes. *Extremophiles* 4:305-313.

Barns, S., Fundyga, R., Jefferies, M. and Pace, N. **1994**. Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. *Proceedings of the National Academy of Sciences* 91:1609-1613.

Barns, S., Delwiche, C., Palmer, J. and Pace, N. **1996a**. Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. *Proceedings of the National Academy of Sciences* 93:9188-9193.

Barns, S., Delwiche, C., Palmer, J., Dawson, S., Hershberger, K. and Pace, N. **1996b.** Phylogenetic perspective on microbial life in hydrothermal ecosystems, past and present. In, *Evolution of Hydrothermal Ecosystems on Earth (and Mars?).* Edited by, Bock, G. and Goode, J. Ciba Foundation Symposium 202, John Wiley & Sons, Great Britain.

Birnboim, H. and Doly, J. **1979**. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* 7:1513-1523.

Blank, C., Cady, S. and Pace, N. **2002**. Microbial composition of near-boiling silica-depositing thermal springs throughout Yellowstone National Park. *Applied and Environmental Microbiology* 68:5123-5135.

Blöchl, E., Rachel, R., Burrgraf, S., Hafenbradl, D., Jannasch, H. and Stetter, K. **1997**. *Pyrolobus fumarii*, gen. and sp. nov. represents a novel group of archaea, extending the upper temperature limit for life to 113°C. *Extremophiles* 1:14-21.

Bohlool, B. and Brock, T. **1974.** Immunofluorescence approach to the study of the ecology of *Thermoplasma acidophilum* in coal refuse material. *Applied Microbiology* 28:11–16.

Boomer, S., Lodge, D., Dutton, B. and Pierson, B. **2002**. Molecular characterization of novel red green nonsulfur bacteria from five distinct hot spring communities in Yellowstone National Park. *Applied and Environmental Microbiology* 68:346-355.

Breitbart, M., Wegley, L., Leeds, S., Schoenfeld, T. and Rohwer, F. **2004**. Phage community dynamics in hot springs. *Applied and Environmental Microbiology* 70:1633-1640.

Brim, H., Venkateswaran, A., Kostandarithes, H., Fredrickson, J. and Daly, M. **2003**. Engineering *Deinococcus geothermalis* for bioremediation of high-temperature radioactive waste environments. *Applied and Environmental Microbiology* 69:4575-4582.

Brochier, C., Gribaldo, S., Zivanovic, Y., Canfolonieri, F. and Forterrre, P. **2005**. Nanoarchaea: representatives of a novel archaeal phylum or a fast-evolving euryarchaeal lineage related to Thermococcales. *Genome Biology* 6:R42.

Brock, T. **1978**. *Thermophilic microorganisms and life at high temperatures,* edited by Starr, M. P. Springer-Verlag, New York, USA.

Brock, T. **1986**. *Thermophiles: general, molecular and applied microbiology*. John Wiley & Sons, Inc., USA.

Brody, J. and Kern, S. **2004**. Sodium boric acid: a Tris-free, cooler conductive medium for DNA electrophoresis. *BioTechniques* 36:214-216.

Buckley, D., Graber, J. and Schmidt, T. **1998**. Phylogenetic analysis of nonthermophilic members of the kingdom crenarchaeota and their diversity and abundance in soils. *Applied and Environmental Microbiology* 64:4333-4339.

Burggraf, S., Larsen, N., Woese, C. and Stetter, K. **1993**. An intron within the 16S ribosomal RNA gene of the archaeon *Pyrobaculum aerophilum*. *Proceedings of the National Academy of Sciences* 90:2547-2550.

Burggraf, S., Heyder, P. and Eis, N. **1997a**. A pivotal Archaea group. *Nature* 385:780.

Burggraf, S., Huber, H. and Stetter, K. **1997b**. Reclassification of the crenarchaeal orders and families in accordance with 16S rRNA sequence data. *International Journal of Systematic Bacteriology* 47:657-660.

Burton, N. and Norris, P. **2000**. Microbiology of acidic, geothermal springs of Montserrat: environmental rDNA analysis. *Extremophiles* 4:315-320.

Carpenter-Boggs, L., Kennedy, A. and Reganold, J. **1998**. Use of phospholipid fatty acids and carbon source utilization patterns to track microbial community succession in developing compost. *Applied and Environmental Microbiology* 64:4062-4064.

Cary, S., Cottrell, M., Stein, J., Camacho, F. and Desbruyères, D. **1997**. Molecular identification and localization of a filamentous symbiotic bacteria associated with the hydrothermal vent Annelid, *Alvinella pompejana*. *Applied and Environmental Microbiology* 63:1124-1130.

Cole, J., Chai, B., Farris, R., Wang, Q., Kulam, S., McGarrell, D., Garrity, G. and Tiedje, J. **2005**. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Research* 1:33(Database Issue):D294-D296.

Deckert, G., Warren, P., Gaasterland, T., Young, W., Lenox, A., Graham, D., Overbeek, R., Snead, M., Keller, M., Aujay, M., Huber, R., Feldman, R., Short, J., Olsen, G. and Swanson, R. **1998**. The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*. *Nature* 392:353-358.

Dempster, E., Pryor, K., Francis, D., Young, J. and Rogers, H. **1999**. Rapid DNA extraction from ferns for PCR-based analyses. *Biotechniques* 27:66-68.

Distel, D., Lane, D., Olsen, G., Giovannoni, S., Pace, B., Pace, N., Stahl, D. and Felbeck, H. **1998**. Sulfur-oxidizing bacterial endosymbionts: analysis of phylogeny and specificity by 16S rRNA sequences. *Journal of Bacteriology* 170:2506-2510.

Donachie, S., Christenson, B., Kunkel, D., Malahoff, A. and Alam, M. **2002**. Microbial community in acidic hydrothermal waters of volcanically active White Island, New Zealand. *Extremophiles* 6:419-425.

Dunbar, J., White, S. and Forney, L. **1997**. Genetic diversity through the looking glass: effect of enrichment bias. *Applied and Environmental Microbiology* 63:1326-1331.

Dunn, M. **1989**. *'Protein purification methods' a practical approach*, edited by Harris, E. and Angal, S. Oxford University Press, New York, printed by Information Press Ltd., Oxford, England.

Eder, W., Ludwig, W. and Huber, R. **1999**. Novel 16S rDNA gene sequences retrieved from highly saline brine sediments of Kebrit Deep, Red Sea. *Archives of Microbiology* 172:213-218.

Eder, W. and Huber, R. **2002**. New isolates and physiological properties of the *Aquificales* and description of *Thermocrinis albus* sp. nov. *Extremophiles* 6:309-318.

Ercolini, D. **2004**. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods* 56:297-314.

Everett, L. **2005**. *Thermophilic Research Work.* Second Year Placement Report, University of Waikato.

Felsenstein, J. **1993**. PHYLIP – phylogeny inference package (version 3.5c).

Ferris, M., Ruff-Roberts, A., Kopczynski, E., Bateson, M. and Ward, D. **1996a**. Enrichment culture and microscopy conceal diverse thermophilic *Synechococcus* populations in a single hot spring microbial mat habitat. *Applied and Environmental Microbiology* 62:1045-1050.

Ferris, M., Muyzer, G. and Ward, M. **1996b**. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Applied and Environmental Microbiology* 62:340-346.

Ferris, M., Nold, S., Revsbech, N. and Ward, D. **1997**. Population structure and physiological changes within a hot spring microbial mat community following disturbance. *Applied and Environmental Microbiology* 63:1367-1374.

Ferris, M. and Ward, D. **1997**. Seasonal distributions of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology* 63:1375-1381.

Ferris, M., Magnuson, T., Fagg, J., Thar, R., Kühl, M., Sheehan, K. and Henson, J. **2003**. Microbially mediated sulphide production in a thermal, acidic algal mat community in Yellowstone National Park. *Environmental Microbiology* 5:954-960.

Fiala, G., Stetter, K., Jannasch, H., Langworthy, T. and Madon, J. **1986**. *Staphylothermus marinus* sp. nov. represents a novel genus of extremely thermophilic submarine heterotrophic archaebacteria growing up to 98°C. *Systematic and Applied Microbiology* 8:106-113.

Fiala, G. and Stetter, K. **1986**. *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100°C. *Archives of Microbiology* 145:56-61.

Gihring, T., Druschel, G., McCleskey, R., Hamers, R. and Banfield, J. **2001**. Rapid arsenite oxidation by *Thermus aquaticus* and *Thermus thermophilus*: field and laboratory investigations. *Environmental Science and Technology* 35:3857-62.

González, J., Sheckells, D., Viebahn, M., Krupatkina, D., Borges, K. and Robb, F. **1999**. *Thermococcus waiotapuensis* sp. nov., an extremely thermophilic archaeon isolated from a freshwater hot spring. *Archives of Microbiology* 172:95-101.

Götz, D. **1998**. *The characterization of three hyperthermophilic archaea from New Zealand hot springs*. Ph.D. Thesis, University of Waikato.

Hafenbradl, D., Keller, M., Dirmeier, R., Rachel, R., Rossnagel, P., Burggraf, S., Huber, H. and Stetter, K. **1996**. Ferroglobus placidus gen. nov., sp. nov., a novel hyperthermophilic archaeum that oxidizes Fe2+ at neutral pH under anoxic conditions. *Archives of Microbiology* 166:308-14.

Handelsman, J. **2004**. Metagenomics: Application of genomics to uncultured microorganisms. *Microbiology and Molecular Biology Reviews* 68:669-685.

Harmsen, H., Prieur, D. and Jeanthon, C. **1997**. Distribution of microorganisms in deep-sea hydrothermal vent chimneys investigated by whole-cell hybridization and enrichment cultures of thermophilic subpopulations. *Applied and Environmental Microbiology* 63:2876-2883.

Haymon, R. **1983**. Growth history of hydrothermal black smoker chimneys. *Nature* 304:695-698.

Henley, R. **1996**. Chemical and physical context for life in terrestrial hydrothermal systems: chemical reactors for the early development of life and hydrothermal ecosystems. In, *Evolution of Hydrothermal Ecosystems on Earth (and Mars?)*. Ciba Foundation Symposium 202. Edited by, Bock, G. and Goode, J. John Wiley & Sons Ltd., Great Britain.

Hensel, R., Matussek, K., Michalke, K., Tacke, L., Tindall, B., Kohlhoff, M., Siebers, B. and Dielenschneider, J. **1997**. *Sulfophobococcus zilligii* gen. nov., spec. nov. a novel hyperthermophilic archaeum isolated from hot alkaline springs of Iceland. *Systematic and Applied Microbiology* 20:102-110.

Hjorleifsdottir, S., Skirnisdottir, S., Hreggvidsson, G., Holst, O. and Kristjansson, J. **2001**. Species composition of cultivated and noncultivated bacteria from short filaments in an Icelandic hot spring at 88°C. *Microbial Ecology* 42:117-125.

Hoek, J., Banta, A., Hubler, F. and Reysenbach, A.-L. **2003**. Microbial diversity of a sulphide spire located in the Edmond deep-sea hydrothermal vent field on the Central Indian Ridge. *Geobiology* 1:119-127.

Hohn, M., Hedlund, B. and Huber, H. **2002**. Detection of 16S rDNA sequences representing the novel phylum 'Nanoarchaeota" indication for a wide distribution in high temperature biotopes. *Systematic and Applied Microbiology* 25:551-554.

Huber, G., Spinnler, C., Gambacorta, A. and Stetter, K. **1989**. *Metallosphaera sedula* gen. nov. and sp. nov. represents a new genus of aerobic, metal-mobilizing, thermoacidophilic archaebacteria. *Systematic and Applied Microbiology* 12:38-47.

Huber, H., Burggraf, S., Mayar, T., Wyschkony, I., Rachel, R. and Stetter, K. **2000a**. *Ignicoccus* gen. nov., a novel genus of hyperthermophilic, chemolithoautotrophic *Archaea*, represented by two new species, *Ignicoccus islandicus* sp. nov. and *Ignicoccus pacificus* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 50:2093-2100.

Huber, H., Hohn, M., Rachel, R., Fuchs, T., Wimmer, V. and Stetter, K. **2002**. A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. *Nature* 417:63-67.

Huber, R., Kristjansson, J. and Stetter, K. **1987**. *Pyrobaculum* gen. nov., a new genus of neutrophilic rod-shaped archaebacteria from continental solfataras growing optimally at 100°C. *Archives of Microbiology* 149:95-101.

Huber, R., Woese, C., Langworthy, T., Kristjansson, K. and Stetter, K. **1990**. *Fervidobacterium islandicum* sp. nov., a new extremely thermophilic eubacterium belonging to the '*Thermotogales*'. *Archives of Microbiology* 154:105-111.

Huber, R., Wilharm, T. Huber, D., Trincone, A., Burggraf, S., König, H., Rachel, R., Rockinger, I., Fricke, H. and Stetter, K. **1992**. *Aquifex pyrophilus* gen. nov. sp. nov., represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacteria. *Systematic and Applied Microbiology* 15:340-351.

Huber, R., Cyba, D., Huber, H., Burggraf, S. and Reinhard, R. **1998**. Sulfur-inhibited *Thermosphaera aggregans* sp. nov., a new genus of hyperthermophilic archaea isolated after its prediction from environmentally derived 16S rRNA sequences. *International Journal of Systematic Bacteriology* 48:31-38.

Huber, R., Sacher, M., Vollmann A., Huber, H. and Rose, D. **2000b**. Respiration of arsenate and selenate by hyperthermophilic archaea. *Systematic and Applied Microbiology* 23:305-314.

Huber, R., Huber, H. and Stetter, K. **2000c**. Towards the ecology of hyperthermophiles: biotopes, new isolation strategies and novel metabolic properties. *FEMS Microbiology Reviews* 24:615-623.

Hudson, J., Morgan, H. and Daniel, R. **1987**. *Thermus filiformis* sp. nov., a filamentous caldoactive bacterium. *International Journal of Systematic Bacteriology* 37:431-436.

Hudson, J., Schofield, K., Morgan, H. and Daniel, R. **1989**. *Thermonema lapsum* gen. nov., sp. nov., a thermophilic gliding bacterium. *International Journal of Systematic Bacteriology* 39:485-487.

Hugenholtz, P., Pitulle, C., Hershberger, K. and Pace, N. **1998**. Novel division level bacterial diversity in a Yellowstone hot spring. *Journal of Bacteriology* 180:366-376.

Hugenholtz, P. and Huber, T. **2003**. Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. *International Journal of Systematic and Evolutionary Microbiology* 53:289-293.

Ibekwe, A., Papiernik, S., Gan, J., Yates, S., Yang, C. and Crowley, D. **2001**. Impacts of fumigants on soil microbial communities. *Applied and Environmental Microbiology* 67:3245-3257.

Itoh, T., Suzuki, K. and Nakase, T. **1998a**. Occurrence of introns in the 16S rRNA genes of members of the genus *Thermoproteus*. *Archives of Microbiology* 170:1157-1163.

Itoh, T., Suzuki, K. and Nakase, T. **1998b**. *Thermocladium modesties* gen. nov., a new genus of rod-shaped, extremely thermophilic crenarchaeote. *International Journal of Systematic Bacteriology* 48:879-887.

Itoh, T., Suzuki, K., Sanchez, P. and Nakase, T. **1999**. *Caldivirga maquilingensis* gen. nov., sp. nov., a new genus of rod-shaped crenarchaeote isolated from a hot spring in the Philippines. *International Journal of Bacteriology* 49:1157-1163.

Itoh, T., Suzuki, K. and Nakase, T. **2002**. *Vulcanisaeta distributa* gen. nov., sp. nov., and *Vulcanisaeta souniana* sp. nov., novel hyperthermophilic rod-shaped crenarchaeotes isolated from hot springs in Japan. *International Journal of Systematic and Evolutionary Microbiology* 52:1097-1104.

Itoh, T., Suzuki, K., Sanchez, P. and Nakase, T. **2003**. *Caldisphaera lagunensis* gen. nov., sp. nov., a new thermoacidophilic crenarchaeote isolated from a hot spring at Mt Maquiling, Philippines. *International Journal of Systematic and Evolutionary Microbiology* 53:1149-1154.

Jackson, C., Churchill, P. and Roden, E. **2001a**. Successional changes in bacterial assemblage structure during epilithic biofilm development. *Ecology* 82:555-566.

Jackson, C., Langner, H., Donahoe-Christiansen, J., Inskeep, W. and McDermott, T. **2001b**. Molecular analysis of microbial community structure in an arsenite-oxidizing acidic thermal spring. *Environmental Microbiology* 3:532-542.

Jahn, U., Summons, R., Sturt, H., Grosjean, E. and Huber, H. **2004**. Composition of the lipids of *Nanoarchaeum equitans* and their origin from its host *Ignicoccus* sp. strain KIN4/I. *Archives of Microbiology* 182:404-413.

Jan, R.-L., Wu, J., Chaw, S.-M., Tsai, C.-W. and Tsen, S.-D. **1999**. A novel species of thermoacidophilic archaeon *Sulfolobus yangmingensis* sp. nov. *International Journal of Systematic Bacteriology* 49:1809-1816.

Janse, I., Bok, J. and Zwart, G. **2004**. A simple remedy against artifactual double bands in denaturing gradient gel electrophoresis. *Journal of Microbiological Methods* 57:279-281.

Jochimsen, B., Peinemann-Simon, S., Völker, H., Stüben, D., Botz, R., Stoffers, P., Dando, P. and Thomm, M. **1997**. *Stetteria hydrogenophila*, gen. nov. and sp. nov., a novel mixotrophic sulphur-dependent *crenarchaeote* isolated from Milos, Greece. *Extremophiles* 1:67-73.

Johnson, D. **1998**. Biodiversity and ecology of acidophilic microorganisms. *FEMS Microbial Ecology* 27:307-317.

Jones, B. and Renaut, R. **1996**. Influence of thermophilic bacteria on calcite and silica precipitation in hot springs with water temperatures above 90-degrees-C evidence from Kenya and New Zealand. *Canadian Journal of Earth Sciences* 33:72-83.

Jones, B., Renaut, R. and Rosen, M. **1999**. Actively growing siliceous oncoids in the Waiotapu geothermal area, North Island, New Zealand. *Journal of the Geological Society* 156:99-103.

Jones, B., Konhouser, K., Renaut, R. and Wheeler, R. **2004**. Microbial silicification in Iodine Pool, Waimangu geothermal area, North Island, New Zealand: implications for recognition and identification of ancient silicified microbes. *Journal of the Geological Society, London* 161:983-993.

Jones, W., Leigh, J., Mayer, F., Woese, C. and Wolfe, R. **1983**. *Methanococcus jannaschii*, sp. nov., an extremely thermophilic methanogen from a submarine hydrothermal vent. *Archives of Microbiology* 136:254-261.

Karner, M., Delong, E. and Karl, D. **2001**. Archaeal dominance in the mesopelagic zone of the Pacific Ocean. *Nature* 409:507-510.

Kashefi, K. and Lovely, D. **2003**. Extending the upper temperature limit of life. *Science* 301:934.

Kjems, J. and Garrett, R. **1985**. An intron in the 23S ribosomal RNA gene of the archaebacterium *Desulfurococcus mobilis*. *Nature* 318:675-677.

Kjems, J. and Garrett, R. **1991**. Ribosomal RNA introns in archaea and evidence for RNA conformational changes associated with splicing. *Proceedings of the National Academy of Sciences* 88:439-443.

Klappenbach, J., Saxman, P., Cole, J. and Schmidt, T. **2001.** rrndb: the ribosomal RNA operon copy number database. *Nucleic Acids Research* 29:181-184.

Kristjansson, J. **1991**. *Thermophilic bacteria*. CRC Press, Inc., USA.

Kurosawa, N., Itoh, Y., Iwai, T., Sugai, A., Uda, I., Kimura, N., Horiuchi, T. and Itoh, T. **1998.** *Sulfurisphaera ohwakuensis* gen. nov. sp. nov., a novel extremely thermophilic acidophile of the order *Sulfolobales*. *International Journal of Bacteriology* 48:451-456.

Kurr, M., Huber, R., König, H., Jannasch, H., Fricke, H., Trincone, A., Kristjansson, J. and Stetter, K. **1991**. *Methanopyrus kandleri*, gen. and sp. nov. represents a novel group of hyperthermophilic methanogens, growing at 110°C. *Archives of Microbiology* 156:239-247.

Kvist, T., Mengewein, A., Manzei, S., Ahring, B. and Westermann, P. **2005**. Diversity of thermophilic and non-thermophilic crenarchaeota at 80°C. *FEMS Microbiology Letters* 244:61-68.

Kysela, D., Palacios, C. and Sogin, M. **2005**. Serial analysis of V6 ribosomal sequence tags (SARST-V6): a method for efficient, high-throughput analysis of microbial community composition. *Environmental Microbiology* 7:356-364.

LaMontagne, M., Michel, F., Jr., Holden, P. and Reddy, C. **2002**. Evaluation of extraction and purification methods for obtaining PCR-amplifiable DNA from compost for microbial community analysis. *Journal of Microbiological Methods* 49:255-64.

Lepage, E., Marguet, E., Geslin, C., Matte-Tailliez, O., Zillig, W., Forterre, P. and Tailliez, P. **2004**. Molecular diversity of new Thermococcales isolates from a single area of hydrothermal deep-sea vents as revealed by randomly amplified polymorphic DNA fingerprinting and 16S rRNA gene sequence analysis. *Applied and Environmental Microbiology* 70:1277-1286.

Liu, W.-T., Marsh, T., Cheng, H. and Forney, L. **1997**. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology* 63:4516-4522.

Ludwig, W., Strunk, O., Westram, R., and 29 other authors. **2004**. ARB: a software environment for sequence data. *Nucleic Acids Research* 32:1363-1371.

Macy, J., Santini, J., Pauling, B., O'Neill, A. and Sly, L. **2000**. Two new arsenate/sulfate-reducing bacteria: mechanisms of arsenate reduction. *Archives of Microbiology* 173:49-57.

Madigan, M., Martinko, J. and Parker, J. **2000**. *Brock Biology of Microorganisms.* Ninth Edition, Prentice-Hall Inc., Upper Saddle River, New Jersey, USA.

Maidak, B., Cole, J., Lilburn, T., Parker, C. Jr., Saxman, P., Farris, J., Garrity, G., Olsen, G., Pramanik, S., Schmidt, T. and Tiedje, J. **2001.** The RDP-II (Ribosomal Database Project). *Nucleic Acids Research* 29:173-174.

Marsh, T. **1999**. Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. *Current Opinion in Microbiology* 2:323-327.

Marteinsson, V., Hauksdóttir, S., Hobel, C., Kristmannsdóttir, H., Hreggvidsson, G. and Kristjánsson, J. **2001**. Phylogenetic diversity analysis of subterranean hot springs in Iceland. *Applied and Environmental Microbiology* 67:4242-4248.

McCliment, E., Vogelsonger, K., O'Day, P., Dunn, E., Holloway, J. and Cary, S. **In Press**. Colonization of nascent, deep sea hydrothermal vents by a novel archaeal and nanoarchaeotal assemblage. *Environmental Microbiology*.

Miroshnichenko, M., Gongadze, G., Lysenko, A. and Bonch-Osmolovskaya, E. **1994**. *Desulfurella multipotens* sp. nov., a new sulphur-respiring thermophilic eubacterium from Raoul Island (Kermadec archipelago, New Zealand). *Archives of Microbiology* 161:88-93.

Mountain, B., Benning, L. and Boerema, J. **2003**. Experimental studies on New Zealand hot spring sinter: rates of growth and textural development. *Canadian Journal of Earth Sciences* 40:1643-1667.

Muyzer, G., Waal, E. and Uitterlinden, A. **1993**. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59:695-700.

Nakagawa, T. and Fukui, M. **2003**. Molecular characterization of community structures and sulfur metabolism within microbial streamers in Japanese hot springs. *Applied and Environmental Microbiology* 69:7044-7057.

Nakagawa, S., Takai, K., Horikoshi. K. and Sako, Y. **2004**. *Aeropyrum camini* sp. nov., a strictly aerobic, hyperthermophilic archaeon from a deep-sea hydrothermal vent chimney. *International Journal of Systematic and Evolutionary Microbiology* 54:329-335.

Nakayama, H., Morinaga, Y., Nomura, N., Nunoura, T., Sako, Y. and Uchida, A. **2003**. An archaeal homing endonuclease I-*Pog*I cleaves at the insertion site of the neighbouring intron, which has no nested open reading frame. *FEBS Letters* 544:165-170.

Nercessian, O., Reysenbach, A.-L., Prieur, D. and Jeanthon, C. **2003**. Archaeal diversity associated with in situ samplers deployed on hydrothermal vents on the East Pacific Rise (13 degrees N). *Environmental Microbiology* 5:492-502.

Nercessian, O., Prokofeva, M., Lebedinski, A., L'Haridon, S., Cary, S., Prieur, D. and Jeanthon, C. **2004**. Design of 16S rRNA-targeted oligonucleotide probes for detecting cultured and uncultured archaeal lineages in high-temperature environments. *Environmental Microbiology* 6:170-182.

Neufield, J., Yu, Z., Lam, W. and Mohn, W. **2004**. Serial analysis of ribosomal sequence tags (SARST): a high-throughput method for profiling complex microbial communities. *Environmental Microbiology* 6:131-144.

Nomura, N., Sako, Y. and Uchida, A. **1998**. Molecular characterization and postsplicing fate of three introns within the single rRNA operon of the hyperthermophilic archaeon *Aeropyrum pernix* K1. *Journal of Bacteriology* 180:3635-3643.

Nomura, N., Morinaga, Y., Kogishi, T., Kim, E., Sako, Y. and Uchida, A. **2002**. Heterogenous yet similar introns reside in identical positions of the rRNA genes in natural isolates of the archaeon *Aeropyrum pernix*. *Gene* 295:43-50.

Nübel, U., Bateson, M., Vandieken, V., Wieland, A., Kühl, M. and Ward, D. **2002**. Microscopic examination of distribution and phenotypic properties of phylogenetically diverse *Chloroflexaceae*-related bacteria in hot spring microbial mats. *Applied and Environmental Microbiology* 68:4593-4603.

Page, R. **1996**. TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* 12:357-358.

Pancost, R., Pressley, S., Coleman, J., Benning, L. and Mountain, B. **2005**. Lipid biomolecules in silica sinters: indicators of microbial biodiversity. *Environmental Microbiology* 7:66-77.

Papke, R., Ramsing, N., Bateson, M. and Ward, D. **2003**. Geographical isolation in hot spring cyanobacteria. *Environmental Microbiology* 5:650-659.

Patel, B., Morgan, H. and Daniel, R. **1985**. *Fervidobacterium nodosum* gen. nov. and spec. nov., a new chemoorganotrophic, caldoactive, anaerobic bacterium. *Archives of Microbiology* 141:63-69.

Patel, B., Morgan, H., Wiegel, J. and Daniel, R. **1987**. Isolation of an extremely thermophilic chemoorganotrophic anaerobe similar to *Dictyoglomus thermophilum* from New Zealand hot springs. *Archives of Microbiology*. 147:21-24.

Prokofeva, M., Miroshnichenko, M., Kostriina, N., Chernyh, N., Kuznetsov, B., Tourova, T. and Bonch-Osmolovskaya, E. **2000**. *Acidilobus aceticus* gen. nov., sp. nov., a novel anaerobic thermoacidophilic archaeon from continental hot vents in Kamchatka. *International Journal of Systematic and Evolutionary Microbiology* 50:2001-2008.

Rainey, F., Donnison, A., Janssen, P., Saul, S., Rodrigo, A., Bergquist, P., Daniel, R., Stackebrandt, E. and Morgan, H. **1994**. Description of *Caldicellulosiruptor saccharolyticus* gen. nov., sp. nov: An obligately anaerobic, extremely thermophilic, cellulolytic bacterium. *FEMS Microbiology Letters* 120:263-266.

Reysenbach, A.-L., Giver, L., Wickham, G. and Pace, N. **1992.** Differential amplification of rRNA genes by polymerase chain reaction. *Applied and Environmental Microbiology* 58:3417-3418.

Reysenbach, A.-L., Wickham, G. and Pace, N. **1994**. Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone National Park. *Applied and Environmental Microbiology* 60:2113-2119.

Reysenbach, A.-L., Ehringer, M. and Hershberger, K. **2000a.** Microbial diversity at 83°C in Calcite springs, Yellowstone National Park: another environment where the *Aquificales* and "Korarchaeota" coexist. *Extremophiles* 4:61-67.

Reysenbach, A.-L., Banta, A., Boone, D., Cary, S. and Luther, G. **2000b.** Microbial essentials at hydrothermal vents. *Nature* 404:835.

Reysenbach, A.-L. and Cady, S. **2001**. Microbiology of ancient and modern hydrothermal systems. *TRENDS in Microbiology* 9:79-86.

Rinehart, J. **1980**. *Geysers and geothermal energy*. Springer-Verlag, New York, USA.

Ronimus, R. **1993**. *Investigation into the control of genome topology in the archaeal isolate AN1*. Ph.D. Thesis, University of Waikato.

Ronimus, R., Parker, L. and Morgan, H. **1997**. The utilization of RAPD-PCR for identifying thermophilic and mesophilic *Bacillus* species. *FEMS Microbiology Letters* 147: 75-79.

Rueckert, A., Ronimus, R. and Morgan, H. **2005**. Development of a rapid detection and enumeration method for thermophilic bacilli in milk powders. *Journal of Microbiological Methods* 60:155-167.

Ruff-Roberts, A., Kuenen, J. and Ward, D. **1994**. Distribution of cultivated and uncultivated Cyanobacteria and *Chloroflexus*-like bacteria in hot spring microbial mats. *Applied and Environmental Microbiology* 60:697-704.

Rusch, A. and Amend, J. **2004**. Order-specific 16S rRNA targeted oligonucleotide probes for (hyper)thermophilic archaea and bacteria. *Extremophiles* 8:357-366.

Sako, Y., Nomura, N., Uchida, A., Ishida, Y., Morii, H., Koga, Y., Hoaki, T. and Maruyama, T. **1996**. *Aeropyrum pernix* gen. nov., sp. nov., a novel aerobic hyperthermophilic archaeon growing at temperatures up to 100°C. *International Journal of Systematic Bacteriology* 46:1070-1077.

Sako, Y., Nunoura, T. and Uchida, A. **2001**. *Pyrobaculum oguniense* sp. nov., a novel facultatively aerobic and hyperthermophilic archaeon growing at up to 97°C. *International Journal of Systematic and Evolutionary Microbiology* 51:303-309.

Sambrook, J. and Russell, D. **2001**. *Molecular Cloning. A Laboratory Handbook.* 3rd Edition. Cold Spring Harbour Laboratory Press, New York, USA.

Santegods, C., Nold, S. and Ward, D. **1996**. Denaturing gradient gel electrophoresis used to monitor the enrichment culture of aerobic chemoorganotrophic bacteria from a hot spring cyanobacterial mat. *Applied and Environmental Microbiology* 62:3922-3928.

Schleper, C., Pühler, G., Klenk, H.-P., Zillig, W. **1996**. *Picrophilus oshimae* and *Picrophilus torridus* fam. nov., gen. nov., sp. nov., two species of hyperacidophilic, thermophilic, heterotrophic, aerobic archaea. *International Journal of Systematic Bacteriology* 46:814-816.

Schleper, C., Jurgens, G. and Jonuscheit, M. **2005**. Genomic studies of uncultivated archaea. *Nature Reviews Microbiology* 3:479-488.

Schrenk, M., Kelley, D., Delaney, J. and Baross, J. **2003**. Incidence and diversity of microorganisms within the walls of an active deep sea sulphide chimney. *Applied and Environmental Microbiology* 69:3580-35929.

Schwieger, F. and Tebbe, C. **1998**. A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. *Applied and Environmental Microbiology* 64:4870-4876.

Segerer, A., Neuner, A., Kristjansson, J. and Stetter, K. **1986**. *Acidianus infernus* gen. nov., sp. nov., and *Acidianus brierleyi* comb. nov.: facultatively aerobic, extremely acidophilic thermophilic sulfur-metabolizing archaebacteria. *International Journal of Systematic Bacteriology* 36:559-564.

Segerer, A., Trincone, A., Gahritz, M. and Stetter, K. **1991**. *Stygiolobus azoricus* gen. nov., sp. nov. represents a novel genus of anaerobic, extremely thermoacidophilic archaebacteria of the order Sulfolobales. *International Journal of Systematic Bacteriology* 41:495-501.

Shock, E. **1996**. Hydrothermal systems as environments for the emergence of life. In, *Evolution of Hydrothermal Ecosystems on Earth (and Mars?).* Ciba Foundation Symposium 202. Edited by, Bock, G. and Goode, J. John Wiley & Sons Ltd., Great Britain.

Shroll, L. **1997**. *The development of a randomly amplification of polymorphic DNA (RAPD) method for the Bacteria, Thermus.* M.Sc. Thesis, University of Waikato.

Simon, H., Dodsworth, J. and Goodman, R. **2000**. Crenarchaeota colonize terrestrial plant roots. *Environmental Microbiology* 2:495-505.

Skirnisdottir, S., Hreggvidson, G., Hjörleifsdottir, S., Marteinsson, V., Petursdottir, S., Holst, O. and Kristjansson, J. **2000**. Influence of sulfide and temperature on species composition and community structure of hot spring microbial mats. *Applied and Environmental Microbiology* 66:2835-2841.

Slobodkin, A., Reysenbach, A.-L., Strutz, N., Dreier, M. and Wiegel, J. **1997**. *Thermoterrabacterium ferrireducens* gen. nov., sp. nov., a thermophilic anaerobic dissimilatory Fe(III)-reducing bacterium from a continental hot spring. *International Journal of Systematic Bacteriology* 47:541-547.

Slobodkin, A., Campbell, B., Cary, S., Bonch-Osmolovskaya, E. and Jeanthon, C. **2001**. Evidence for the presence of thermophilic Fe(III)-reducing microorganisms in deep-sea hydrothermal vents at 13°N (East Pacific Rise). *FEMS Microbiology Ecology* 36:235-243.

Spear, J., Walker, J., McCollom, T. and Pace, N. **2005**. Hydrogen and bioenergetics in the Yellowstone geothermal ecosystem. *Proceedings of the National Academy of Sciences* 102:2555-2560.

Stetter, K., Thomm, M., Winter, J., Wildgruber, G., Huber, H., Zillig, W., Jane-Covic, D., König H., Palm, P. and Wunderl, S. **1982**. Validation List N° 8. *International Journal of Systematic Bacteriology* 32:266-268.

Stetter, K., König, H. and Stackebrandt, E. **1983**. Pyrodictium gen. nov., a new genus of submarine disc-shaped sulphur reducing archaebacteria growing optimally at 105 °C. *Systematic and Applied Microbiology* 4:535-551.

Stetter, K. and Huber, H. **1986**. Validation List N° 22. *International Journal of Systematic Bacteriology* 36:573-576.

Stetter, K. **1996**. Hyperthermophilic prokaryotes. *FEMS Microbiology Reviews* 18:149-158.

Stetter, K. **1998a**. Hyperthermophiles: Isolation, classification and properties. In, *Extremophiles: Microbial life in extreme environments* 1-24. Edited by Horikoshi, K. and Grant, W. Wiley-Liss, John Wiley & Sons, Inc., New York, USA.

Stetter, K. **1998b**. *Archaeglobus fulgidus* gen. nov., sp. no.,: a new taxon of extremely thermophilic archaebacteria. *Systematic and Applied Microbiology* 10:172-173.

Stetter, K. **2001**. Genus VII. *Thermodiscus* gen. nov. In: Bergey's Manual of Systematic Bacteriology, second edition, vol. 1 (The *Archaea* and the deeply branching and phototrophic *Bacteria*). Edited by, Boone, D., Castenholtz, R. and Garrity, G. Springer-Verlag, New York, USA.

Strathmann, M., Griebe, T. and Flemming, H.-C. **2000**. Artificial biofilm model – a useful tool for biofilm research. *Applied Microbiology and Biotechnology* 54:231-237.

Suzuki, M. and Giovannoni, S. **1996**. Bias caused by template annealing in the amplification mixtures of 16S rRNA genes by PCR. *Applied and Environmental Microbiology* 62:625-630.

Takacs, C. D., Ehringer, M., Favre, R., Cermola, M., Eggertsson, G., Palsdottir, A. and Reysenbach, A.-L. **2001**. Phylogenetic characterization of the blue filamentous bacterial community from an Icelandic geothermal spring. *FEMS Microbiology Ecology* 35:123-128.

Takai, K. and Horikoshi, K. **1999**. Molecular phylogenetic analysis of archaeal intron-containing genes coding for rRNA obtained from a deep-subsurface geothermal water pool. *Applied and Environmental Microbiology* 65:5586-5589.

Takai, K. and Sako, Y. **1999**. A molecular view of archaeal diversity in marine and terrestrial hot water environments. *FEMS Microbiology Ecology* 28:177-188.

Takai, K., Komatsu, T., Inagaki, F. and Horikoshi, K. **2001**. Distribution of archaea in a black smoker chimney structure. *Applied and Environmental Microbiology* 67:3618-3629.

Van der Meer, M., Schouten, S., de Leeuw, J. and Ward, D. **2000**. Autotrophy of green non-sulphur bacteria in hot spring microbial mats: biological explanations for isotopically heavy organic carbon in the geological record. *Environmental Microbiology* 2:428-435.

Völkl, P., Huber, R., Drobner, E., Rachel, R., Burggraf, S., Trincone, A. and Stetter, K. **1993**. *Pyrobaculum aerophilum* sp. nov., a novel nitrate-reducing hyperthermophilic archaeum. *Applied and Environmental Microbiology* 59:2918-2926.

Wang, G. and Wang, Y. **1997**. Frequency of formation of chimeric molecules as a consequence of PCR coamplification of 16S rRNA genes from mixed bacterial genomes. *Applied and Environmental Microbiology* 63:4645-4650.

Ward, D., Weller, R. and Bateson, M. **1990**. 16S rRNA sequences reveal uncultured inhabitants of a well-studied thermal community. *FEMS Microbiology Reviews* 75:105-116.

Waters, E., Hohn, M., Ahel, I., Graham, D., Adams, M., Barnstead, M., Beeson, K., Bibbs, L., Bolanos, R., Keller, M., Kretz, K., Lin, X., Methur, E., Ni, J., Podar, M., Richardson, T., Sutton, G., Simon, M., Söll, D., Stetter, K., Short, J. and Noordewier, M. **2003**. The genome of *Nanoarchaeum equitans*: Insights into early archaeal evolution and derived parasitism. *Proceedings of the National Academy of Sciences* 100:12984-12988.

Whitaker, R., Grogan, D. and Taylor, J. **2003.** Geographic barriers isolate endemic populations of hyperthermophilic archaea. *Science* 301:976-977.

Williams, H. and McBirney, A. **1979**. *Volcanology*. Freeman, Cooper and Company, San Francisco, USA.

Wimpenny, J., Manz, W. and Szewzyk, U. **2000**. Heterogeneity in biofilms. *FEMS Microbiology Reviews* 24:661-671.

Yamamoto, H., Hiraishi, A., Kato, K., Chiura, H., Maki, Y. and Shimizu, A. **1998**. Phylogenetic evidence for the existence of novel thermophilic bacteria in hot spring sulfur-turf microbial mats in Japan. *Applied and Environmental Microbiology* 64:1680-1687.

Zillig, W., Stetter, K., Schäfer, W., Janekovic, D., Wunderl, S., Holz, I. and Palm, P. **1982**. Validation List N° 8. *International Journal of Systematic Bacteriology* 32:266-268.

Zillig, W., Gierl, A., Schreiber, G., Wunderl, S., Janekovic, D., Stetter, K. and Klenk, H. **1983a**. The archaebacterium *Thermophilum pendens* represents a novel genus of the thermophilic, anaerobic sulfur respiring *Thermoproteales*. *Systematic and Applied Microbiology* 4:79-87.

Zillig, W., Holz, I., Janekovic, D., Schäfer, W. and Reiter, W. **1983b**. The archaebacterium *Thermococcus celer* represents a novel genus within the thermophilic branch of the archaebacteria. *Systematic and Applied Microbiology* 4:88-94.

Zillig, W., Stetter, K., Prangishvilli, D., Schäfer, W., Wunderl S., Janekovic D., Holz, I. and Palm, P. **1983c**. Validation List N° 10. *International Journal of Systematic Bacteriology* 33:438-440.

Zillig, W., Holz, I., Janekovic, D., Klenk, H.-P., Imsel, E., Trent, J., Wunderl, S., Forjaz, H., Countinho, R. and Ferreira, T. **1990**. *Hyperthermus butylicus,* a hyperthermophilic sulfur-reducing archaebacterium that ferments peptides. *Journal of Bacteriology* 172:3959-3965.