The Phylogeny of Prokaryotes Associated with Australia's Great Artesian Basin

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the School of Biomolecular and Biomedical Sciences, Faculty of Science, Griffith University.

Mark D. Spanevello, B. App. Sci. (Biotech.) Hons
School of Biomolecular and Biomedical Science
Faculty of Science and Technology
Griffith University, Nathan Campus
Queensland, Australia

November, 2001.

The Phylogeny of Prokaryotes Associated with Australia's Great Artesian Basin

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the School of Biomolecular and Biomedical Sciences, Faculty of Science, Griffith University.

Mark D. Spanevello, B. App. Sci. (Biotech.) Hons
School of Biomolecular and Biomedical Science
Faculty of Science and Technology
Griffith University, Nathan Campus
Queensland, Australia

November, 2001.

STATEMENT OF ORIGINALITY

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Mark D. Spanevello

ACKNOWLEDGMENTS

I thank Associate Professor Bharat Patel for the opportunity to work with him. His advice, support and assurance gave me enthusiasm and confidence during my research.

I appreciate the advice, assistance and friendship of Mr Ben Mijts, Dr David Innes, Dr Hok-Sin (Tony) Woo, Dr Kathy Andrews, Ms Sungwan Kanso, Mr Marwan Abu-Halaweh, Mr Lyle McMillen, Mrs Kathy Hampson, and Ms Brenda Cheung throughout the course of my PhD. The visitors from France, Dr Bernard Ollivier, Dr Jean-Luc Cayol, Dr Cathy Joulian, and Ms Monica Bonilla are mentioned for their friendship and discussions.

I acknowledge the Department of Primary Industries and the staff at Terrick Terrick Stud for their help and assistance on site.

I gratefully acknowledge the financial support from The Australian Research Council Project Grant for my Ph.D. program and the internal funding provided by Griffith University.

All my friends who continued asking "So, how far to go now?" are mentioned for their ongoing companionship.

Finally, I thank my family for their continued interest and encouragement in all that I have done and will do, and my wife Felicity for her enduring patience, love and support during this time.

PUBLICATIONS AND PROCEEDINGS ARISING FROM THIS THESIS

PUBLICATIONS IN PREPARATION

Spanevello, M. and Patel, B.K.C. *Thermoaerobacter subterreaneus* sp. nov., from the subterranean waters of the Great Artesian Basin of Australia, *International Journal of Systematic and Evolutionary Bacteriology*.

Spanevello, M. and Patel, B.K.C. Molecular ecology of 5 different microbial communities colonizing the outflow of a subterranean Great Artesian Basin aquifer bore, *Applied and Environmental Microbiology*

Spanevello, M. and Patel, B.K.C. The phylogenetic diversity of *Thermus* and *Meiothermus* from microbial mats of an Australian subsurface aquifer runoff channel, *FEMS Microbiology Ecology*.

CONFERENCE PROCEEDINGS

Spanevello M., Patel, B.K.C. and Ollivier, B. (1998). Microbial diversity of the thermal waters emitted from the deep subterrestrial aquifer, the Great Artesian Basin of Australia. Thermophiles '98, 6-11 September, Brest, France

Spanevello, M. and Patel, B.K.C. (2000). The molecular community diversity of the Great Artesian Basin of Australia. Ninth International Congress for Culture Collections, 23-28 July, Brisbane, Australia.

ABSTRACT

The Great Artesian Basin of Australia is the largest freshwater artesian basin in the world. It underlies arid and semi-arid regions of Queensland, New south Wales, South Australia and the Northern territory, approximately 20% of the Australian continent. Temperatures of its' waters range from 30ºC to over 100ºC and over 5000 bores access its waters and empty into open drainage systems for agricultural irrigation and stock watering purposes. The Great Artesian Basin of Australia has great economic and agricultural importance, but prokaryotic communities persisting in the bore waters influence its' quality.

To gain an understanding of these prokaryotic communities, a culture-independent study was conducted on microbial communities present in the outflow of the New Lorne bore (registered bore number 17263). Five distinct prokaryotic communities existing at different temperatures (88ºC, 75ºC, 66ºC, 57ºC, and 52ºC) were selected and total genomic DNA was extracted. PCR-amplified 16S rRNA genes were subsequently cloned and a total of 64 clones from the 88ºC community and 96 clones from the other communities were examined. Partial sequences identified phylotypes that were then fully sequenced and analysed phylogenetically. The analysis revealed identical phylotypes existing in adjacent communities, as well as an increase in the phylogenetic diversity as water temperature decreased. Sequences identified belonged to species spanning the full diversity of the *Bacterial* domain, including *Hydrogenobacter*, *Thermus*, *Meiothermus*, *Chloroflexus*, *Cytophaga*, *Planctomycetes*, *Rhodothermus*, *Bacillus*, *Clostridium*, *Nitrospira*, *Verrucomicrobium*, *Acidobacterium*, α-, β-, γ-, and δ-*Proteobacteria*. Clones recovered also grouped with taxa with no isolated representatives. Of the libraries, 24 phylotypes from 6 phyla had a similarity of 96% or more to cultured isolates and comprised 73% of all clones analysed. 34 phylotypes from 11 phyla had less than 96% similarity to cultured isolates, or were related to previously cloned 16S rRNA gene sequences, and comprised 27% of the clones analysed. This shows the prokaryotic ecology of the Great Artesian Basin environment includes a diverse range of many uncultured, novel species.

Previous studies on isolates of *Thermus* and *Meiothermus* have revealed a relationship between the taxonomic groups and the geographical sites of isolation. A survey of 14 *Thermus* and 2 *Meiothermus* isolates and 16S rRNA gene clone data from the New Lorne bore extends the geographical diversity of these two genera. *Thermus* was isolated from all four mat samples and were most dominant in the red mat at 66ºC. *Meiothermus* was only isolated from the red mat at 66ºC. 16S rRNA gene sequence analysis revealed that 13 of the 14 *Thermus* isolates were closely related to *T. igniterrae* (100% similarity) and one isolate was closely related to *Thermus* strain SRI-96 (99.1% similarity). Both *Meiothermus* had 100% similarity with *Meiothermus ruber*. The 16S rRNA gene study of the environment showed that *Thermus* dominated the grey mat (75ºC) followed by the red mat (66ºC) and green mat (57ºC), but was absent from the brown mat (52ºC). Four *Thermus* phylotypes were identified with *T. scotoductus* the most dominant, followed by *T. igniterrae*, *Thermus* strain SRI-248, and *T. oshimai*. *T. scotoductus* dominated over *T. igniterrae* in the grey mat library, and, only marginally, in the red mat. Phylotypes belonging to the genus *Meiothermus* were identified in the red, green and brown mats, but not in the grey coloured mats with 2 distinct phylotypes related to *M. ruber* and *M. cerberus*. The *M. ruber* phylotype was dominant in the red mat and the *M. cerberus* phylotype was dominant in the brown mat with *M. ruber* only slightly dominant over *M. cerberus* in the green mat. Based on 16S rRNA gene sequence analysis, isolates and clones were most similar to those obtained from hot springs in Iceland, perhaps demonstrating a ecological similarity between the Great Artesian Basin of Australia and Iceland's thermal environments. Similarity of biodiversity was low between *Thermus* and *Meiothermus* species identified from the Great Artesian Basin and other well-studied thermal environments such as Yellowstone National Park, USA and New Zealand.

From enrichment studies, a strictly aerobic, thermophilic, Gram-positive, spore-producing rod-shaped bacterium (2 - 10μm x 0.3μm), designated isolate C21^T (^{T} = type strain) was isolated from a sediment sample collected from the run-off channel of the New Lorne bore accessing the Great Artesian Basin of Australia. Isolate C21^T grew optimally at 70ºC

(temperature range for growth between of 55ºC and 80ºC) and a pH of 8.5 (pH growth range between 6 and 10.5) with a generation time of 90 minutes. The isolate is strictly heterotrophic and grew on yeast extract and/or tryptone as sole carbon and energy source(s). The growth of isolate C21^T was not improved with the addition of a variety of carbohydrates (sucrose, cellobiose, glucose, dextrin, amylopectin, chitin, xylan, carboxymethylcellulose, inositol, arabinose, mannose, fructose, gelatin, starch, amylose, galactose, dextrose, xylose, maltose, L-sorbose, and raffinose), organic acids (lactic acid, pyruvic acid, and benzoic acid), or casamino acids without either yeast extract or tryptone. The G+C content of the chromosomal DNA of isolate C21^T was 71mol%. A phylogenetic analysis of the 16S rRNA gene of isolate C21^T indicated that it was a member of the phylum *Firmicutes* clustering with *Thermaerobacter marianensis* (similarity of 98%). However, isolate C21^T differed from *T. marianensis* in a number of key physiological and phenotypic properties and based on the evidence isolate C21^T is designated *Thermaerobacter subterranea* sp. nov. (type strain C21^T = ATCC BAA-137).

To further understand the prokaryotic ecology of the Great Artesian Basin, the development of real-time PCR to detect and quantify environmental isolates of *Caloramator* was undertaken. Thermophilic isolates from the genus *Caloramator* within the phylum *Firmicutes* are readily isolated from drainage systems of the Great Artesian Basin of Australia. Adjacent hybridisation probes were designed to identify *Caloramator* strains. The real-time PCR was optimised by manipulating the PCR and the concentrations of the adjacent hybridisation probes. Real-time PCR enabled the detection of as little as 6fg of DNA in the 45 PCR cycles. The development of real-time PCR will provide the foundation of applying this technique to identify and monitor other, perhaps detrimental, members of prokaryotic communities in the environment.

The information provided by the examination of these communities has improved our understanding of the culturable and non-culturable members of the prokaryotic communities of the Great Artesian Basin of Australia and provides the basis for further ecological studies of the Great Artesian Basin.

TABLE OF CONTENTS

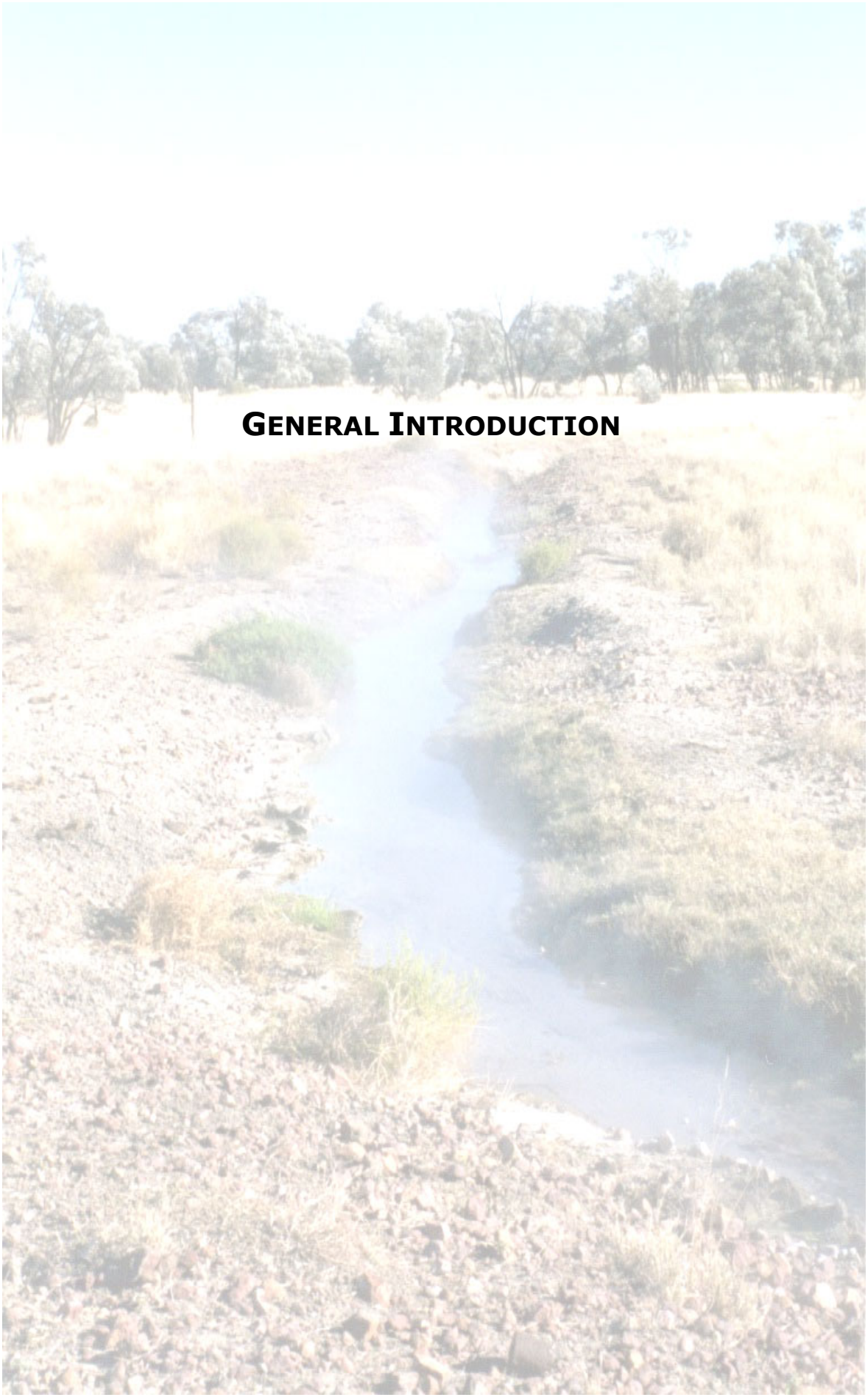
List of Figures

LIST OF TABLES

ABBREVIATIONS

A_{260}	absorbency at 260nm
A_{660}	absorbency at 660nm
aa	amino acid
ATP	adenosine 5'-triphosphate
ADP	adenosine 5'-diphosphate
blastn	basic local alignment search tool for nucleic acids
bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
cfu	colony forming unit
CTAB	hexadecyltrimethyl ammonium bromide
DGGE	denaturing gradient gel electrophoresis
dH_2O	deionised water
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dNTP	deoxynucleoside triphosphate
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
dTTP	deoxythymidine triphosphate
EDTA	ethylenediamine-tetraacetic acid
F	Faraday
FISH	fluorescent *in situ* hybridisation
FITC	fluorescein isothiocyanate
FRET	fluorescence resonance energy transfer
GAB	Great Artesian Basin
g	gram
hrs	hours
IPTG	isopropyl-β-D-thiogalactopyranoside
J	joules
JCM	Japan Collection of Microorganisms
L	litre

LB	Luria Bertani
M	molar
m	metre
mol	mole
mRNA	messenger ribonucleic acid
MW	molecular weight
N.D.	none detected
nt	nucleotide
Ω	ohm
PCR	polymerase chain reaction
rDNA	ribosomal deoxyribonucleic acid
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RNase	ribonuclease
RT	reverse transcriptase
S	siemens
str	strain
SSU	small sub unit
T	type strain
TAE	tris/acetate/ethylenediamine tetra-acetic acid (buffer)
Taq	*Thermus aquaticus* (DNA polymerase)
TE	tris/ethylenediamine tetra-acetic acid (buffer)
TEM	transmission electron microscopy
T_M	melting temperature
Tris	tris(hydroxymethyl)aminomethane
Tris-Cl	Tris-chloride (buffer)
tRNA	transfer ribonucleic acid
TYEG	trypticase peptone yeast extract glucose (media)
UPGMA	unweighted pair group method using arithmetic averages
UV	ultraviolet
V	volts
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside



GENERAL INTRODUCTION

INTRODUCTION

The phylogeny of prokaryotes has been greatly elucidated recently due to the large increases in studies of environmental communities. The use of the 16S rRNA gene as a molecular identification tool has enabled the characterisation of prokaryotic communities from a wide variety of ecosystems. Thermophilic communities (those that survive at high temperatures) are of particular importance as the search for the ancestry of modern life continues. Thermophilic members of the domains *Bacteria* and *Archaea* are the most deep branching organisms known, and are assumed to be the closest living relatives of the origin of life. In addition to providing phylogenetic data, prokaryotes surviving in extreme environments possess unique cellular adaptations that can be exploited biotechnologically.

Thermophilic communities from a wide variety of ecosystems have been examined including those associated with hot springs at Yellowstone National Park, USA, New Zealand, and Iceland, and marine communities associated with hydrothermal vents. The hydrochemistry of these sites is comparable, while the hydrochemistry of the Great Artesian Basin of Australia is unusual. Although the amount of research on the prokaryotic diversity of the Great Artesian Basin of Australia is relatively low, it has revealed a number of novel isolates that show that it provides a unique ecosystem for prokaryotes.

RESEARCH OBJECTIVES

The aim of this thesis is to characterise the prokaryotic communities associated with the thermal environment of the Great Artesian Basin of Australia. A literature review of the current status of prokaryotic diversity and approaches for studying prokaryotic communities is presented to highlight techniques and associated complications when characterising prokaryotic communities. The particular site of study (the New Lorne bore) was chosen due to a high outflow temperature (89ºC) and the presence of a open drain runoff system that allows a unique temperature gradient to

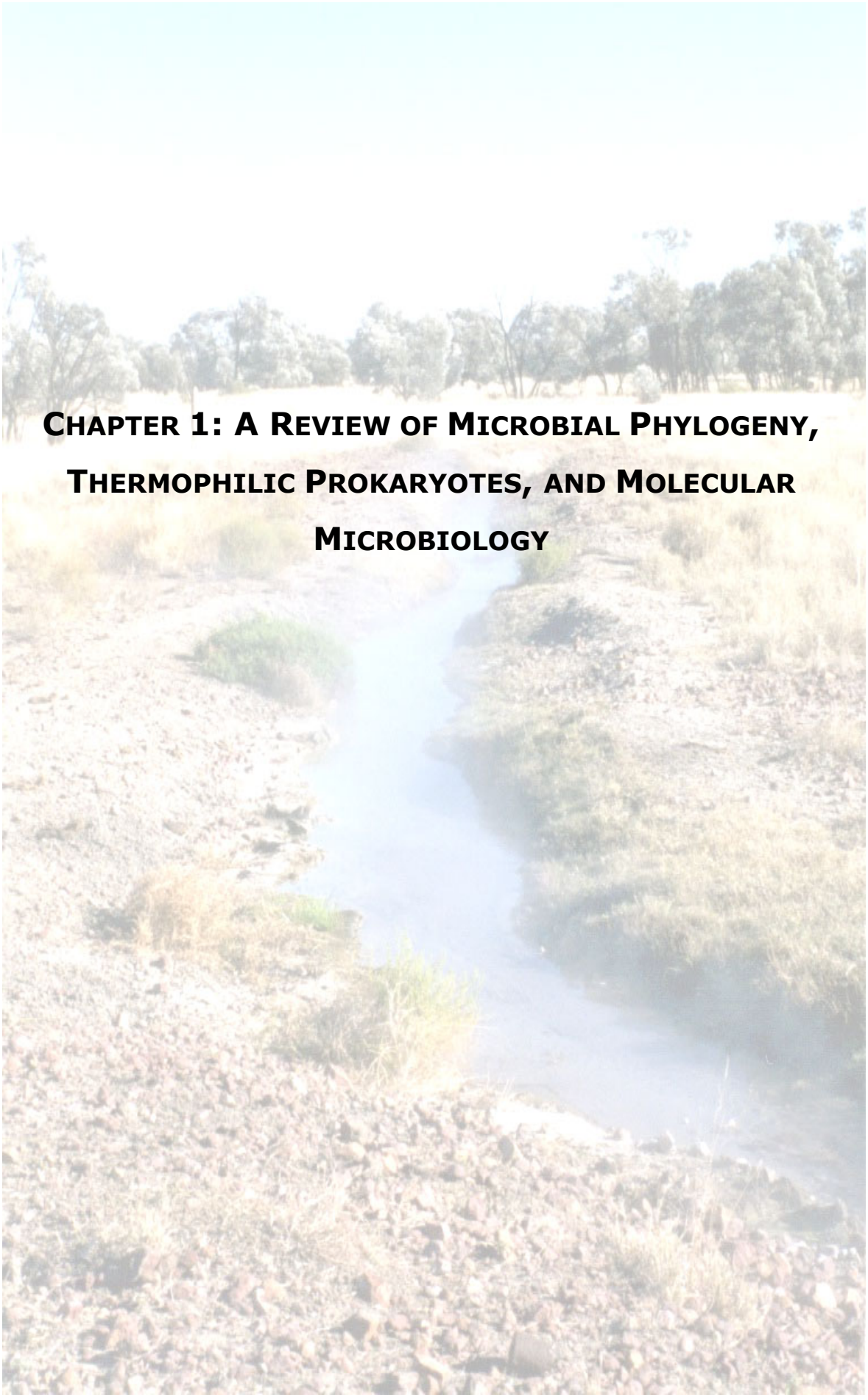
form. This temperature gradient allows the growth of associated, yet spatially distinct prokaryotic communities to develop.

To describe the prokaryotic communities present in this thermal environment, a combination of culture-independent and culture-dependent methods are used. A molecular approach based on the retrieval of 16S rRNA gene sequence data is used. This approach circumvents the inherent biases associated with traditional enrichment and isolation techniques that are used in characterising pure cultures. The communities present were phylogenetically characterised using 16S rRNA gene sequence data.

To further understand the ecological nature of the Great Artesian Basin, a survey of *Thermus* and *Meiothermus* isolates and 16S rRNA gene sequence data from the Great Artesian Basin is compared to other world-wide populations of *Thermus* and *Meiothermus.* As thermal environments are discontinuous throughout the world, it is theorised that these environments provide a unique opportunity to study the divergent evolution of prokaryotic strains that develop in separate thermal ecosystems. An analysis of the 16S rRNA gene sequence data presented increases our knowledge of the geographical restrictions of these species, and species selection by the environment.

Further enrichment studies isolated a novel obligately aerobic, Gram-positive *Bacterial* species, *Thermaerobacter subterraneus* str. C21. A complete characterisation of this isolate is carried out.

The real-time identification during PCR of environmental isolates belonging to the genus *Caloramator* is the first step in applying this new technology to microbial ecology. *Caloramator* is widespread in thermal environments and easily isolated. The use of real-time PCR has shown that it is very sensitive and specific. The development of new probes will enable the rapid identification and quantification of different members within prokaryotic communities, greatly enhancing the understanding of the ecology of prokaryotes.



Chapter 1: A Review of Microbial Phylogeny, Thermophilic Prokaryotes, and Molecular Microbiology

1.1 DIVERSITY: THE NEED FOR CLASSES

The classification of life is important in three aspects. It enables the prediction of characteristics for certain groups. It provides a basis for identification systems for new isolates, and it provides information regarding the origins and evolutionary pathways for life.

The use of *a priori* characters, that is, a character set chosen by the researcher, is purely subjective. Under this system, morphological characteristics were originally used to categorise living organisms into one of five kingdoms. The kingdom of *Monera* included all prokaryotes and the eukaryotes were divided into four other kingdoms. All single-celled eukaryotes were placed in the kingdom of *Protista*. The three other kingdoms of *Fungi*, *Plantae* and *Animalae* were comprised of multicellular eukaryotes differing in their mode of nutrition: absorption, autotrophism, or ingestion respectively. This system of classification was suitable for all organisms that showed great morphological diversity. With prokaryotes, however, this system was problematic due to their small size and low morphological diversity.

There are two schools of thought regarding the classification of prokaryotes – the phenetic and phylogenetic classification systems (Stackebrandt, 1988).

1.1.1 THE PHENETIC CLASSIFICATION SYSTEM

Phenetic classification relies on the physiological and genealogical similarities between organisms with no respect to the evolutionary pathways. It relies on a polythetic approach. Groupings have a high similarity in all measurable characters and the absence of a specific character will not remove individuals from a group. Traditional phenetic classification relied on observable characteristics like colony morphology and cell shape. As our knowledge of the diversity of the prokaryotic world increased, more information was required to correctly classify and identify isolates. Chemotaxonomic or molecular methods (Section 1.2.2) introduced

included metabolite and enzyme analysis, and DNA-DNA homology. This eliminated much of the uncertainty present in the systematics of prokaryotes.

1.1.2 THE PHYLOGENETIC CLASSIFICATION SYSTEM

Phylogenetic classification is based on the genealogical ancestry of organisms. Phylogenetic classification will mirror phenetic classification if there is no parallel or convergent evolution and the rate of change proceeds constantly along all lines of descent (Kyrpides & Olsen, 1999).

Phylogenetic classification was particularly advantaged with the introduction of PCR and DNA sequencing. Studying the sequence of conserved genes, Woese proposed that all life could be divided into the three domains of *Archaea*, *Bacteria*, and *Eukarya* (Winker & Woese, 1991; Woese *et al*., 1990). Table 1.1 shows a comparison of characters for the three domains. Figure 1.1 illustrates the phylogenetic relationship between the domains. The previous kingdom of *Monera* was split into *Archaea* and *Bacteria*, and the other four kingdoms of *Protista*, *Animalae*, *Plantae*, and *Fungi* were grouped into the domain *Eukarya*. It became possible to now study the phylogenetic and evolutionary relationships between prokaryotes easily.

Due to the high information content used to determined the phenetic relationship, it is more practical to the researcher, however, evolutionary pathways are not shown. On the other hand, phylogenetic classifications do not seem to be any more stable or predictive that phenetic classifications (Hartford & Sneath, 1988). To provide as much information as possible, both approaches need to be combined in classifying microorganisms (Stackebrandt, 1988). There is little ambiguity between the prokaryotic taxa whether defined phenetically or phylogenetically, so the use of both phenetic and phylogenetic data is preferred when characterising prokaryotes.

The discovery and use of DNA phylogenetic markers enables researchers to unambiguously compare DNA sequences and enhances the accuracy of the classification of prokaryotes. These markers are genes that are ubiquitous,

functionally conserved, and evolve with a constant rate of change e.g. rRNA and ATPase genes. Currently, a single gene sequence can identify the phylogenetic position of an unknown isolate. Hence assumptions about its phenotype and ecological importance in its environment can be made as its phenetic description can be deduced from its phylogenetic position (Bond *et al*., 1995; Hugenholtz *et al*., 1998a; Moffett *et al*., 2000; Reysenbach *et al*., 2000).

Table 1.1: Summary of the major differences between *Bacteria*, *Archaea* and *Eukarya*

Character	***Bacteria***	***Archaea***	***Eukarya***
Membrane bound nucleus	No	No	Yes
Circular and covalently closed DNA	Yes	Yes	No
Histones	No	Yes	Yes
Cell wall containing muramic acid	Yes	No	No
Membrane Lipid	Ester-linked	Ether-linked	Ester-linked
Ribosome	70S	70S	80S
Capping and poly-A tailing of mRNA	No	No	Yes
Initiator tRNA	Formyl-methionine	Methionine	Methionine
Introns in tRNA	No	Yes	Yes
Operons	Yes	Yes	No
Ribosomes sensitive to diptheria toxin	No	Yes	Yes
RNA Polymerase	One	Several	Three
Sensitive to: kanamycin, chloramphenicol, and streptomycin.	Yes	No	No
Chemolithotrophy	Yes	Yes	No
Growth above 80ºC	Yes	Yes	No

Adopted from Madigan *et al*. (2000)

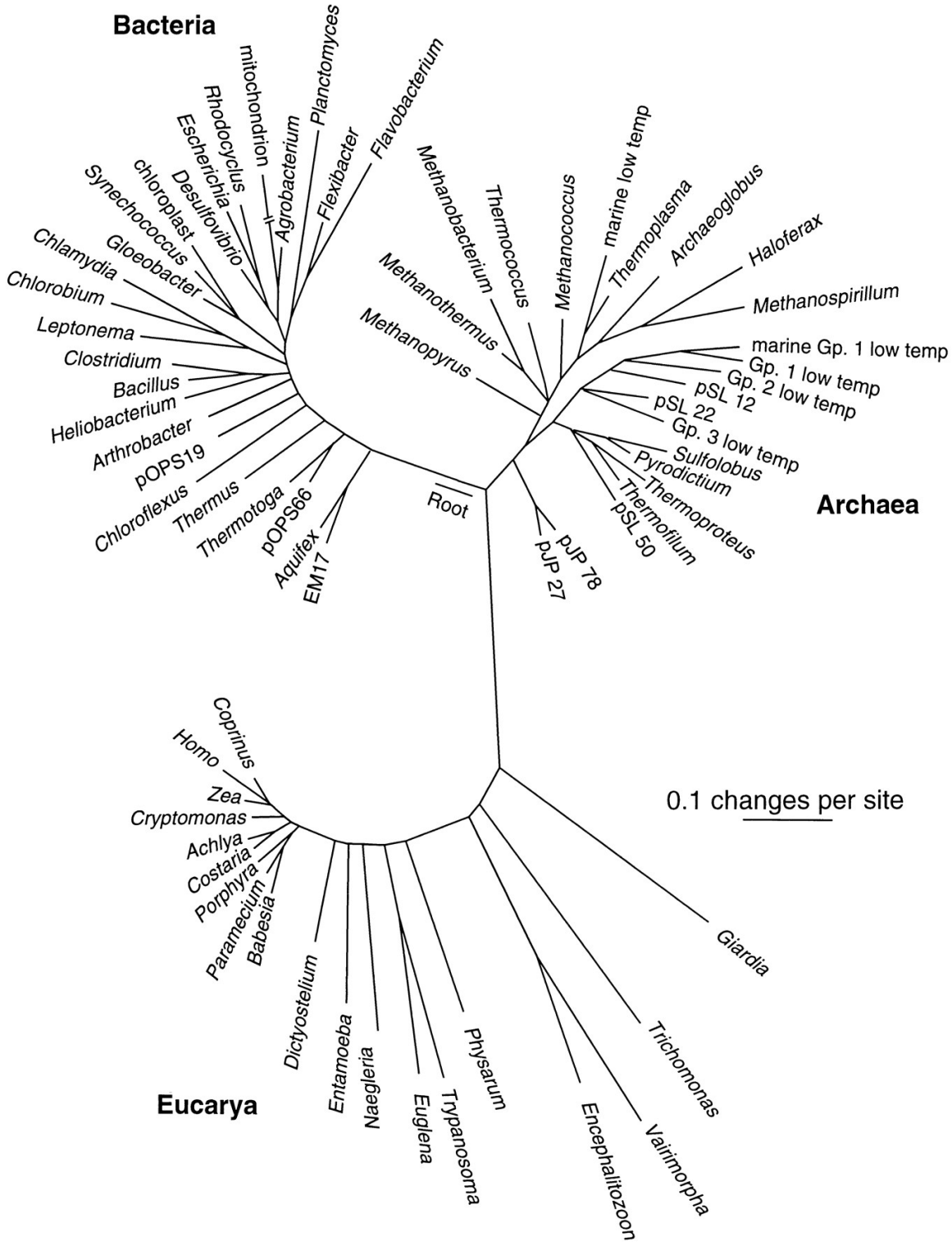


Figure 1.1: The Three Domain Tree of Life

The Three Domain Tree of Life based on the phylogenetic analysis of 16S rRNA gene sequence data. It shows a definitive separation of the domains of *Bacteria*, *Archaea*, and *Eukarya*. Taken from Pace (1997).

1.2 TAXONOMY AND PHYLOGENY

1.2.1 NUMERICAL TAXONOMY

Sneath & Sokal (1973) define numerical taxonomy as `the grouping by numerical methods of taxonomic units into taxa on the basis of their characteristics'. It requires the study of as many aspects of the organism as possible, and for prokaryotes includes such characters as colony morphology, cell morphology, growth characteristics, biochemistry, inhibitory tests, substrate utilisation, serology, chemotaxonomy, molecular genetics and bacteriophage typing. Computational methods are used to calculate similarity between different strains. The similarity is then used to order organisms into groups with high comparability. Hierarchical methods are then employed to place organisms into species, then genera, families, etc. Numerical taxonomy is based on a phenetic classification approach.

1.2.2 CHEMOTAXONOMY

Chemotaxonomy is a classification system based on cell chemical variations between organisms. It is a reflection of the genetic and morphological traits and is less subjective than some character studies used in numerical taxonomy (Hensel *et al*., 1986). It includes analysis on the chromosomal DNA (base composition, DNA-DNA reassociation, RFLP), rRNA (sequence, DNA-rRNA hybridisation), proteins (sequence, electrophoresis patterns), cell wall (peptidogylcan structure, polysaccharides, teichoic acids), membranes (fatty acids, polar lipids, mycolic acids) and metabolic end-products (fatty acids). In some cases an examination of the whole cell (prolysis followed by mass spectrometry) is used. Most chemotaxonomic methods are able to differentiate to the species level while being able to delineate the major divisions present. It is the ability of chemotaxonomical methods to offer fine specificity while covering wide taxonomic diversity that shows its' value in microbial systematics.

One of the major drawbacks of chemotaxonomy is that cell components are affected by environmental fluctuations e.g. lipid content of the cell wall is largely affected by temperature and/or salt concentration. The informative

content of the DNA is largely not affected by environmental changes. It offers the only opportunity to classify large numbers of organisms regardless of growth conditions.

A combination of numerical taxonomy and chemotaxonomy, termed polyphasic taxonomy (Vandamme *et al*., 1996) must be used to develop a taxonomic system for prokaryotes.

Phenotypic data e.g. cell morphology and structure is of limited use for phylogenetic purposes because the choice of early versus derived characters is purely subjective. Molecular phylogenies of genes or gene families can trace evolution or show evidence for the lateral transfer of genes. Given a constant rate of change, divergent evolution, and no lateral gene transfer, homologous sequences of DNA, RNA or proteins represent sequences from a common ancestor. Phylogenetic relatedness is given by the homology between sequences. A high homology indicates high relatedness and recent divergence, whereas low homology indicates low relatedness and an older divergence. The process of obtaining a phylogenetic tree used for classification is similar irrespective of the data set containing protein or DNA sequences.

1.3 PHYLOGENETIC ANALYSIS

1.3.1 SEQUENCE ALIGNMENT

To be able to construct phylogenies based on gene sequences, identical characters must be compared across all individuals. The sequence data must be aligned so that maximum homology is obtained. Gaps are inserted to allow for insertions or deletions. The number of mismatches therefore reflects the degree of divergence. There is no allowance for back mutations or multiple substitutions with regard to distantly related sequences. The conservative secondary structure of rRNA molecules (Figure 1.2) enables the straightforward identification of congruent regions within the gene. Alignment of these analogous regions facilitates the alignment of the remainder of the gene.

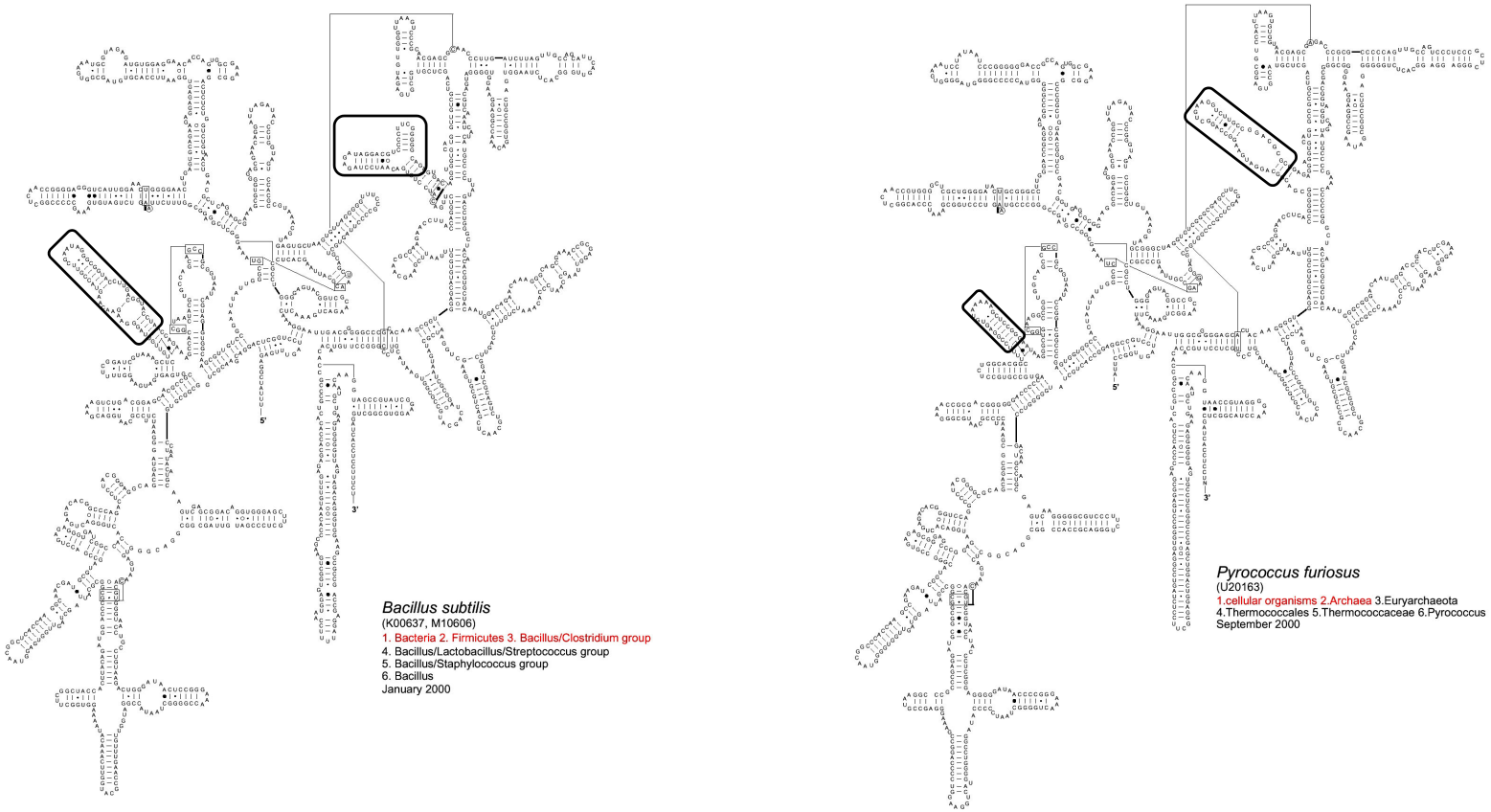


Figure 1.2: A comparison of the secondary structures for *Bacillus subtillus* (*Bacteria*) and *Pyrococcus furiosus* (*Archaea*)

Examples of secondary structure difference are boxed. The similarity of secondary structure allows the easy identification of comparative regions and facilitates alignment. Taken from www.rna.icmb.utexas.edu (Gutell *et al*., 2001).

1.3.2 PHYLOGENETIC RECONSTRUCTION

There are several methods to create a phylogenetic tree once the data has been aligned. Three most commonly used reconstructive methods are the distance matrix, maximum parsimony and maximum likelihood methods.

1.3.2.1 DISTANCE MATRIX METHODS

The distance matrix methods such as unweighted pair group method using arithmetic averages (UPGMA) clustering are based on algorithmic approaches (Saitou & Imanishi, 1989; Saitou & Nei, 1987). They are generally heavily influenced by the rates of evolution and divergence. Distance matrix methods are usually combined with neighbour analysis to determine the best possible tree. Distance matrix methods were developed from a phenetic base and led to a phenetic classification system. Distance matrix methods, however, have been applied to prokaryotic phylogeny using sequences from DNA, RNA and proteins. A major limitation of distance matrix methods is if evolution rates are neither constant nor divergent, this system will not mirror the phylogenetic classes (Grishin, 1999).

1.3.2.2 MAXIMUM PARSIMONY METHODS

Maximum parsimony analysis relies on the assumption that the true phylogeny requires the fewest mutations that accounts for the observed differences between individual sequences. The data set is reduced to only informative sites i.e. individual sites that favour only some of the possible trees. Minimum substations are calculated for each possible tree, and the tree with the least substitutions is the maximum parsimony tree. Maximum parsimony methods will give misleading results if the amount of evolution is unequal in the branches of the phylogenetic tree (Felsenstein, 1978).

1.3.2.3 MAXIMUM LIKELIHOOD METHODS

Maximum likelihood analysis calculates the possibility of observing the data set given a tree and a mathematical model for evolution. There are many

models that can be applied in this analysis e.g. the Markov chain model (Felsenstein, 1981; Schadt *et al*., 1998; Thorne *et al*., 1992). Since the model is invariant for all comparisons, the tree that maximises the probability that it fits the data is the maximum likelihood phylogeny. Maximum likelihood is the most statistically reliable method for phylogenetic inference. Limitations are based on the mathematical model used for evolution, but with the increase in computing speed and power, more complex models are being introduced (Schadt *et al*., 1998).

1.3.3 CONFIDENCE IN ANALYSIS

There are a number of methods available that infer statistical confidence for phylogenies produced. Felsenstein (1985) first applied bootstrapping to phylogenetic analysis. This test can be applied to many data sets or algorithms. It has the ability to test the monophyletic nature of groups of sequences (Brown, 1994).

Deeper branches observed in phylogenetic analysis may not reflect earlier divergence, but may represent a faster rate of evolution. To check that evolution rates are constant for all, a comparison to an outgroup is required (Kollman & Doolittle, 2000). The outgroup is a distant relative of the organisms studied and prior research shows that it has diverged prior to the group. If the organisms have evolved at a similar rate, they will show a similar level of homology to the outgroup. Once shown, an earlier branch point will identify earlier divergence (Baldauf *et al*., 1996).

1.4 SEQUENCES USED IN PHYLOGENY

In choosing which sequences to study several considerations must be made. The sequences must belong to genes that are highly conserved with a specific function. They must show a ubiquitous distribution and divergent evolution with no lateral gene transfer. Protein sequences play an important part in molecular phylogeny. rRNA genes, however, fulfil all these criteria, and for this reason are most widely used.

1.4.1 GENE SEQUENCES AND PHYLOGENY

There are three rRNA genes used in phylogenetic analysis. The 5S rRNA gene has been used in a number of studies (Bulygina *et al*., 1990; Hori & Osawa, 1979; Rogers *et al*., 1985; Stahl *et al*., 1985). However, due to its small size (≈120bp) the phylogenetic information gained is limited. The 16S rRNA gene has an approximate size of 1600bp. Numerous studies on the 16S rRNA gene have shown a variety of conserved regions within the gene (Lane *et al*., 1985; Winker & Woese, 1991). These conserved regions enable the full sequence to be determined easily and quickly via PCR. The increased use of the 16S rRNA gene to study phylogeny has led to large increases in sizes of 16S rRNA gene databases. The RDP has grown from 10,880 aligned SSU rRNA gene sequences on September 17, 1999 (v7.1) (Maidak *et al*., 1999) to 19,833 aligned SSU rRNA gene sequences on June 1, 2000 (v8.0) (Maidak *et al*., 2000). The 23S rRNA gene is less used. It is approximately 3000bp in length and although it contains more phylogenetic information, a lower number of conserved regions hinder the easy determination of its full sequence.

Phenograms based on rRNA gene sequences are providing a comprehensive overview of the relationships between prokaryotes. It confirms the unifying concepts of genus and higher ranked taxa, while showing possible evolutionary pathways linking organisms (Doolittle, 1999).

Recent studies have shown that pure isolates may possess and express different 16S rRNA genes with sequence similarities below 95% (Amann *et al*., 2000; Oren *et al*., 1999). This has implications in overestimating environmental prokaryotic diversity when using the 16S rRNA gene as a tool.

1.4.2 PROTEIN SEQUENCES AND PHYLOGENY

Cytochrome c sequences were one of the first protein sequences analysed (Margoliash & Smith, 1965). This research showed a distinct correlation between the fossil evolutionary record and the phylogenetic analysis undertaken of these sequences. Phylogeny based on protein sequences has

not had a large effect on prokaryote systematics. This is mainly due to the relatively small number of sequences available for comparison. The sequencing of proteins have also led to the discovery of protein families (e.g. globins), subfamilies (e.g. myoglobins and haemoglobins) and further variations (α-, β-, and δ-haemoglobins). For a correct phylogenetic analysis orthologous proteins must be compared i.e. α-haemoglobins must be compared to α-haemoglobins. Incorrect conclusions about the phylogeny of proteins will occur if paralogous proteins are compared i.e. α-haemoglobin to β-haemoglobins. Comparisons of paralogous proteins are important in phylogeny as they enable the definition of the root of the universal tree (Forterre & Philippe, 1999; Kollman & Doolittle, 2000).

Proteins such as EF-Tu (349aa) and the ATPase β-subunit (460aa) have also been used in phylogenetic studies (Baldauf *et al*., 1996; Ludwig *et al*., 1993). The amount of informational content carried in proteins is much less that in gene sequences due to the much shorter length of proteins. The phylogenetic relationships observed using protein sequences support the prokaryotic phylogeny as determined by 16S rRNA gene analysis (Ludwig & Schleifer, 1994). Any groups that did not reflect 16S rRNA gene phylogeny are usually explained by the reduced informative content of protein sequences.

The study of phylogenetic markers such as the 16S rRNA gene do not provide information regarding the potential physiological differences between closely related prokaryotes that may have an ecological effect. Studies on dissimilatory sulfate reductase genes have shown a similar phylogeny to that obtained with analysis of 16S rRNA genes. However, they reveal a greater genetic diversity that is very likely of ecological significance (Chang *et al*., 2001; Wagner *et al*., 1998).

1.4.3 GENOME SEQUENCES AND PHYLOGENY

The increase in interest in whole genome sequencing has added another dimension to phylogenetic analysis. At the time of writing, 49 microbial genomes (7 *Archaea*, 38 *Bacteria*, and 4 *Eukarya*) have been published with more than 150 currently under research (www.tigr.org). Research so far

indicates that there are few genes present across all genomes that are sufficiently similar to analyse (Ludwig & Schleifer, 1999). The topologies of trees defined using genes of different function follow closely that of 16S rRNA gene phylogeny (Snel *et al*., 1999). The inconsistencies observed however do raise questions. The most likely cause is the effect of horizontal gene transfer. The horizontal transfer of 16S rRNA genes cannot be excluded, but evidence leads to the conclusion that rRNA genes are not as transferable as other genes (Aravind *et al*., 1998; Boucher & Doolittle, 2000; Eisen, 2000; Martin, 1999).

1.5 DIVERSITY OF PROKARYOTES

The diversity within the prokaryotic domains is much greater than the eukaryotic group as prokaryotes are not restricted to a relatively limited environmental niche. Metabolically, prokaryotes are broadly broken into four groups. Photoautotrophs harness light energy to synthesis organic molecules from carbon dioxide. Photoheterotrophs use light energy to create ATP, but obtain carbon from organic sources. Chemoautotrophs obtain carbon from carbon dioxide and oxidise inorganic compounds to create ATP. Chemoheterotrophs consume organic compounds for both energy and carbon-sources. The majority of isolated prokaryotes are chemoheterotrophs. The greatest variety of metabolic pathways is within the chemoautotrophic group.

With prokaryotes possessing a wide range of nutritional modes, prokaryotic life is not limited to such "constrained" environments as eukaryotes. Wide ranges of nutritional modes allow the colonisation of more extreme environments. Their adaptations allow for growth at low or high temperatures (psychrophiles and thermophiles respectively), low and high pH (alkalophiles and acidophiles respectively), high salinity (halophiles), high pressure (barophiles), high substrate concentration (osmophiles), and low water availability (xerophiles). Prokaryotes adapted to toxic and recalcitrant compounds have also been described.

1.5.1 THE *BACTERIAL* DOMAIN

The *Bacterial* domain was first described as being comprised of around 12 natural divisions (Woese, 1987). With more 16S rRNA gene information becoming available, the current view contains 36 divisions with about a third of these characterised only by environmental sequences (Hugenholtz *et al*., 1998a) (Figure 1.3). Some of the major divisions with isolated and characterised representatives are discussed below.

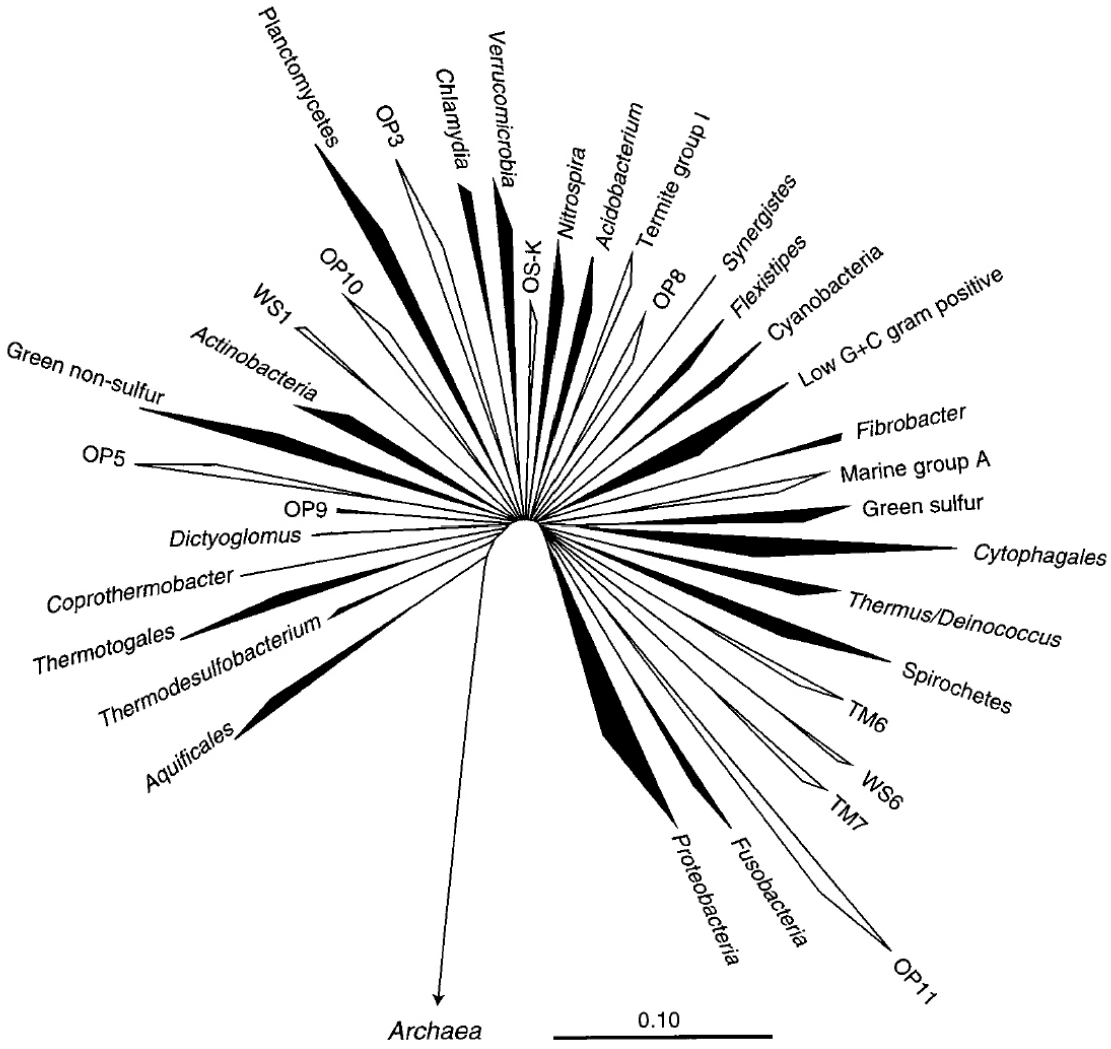


Figure 1.3: Phylogenetic divisions within the domain *Bacteria*

Evolutionary distance tree of the *Bacterial* domain showing currently recognized divisions and candidate divisions. Division-level groupings of two or more sequences are depicted as wedges. The depth of the wedge reflects the branching depth of the representatives selected for a particular division. Divisions that have cultivated representatives are shown in black; divisions represented only by environmental sequences are shown in outline. The scale bar indicates 0.1 change per nucleotide. Taken from Hugenholtz *et al*. (1998a).

1.5.1.1 *AQUIFICAE* (THERMOPHILIC HYDROGEN OXIDISERS)

The thermophilic hydrogen oxidisers contain hyperthermophilic and thermophilic, obligately chemolithotrophic autotrophs. They are microaerophilic and reside only in thermal environments. The complete genome sequence of *Aquifex aeolicus* shows homology with thermophilic *Archaea.* 16S rRNA gene sequence analysis indicates that it is the oldest lineage within the *Bacterial* domain (Bocchetta *et al*., 2000; Burggraff *et al*., 1992; Reysenbach *et al*., 2000). The physiological properties of both *Aquifex* and its closest *Archaeal* relative suggest a common ancestor required H_2 as an electron donor for metabolism (Huber *et al*., 1992).

1.5.1.2 *THERMOTAGALES*

The *Thermotagales* are strictly anaerobic, thermophilic and chemoheterotrophic with a fermentative metabolism (Fardeau *et al*., 1997). *Thermotogales* are named after a unique morphological feature – the presence of an outer sheath (or "toga") covering the cell. In *Fervidobacterium* species this sheath is distended at one pole (Patel *et al*., 1985). They are found in thermal terrestrial aquatic and marine environments.

1.5.1.3 *DEINOCOCCUS*/*THERMUS*

This division contains only three genera: *Thermus*, *Meiothermus*, and *Deinococcus*. *Deinococcus* is well known for its' ability to withstand high levels of radiation and a lack of an outer cell membrane (Ferreira *et al*., 1997). Phylogenetic analysis reveals a close association with the Gram-positive bacteria. *Thermus* and *Meiothermus* are well known thermophilic ecosystem inhabitants being isolated from Iceland, USA, Portugal, Australia, Italy and New Zealand (Brock & Freeze, 1969; Chung *et al*., 2000; Loginova *et al*., 1984; Manaia & da Costa, 1991; Santos *et al*., 1989). Recent work on *Thermus* isolates indicates that there is a geographical limitation on distribution of strains around the world (Moreira *et al*., 1995; Moreira *et al*., 1997). They are obligately aerobic with a chemoorganotrophic metabolism.

All genera within this group share an atypical cell wall in which diaminopimelic acid in the peptidoglycan is replaced by ornithine.

1.5.1.4 GREEN NON-SULFUR BACTERIA

The green non-sulfur bacteria are defined more by sequence analysis than numerical taxonomy. *Chloroflexus auranticus* is the most known species from this kingdom and has similarities to both the green sulfur bacteria and the *Proteobacteria*. It contains *bacteriochlorophyll c* in chlorosomes, like green sulfur bacteria, however its structure of *bacteriochlorophyll a* resembles that of the *Proteobacteria*. It is theorised that *Chloroflexus* may be very similar to a photosynthetic ancestor, but received chlorosome genes by lateral transfer (Gupta *et al*., 1999). *Chloroflexus* is unique in that it can carry out photoautotrotrophy and photoheterotrophy as well as chemoorganotrophy.

1.5.1.5 *PLANCTOMYCETES*

The *Planctomycetes* are a group of morphological distinct organisms. Their cell wall lacks peptidoglycan and is proteinaceous in nature. Primarily aquatic organisms, this group are typically facultatively aerobic possessing a chemoorganotrophic metabolism. Phylogenetic studies reveal a low homology with all other bacterial lineages and a rapidly evolving genome is thought to be the cause (Madigan *et al*., 2000).

1.5.1.6 *CHLAMYDIA*

The obligatory intracellular parasites belonging to the division *Chlamydia* have very limited biosynthetic capabilities. The relatively small genome of *Chlamydia trachomatis* (1Mbp) reflects it restrictive metabolic abilities and the eukaryotic nature of some genes suggest the horizontal transfer of eukaryotic genes into its genome (Madigan *et al*., 2000).

1.5.1.7 *CYANOBACTERIA*

The *Cyanobacteria* are distinguished by the common trait of *chlorophyll a*. Sequence analysis of *Cyanobacteria* show that their closest relatives are the chloroplasts, indicating that they share a common ancestor. The *Cyanobacteria* are generally obligately aerobic phototrophs. They are found in aquatic environments, and are often the dominant phototroph in hot spring environments (Ruff-Roberts *et al*., 1994; Ward *et al*., 1998).

1.5.1.8 GREEN SULFUR BACTERIA

The green sulfur bacteria are a phylogenetically coherent group of non-motile anoxygenic phototrophic bacteria. They are chemolithotrophic utilising H_2S as an electron donor. They use *bacteriochlorophyll a* to photosynthetically convert energy to ATP. Chlorosomes present near the cytoplasmic membrane contain one of the *bacteriochlorophylls c*, *d*, or *e* that act as light harvesting centres. Due to the effectiveness of the chlorosome, they require less light energy, and as a consequence are often found at greater depths than other photosynthetic bacteria (Madigan *et al*., 2000).

1.5.1.9 *FLAVOBACTERIUM*/*CYTOPHAGA*/*BACTEROIDES*

This prokaryotic assembly range from obligate aerobic to obligate anaerobic bacteria. *Bacteroides* are obligately anaerobic and are thought to be dominant in the intestinal gut of animals. *Flavobacterium* species are obligate aerobic prokaryotes that are generally found in aquatic habitats. Their chemoheterotrophic metabolism is limited to a small number of simple sugars (Madigan *et al*., 2000).

Cytophaga species are obligate aerobes and widespread in the environments of soil and water. They are able to degrade polysaccharides and move by a characteristic gliding motility. Their degradative activity is responsible for the majority of oxic degradation of cellulose (Madigan *et al*., 2000).

1.5.1.10 *SPIROCHAETES*

Spirochaetes form a morphologically and phylogenetically coherent group within the *Bacterial* domain. The coiling of endoplasmic flagella around the protoplasmic cylinder forms their characteristic spiral shape. *Spirochaetes* are found in aquatic habitats or as animal pathogens (Kudo *et al*., 1998).

1.5.1.11 GRAM-POSITIVE *BACTERIA*

The Gram-positive *Bacteria* form a distinct line of descent within the *Bacterial* domain. A major division separates the High G+C content Gram-positive groups from the Low G+C content groups. The High G+C content group is primarily aerobic rods to filament-shaped cells inhabiting soil and plant environments. *Mycobacterium* species are distinct due to the unique capability to produce mycolic acids. Another important member of this group is *Streptomyces*. Primarily soil organisms, they are best known for their ability to produce antibiotics. They have a versatile metabolism, being able to grow chemoheterotrophically on a wide variety of simple and compound substances (Roller *et al*., 1994).

The Low G+C content group is dominated by the two classes of *Bacilli* and *Clostridia*. The group is heterogeneous with members of the class *Bacilli* having values of G+C content that ranges over 40 percentile units. The endospore-formers are mainly soil inhabitants with wide metabolic activities. Physiological traits range from obligately aerobic, facultatively or obligately anaerobic, polysaccharide degradation, fermentation variety, acidophiles, alkalophiles, cocci to rod shaped-cells, and thermophiles (Collins *et al*., 1994; de Bartolomeo *et al*., 1991).

1.5.1.12 THE *PROTEOBACTERIA*

The *Proteobacterial* division is the most diverse among the *Bacterial* domain. Anoxygenic photosynthesis is prevalent within the *Proteobacterial* phylum. Photosynthesis is inhibited by oxygen as it represses photopigment synthesis. Their classification has been established by numerous physiological and phylogenetic studies (Gupta, 2000).

The α- and β- groups are heterogeneous groups with most being able to utilise a variety of organic compounds as an electron donor. Photoautotrophic growth is also possible. γ-*Proteobacteria* includes the purple sulfur bacteria and *Enterobacteriaceae*. The purple sulfur bacteria use H_2S as an electron donor for CO_2 reduction during photosynthesis. The purple bacteria are found in illuminated anoxic areas of aquatic habitats. The δ-*Proteobacterial* members are diverse and contain anaerobic sulfate reducers such as *Desulfovibrio* and *Desulfobacter* and the fruiting myxobacteria. *Campylobacter* and *Helicobacter* are two genera within the ε-*Proteobacteria*. Members of the ε-*Proteobacteria* are few in number, and are dominated by human disease causing prokaryotes.

As photosynthesis is widespread throughout the *Proteobacterial* lineage, it is thought that the last common ancestor was photosynthetic. This ability was lost through evolution and replaced by chemoautotrophic capabilities. In addition to the physiological activities mentioned, metabolic groups of the *Proteobacteria* include the sulfur and iron oxidising bacteria, hydrogen oxidizing bacteria, methanotrophic and methylotrophic bacteria, acetic acid bacteria and nitrogen fixers.

1.5.2 THE *ARCHAEAL* DOMAIN

By 16S rRNA gene sequence analysis, it has been shown that there is three main lines of descent within the *Archaeal* Domain: the *Korarchaeota*; the *Euryarchaeota*; and the *Crenarchaeota* (Barns *et al*., 1996) (Figure 1.4).

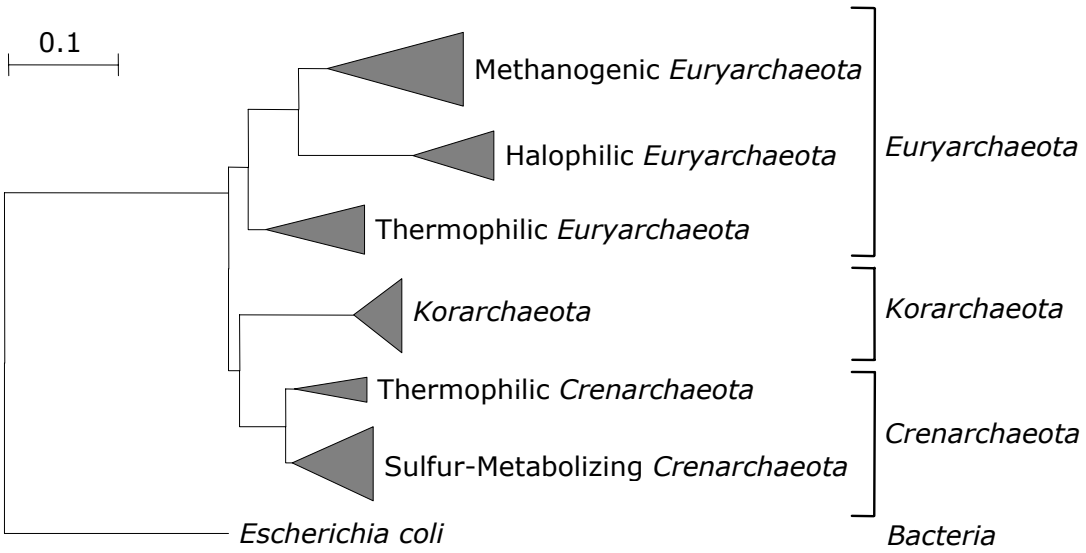


Figure 1.4: Phylogenetic divisions within the domain *Archaea*

Evolutionary distance tree of the *Archaeal* domain showing currently recognised divisions and candidate divisions. *Crenarchaeota* are represented by: *Thermoproteus neutrophilus* JCM 9278^T (AB009618); *Thermofilum pendens* str. Hvv3 DSM 2475^T (X14835*); Staphylothermus marinus* str. F1 DSM 3639^T (X99560); *Thermosphaera aggregans* str. M11TL DSM 11486^T (X99556); *Sulfolobus solfataricus* str. P1 DSM 1616^T (X90478); and *Sulfurisphaera ohwakuensis* str. TA-1 IFO 15161^T (D85507). *Euryarchaeota* are represented by: *Methanoplanus limicola* str. M3 DSM 2279^T (M59143); *Methanomicrobium mobile* str. BP DSM 1539^T (M59142); *Methanococcoides methylutens* str. TMA-10 DSM 2657^T (M59127); *Methanosarcina mazei* str. C16 ATCC 43340^T (M59138); *Haloferax volcanii* str. DS-2 ATCC 29605^T (K00421); *Halorubrum lacusprofundi* JCM 8891^T (U17365); *Natrinema pellirubrum* NCIMB 786^T (AJ002947); *Archaeoglobus fulgidus* str. VC-16 DSM 4304^T (X05567); *Thermococcus chitonophagus* str. GC74 DSM 10152^T (X99570); and *Methanopyrus kandleri* str. av19 DSM 6324^T (M59932). *Korarchaeota* are represented by: Unidentified *Korarchaeote* SRI-306 (AF255604); Unidentified *Korarchaeote* pJP78 (CNBRG16SD); Unidentified korarchaeote pBA5 (AF176347); and Unidentified korarchaeote pJP27 (CNBRG16SK). The evolutionary distance tree is based on an unambiguous data set containing 1179bp. Scale bar represents 10 nucleotide substitutions per 100bp. GenBank accession numbers are contained within parenthesis. The phylogenetic analysis was carried out as detailed in Section 2.15.

1.5.2.1 *KORARCHAEOTA*

The *Korarchaeota* are represented only by clone sequences, as no members of this group have been isolated. All sequences have been retrieved from geothermal habitats i.e. Yellowstone National Park (Reysenbach *et al*., 2000).

1.5.2.2 *EURYARCHAEOTA*

Three main physiological groups dominate the *Euryarchaeota*.

1.5.2.2a Halophilic *Euryarchaeota*

The halophilic *Euryarchaeota* such as *Haloferax* and *Natrinema* are uniquely adapted to hypersaline environments with many requiring salt concentrations near saturation (32%). Their physiology is quite diverse with most having an obligate aerobic, chemoorganotrophic metabolism. However, denitrification and autotrophism is also found. Their ability to withstand high salt concentration stems from their ability to intracellularly accumulate compatible solutes e.g. amino acids or K^+ ions (Benlloch *et al*., 1995).

1.5.2.2b Methanogenic *Euryarchaeota*

Based on 16S rRNA gene sequence analysis, the methanogenic *Euryarchaeota* are a phylogenetically diverse group of microorganisms and include the genera of *Methanoplanus* and *Methanomicrobium*. However, their metabolic characteristics are similar. They all derive methane from a variety of small compounds. Enzymes required for this task are very oxygen-sensitive, hence methanogenic *Euryarchaeota* are obligatory anaerobic. Methanogenesis is limited to the *Archaea*. They are found in a variety of environments ranging from intestinal tracts, anoxic sediments to geothermal environments (Schäfer *et al*., 1999).

1.5.2.2c Thermophilic *Euryarchaeota*

The thermophilic *Euryarchaeota* consist of a number of divergent species. *Thermoplasma* and *Picrophilus* are unique in their physiology. Both are thermophilic and extremely acidophilic. Hyperthermophilic *Euryarchaeota* includes *Thermococcus*, *Pyrococcus*, *Methanopyrus*, and *Archaeoglobus*. All are found to inhabit environments near hydrothermal vents with chemoorganotrophic or chemolithotrophic metabolisms (Barns *et al*., 1996; Hugenholtz *et al*., 1998b; Keller *et al*., 1995).

1.5.2.3 *CRENARCHAEOTA*

The *Crenarchaeota* is a diverse group of species that inhabit two extremes of the Earth's environments. 16S rRNA gene sequence analysis has shown that *Crenarchaeota* are found in a wide variety of non-thermal habitats including marine and rice paddies (Schleper *et al*., 1997). The better known groups of the *Crenarchaeota* are hyperthermophilic with members largely being obligately anaerobic. Members of the *Crenarchaea* include *Thermofilum*, *Thermosphaera*, and *Sulfolobus*. Their metabolisms are generally chemoorganotrophic or chemolithotrophic and members have been isolated from both marine and terrestrial volcanic habitats (Hugenholtz *et al*., 1998b).

1.6 THERMOPHILIC PROKARYOTES

Thermophiles dominate the deeper branches of the Three Domain Tree of Life. However, they are not limited to these groups and are present throughout both *Archaeal* and *Bacterial* domains. The isolation and characterisation of the first true thermophile, *Thermus aquaticus* (Brock & Freeze, 1969), sparked an increase in the study of thermophilic microbial ecology and physiology.

1.6.1 THERMOPHILY AND ITS ADAPTATIONS

Temperature is one of the most important factors that affect life on our planet. Characterisation of microorganisms with respect to the temperatures at which it grows is fundamental in prokaryote systematics. With respect to temperature, microorganisms have been described as belonging to one of three groups - psychrophiles, mesophiles and thermophiles.

There is a specific range of temperature at which growth of microorganisms is possible. Growth starts at a minimum temperature ($T_{MIN)}$ and reaches an optimum at temperature T_{OPT}, 15 to 20ºC higher than T_{MIN}. Growth stops at a maximum temperature (T_{MAX}), 5 to 10ºC higher that T_{OPT}. The difference between T_{MIN} and T_{MAX} rarely exceeds 30 to 40ºC. The T_{MIN}, T_{OPT}, and T_{MAX}

values are characteristic for individual isolates, and are used to broadly describe the prokaryote. Organisms with T_{OPT} <20ºC are described as psychrophiles. Mesophiles have a T_{OPT} between 20ºC and 50ºC and thermophiles have T_{OPT} >50ºC (Figure 1.5). Although these are discrete brackets, it should be seen as a continuum, and many species are described as a combination of two groups e.g. facultative thermophile. Thermophilic prokaryotes with a T_{OPT} between 60ºC and 80ºC are hyperthermophiles, and extreme thermophiles have a T_{OPT} >80ºC.

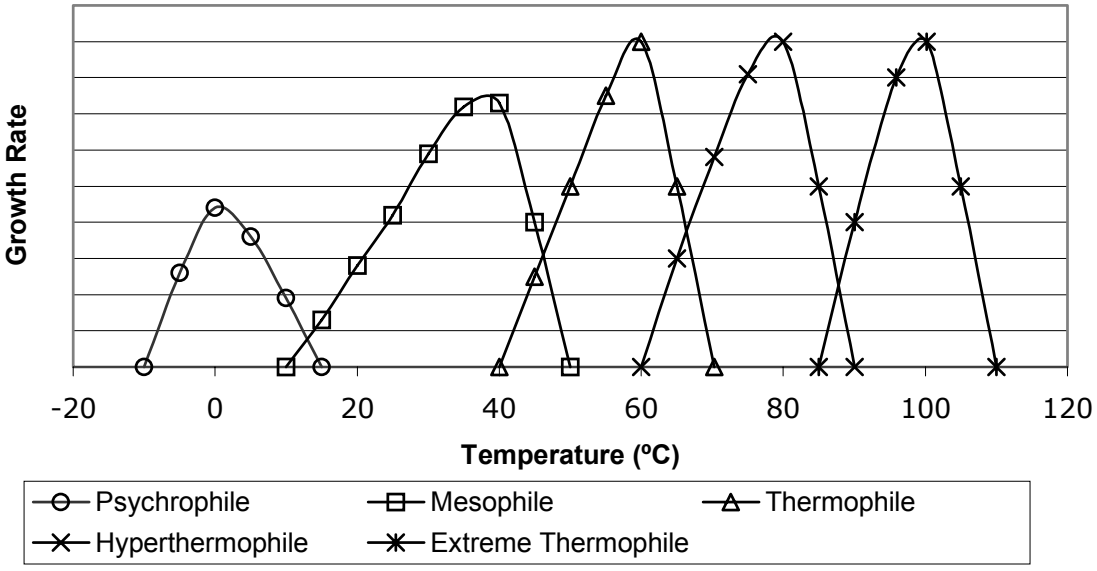


Figure 1.5: Typical growth profiles for a range of prokaryotes

1.6.1.1 ADAPTATIONS TO HIGH TEMPERATURES

The temperature to which growth is limited is currently unknown. Most popularly, it is believed to be around 140ºC, although currently the most thermophilic species is *Pyrolobus fumarii* with a T_{MAX} of 113°C (Blochl *et al*., 1997). The ability of an organism to grow at a temperature is limited by the stability of its constituents. Thermophilic prokaryotes have adaptations that ensure their cell constituents are stable at high temperatures

1.6.1.1a Monomer Adaptations to High Temperatures

Low molecular weight metabolites and cofactors have relatively short half-lives at high temperatures. Studies have shown that the stability of certain metabolites like ATP are greatly affected by pH and the presence of ions and

manipulation of the chemical microenvironment can aid in the stability of these compounds (Cowan, 1997).

Some metabolic intermediates are also extremely heat-labile, and an increase in catalytic efficiency of enzymes would ensure such intermediates are transformed before they are denatured. Comparisons with mesophilic enzymes, however, show comparable catalytic efficiencies for most enzymes and increasing the catalytic efficiency of thermophilic enzymes may not be a widespread occurrence.

Physical-associations of enzymes may prevent metabolite degradation by the channelling of intermediates. Intermediate-enzyme associations increase the stability of the metabolite, and sequential enzyme associations ensure a high throughput and reduce the intracellular concentration of the intermediate.

The use of alternate metabolites is an additional means that may aid thermal stability of the cell. ATP is the popularly considered the main source of cellular energy for organisms. However, ATP has a relatively short half-life. The use of other phosphorylated compounds in certain metabolic pathways e.g. ADP in *Pyrococcus* or pyrophosphate in *Thermoproteus* bypass the need for high cellular concentrations of ATP.

1.6.1.1b Lipid and Membrane Adaptations to High Temperatures

The maintenance of the membrane is a further consideration of extreme thermophiles. Thermophilic *Archaea* have unique adaptations that allow their membrane to maintain its functions at very high temperatures. The more stable ether linkage replaces the ester linkage found in the *Bacterial* and *Eukaryal* domains. In hyperthermophilic *Archaea*, the presence of transmembrane C_{40} phytanyl chains has the effect of reducing the membrane into a monolayer. The phytanyl chains also show cyclic structures that aid in membrane packing and reducing the fluidity at high temperatures. In some thermophilic *Bacteria* ether linkages have been identified indicating the possibility that this characteristic may be a definitive thermophilic adaptation (Langworthy & Pond, 1986). *Bacteria* are

known to modify the types of lipids contained within their membrane relative to the temperature by increasing the length, branching and saturation of the lipid molecule at high temperature (Reizer *et al*., 1985).

1.6.1.1c Protein Adaptations to High Temperatures

Continued studies on thermostable proteins have been shown that there is no pattern that confers thermal stability. It has shown that mesophilic enzymes are more active than their thermal relatives, however the stability of the mesophilic enzymes is much lower.

The structure of proteins is determined by a variety of forces including hydrogen bonds, Van der Waals interactions, salt bridges, and the hydrophobic effect, while the conformational entropy largely governs the denaturation of proteins. The energy of both these stabilising and destabilising forces is in the order of 1 MJ mol^{-1}. The difference between these two forces is known as the conformational stability (ΔG) and is usually around 50 kJ (Jaenicke, 1996). Single amino acid substitutions can increase the ΔG by up to 25kJ without any effect on the protein's conformation. It is this relatively large increase in ΔG caused by little change in the protein sequence that can have such a large effect on protein thermostability. These subtle changes minimise the surface energy and the hydration of apolar surface groups while maximising core packing and burying hydrophobic residues. Increases in the number of salt bridges and salt bridge networks also aid in thermal stability (Ladenstein & Antranikian, 1998).

Thermal stability is also correlated with protein flexibility (Cowan, 1997; Vieille *et al*., 1996). As protein flexibility decreases, the ΔG increases, reducing its susceptibility to denaturation. A decrease in flexibility has a detrimental effect on enzyme activity. Mesophilic enzymes are much more flexible than thermal enzymes, and at any temperature mesophilic enzymes have a higher specific activity than their thermophilic relatives have (Shoichet *et al*., 1995). Higher conformational stability also reduces cellular turnover of the protein.

Spontaneous protein degradation caused by amidation, succinimide formation and oxidation of side chains also increases with temperature. However, it has been shown that these reactions are reduced on correctly folded proteins.

1.6.1.1d DNA and RNA Adaptations to High Temperatures

The instability of the DNA helix at high temperatures can be overcome in a number of ways. The accumulation of salts like potassium di-inositol-1,1'-phosphate in *Pyrococcus woesei* has been shown to stabilise the DNA helix, and also aids in protein conformational stability at high temperatures (Scholz *et al*., 1992). Polycationic polyamines also increase the melting temperature of DNA and are found in many sulfur-dependent *Archaea*.

DNA topology plays an important part in maintaining the durability of the DNA at high temperature. Positive and negative supercoiling has been shown to exist in hyperthermophilic *Archaea* and in some hyperthermophilic *Bacteria* (de la Tour *et al*., 1990; Guipaud *et al*., 1997). Evidence of DNA associating with cationic proteins to form nucleosome-like structures is also shown. The thermal stability of the chromosomal DNA is increased by compaction.

The degradation of DNA, like proteins, also increases with temperature. To combat this, it has been shown that some hyperthermophilic prokaryotes possess homologues of DNA repair systems, including possible homologous recombination SOS repair, excision repair and uracil DNA glycosylase activity.

Transfer RNA (tRNA) must be able to maintain its functional activity in the absence of any molecular associations. The most commonly employed strategy is post-transcriptional modification e.g. methylation. Nucleoside modification is usually localised to regions that aid in structural rigidity. It has been shown that a greater number of modification is found in *Pryodicitium occultum* (T_{OPT} = 105ºC) than in *Thermoplasma acidophilum* (T_{OPT} = 55ºC). Temperature studies on *Pyrococcus furiousus* show an increase in the relative abundance of base modification relative to growth

temperature (Edmonds *et al*., 1991). These results suggest that post-transcriptional modification of nucleosides is an important method for thermal stability in prokaryotes.

1.6.1.2 BIOTECHNOLOGY OF THERMOPHILIC PROKARYOTES

The biotechnological exploitation of thermophilic prokaryotes has come to the fore recently. Inclusion of biological catalysts in industrial applications has been predominant as man looks towards more environmentally friendly solutions. Many industries that employ biocatalysts gain from running these applications at high temperatures. Higher reaction rates, better solubility and diffusion, and greater fluidity are all results of higher temperatures. However, to enable biological catalysts to be efficient, their stability and activity at these high temperatures must be assured.

Research into thermophilic organisms as sources for these biocatalysts has shown that new sources may be better suited for current applications. It also uncovers new possibilities that were previously unthought of. e.g. xylanases in paper pulp bleaching (Viikari, 1994). The discoveries of novel thermophilic *Archaea* and *Bacteria* growing above 100ºC have pushed the boundaries of life, and their potential, to new extremes.

1.6.2 THE ECOLOGY OF THERMOPHILES

1.6.2.1 THERMAL ENVIRONMENTS

Thermal natural environments are found in isolated pockets throughout the world and are usually described as high-temperature solfatara fields or freshwater hot springs. The neighbouring environments have a much lower temperature and temperature gradients are formed. Distinct temperature zones are usually evident where phototrophic communities are present. Thermal environments are broadly broken into high temperature acidic solfatara fields and low temperature freshwater hot fields.

1.6.2.1a Acidic Solfatara Fields

Volcanic heating of soils and aquatic environments results in high temperature acidic solfataric fields. Temperatures of these environments can reach as high as 350ºC and have a pH as low as 2. Solfataric soils consist of an upper acidic oxygenic layer with ferric iron (Fe^{3+}) and a lower neutral, anoxic layer with ferrous iron (Fe^{2+}). Major gases expelled by these environments are steam, CO_2 and H_2S. These weak acids allow the subsurface to remain near neutrality. On the surface, however, the H_2S is oxidised to sulfur, and then to sulfuric acid, lowering the pH to 2.

Solfataric boiling mud and hot springs have been studied in New Zealand (Jones *et al*., 1999; Saul *et al*., 1999), United States of America (Huber *et al*., 1998; Hugenholtz *et al*., 1998b; Reysenbach *et al*., 2000), Japan (Yamamoto *et al*., 1998) and Italy (Canganella & Trovatelli, 1995; Tenreiro *et al*., 1997). Most of these mats are dominated by photolithotrophic metabolisms with *Cyanobacteria* and chemolithotrophs (both *Archaea* and *Bacteria*) being the dominant species.

Research into the prokaryotic communities from various hot springs at Yellowstone National Park, U.S.A., show that a wide diversity of prokaryotic life exist simultaneously, including *Archaea* and *Bacteria* (Hugenholtz *et al*., 1998b; Reysenbach *et al*., 2000; Reysenbach *et al*., 1994). Examinations of thermophilic communities from Japan do not show the presence of *Archaea*, but deep branching *Bacteria* like *Aquificales* sequences are identified with *Thermus* being easily isolated (Oshima & Imahori, 1971; Yamamoto *et al*., 1998).

Hydrothermal vent systems occur when volcanic activity breaks through the Earth's crust in deep ocean water. Although the temperature is very high, the pressure at that depth ensures that water remains in its liquid form. As with hot springs on the surface, these waters have high levels of dissolved minerals including sulfur and chemolithotrophy is the dominant form of metabolism. As the high temperature magma mixes with the cold oxygenic seawater, metal sulphides precipitate, causing chimneys to form and H_2S is oxidised to other sulfur-based compounds. Due to the high levels of sulfur

compounds present many of the prokaryotic species identified from these environments have a sulfur-dependent metabolism (Moyer *et al*., 1995; Prieur, 1997; Takami *et al*., 1997; Taylor *et al*., 1999). Many other species have been identifed from these environments including *Thermus*, *Thermosipha* and *Thermococcus* (Godfroy *et al*., 1997; Marteinsson *et al*., 1999; Takai & Horikoshi, 2000).

Oil and petroleum reservoirs are dominated by fermentative or sulfur-metabolising prokaryotes and methanogens (Grassia *et al*., 1996; Greene *et al*., 1997; Magot *et al*., 2000; Nilsen *et al*., 1996; Voordouw *et al*., 1996). These prokaryotes are implicated in the degradation of long chain hydrocarbons and the corrosion of piping. Other terrestrial subsurface environments have revealed the presence of novel prokaryotes e.g. *Bacillus infernus* (Boone *et al*., 1995).

1.6.2.1b Freshwater Hot Fields

Freshwater hot springs are usually located outside volcanically active areas. They are passively heated by deep magma chambers and reach temperatures of up to 150ºC. The water usually contains low levels of dissolved minerals, but high levels of dissolved CO_2 and silicates, buffering the system to a pH around 9-10 (Kristjánssen & Hregguidssen, 1995). Due to the alkaline pH, any sulfur that is present is in the form H_2S.

In Iceland, studies on an alkaline hot spring environments reveal diverse populations of *Thermus* (Chung *et al*., 2000; Kristjánsson *et al*., 1994). Indian hot springs have revealed the presence of novel isolates (Chrisostomos *et al*., 1996). Another freshwater thermal environment is the Great Artesian Basin of Australia. Limited studies on freshwater hot fields show a wide diversity of *Bacteria* present including the deep branching *Aquificales* and *Thermus*, members of the *Proteobacteria*, *Bacillus* and *Clostridium*. As yet, *Archaea* are not known to widely populate this thermal environment.

1.6.2.1c The Great Artesian Basin of Australia

The Great Artesian Basin of Australia is a freshwater hot spring. It is one of the largest artesian groundwater basins in the world. It underlies approximately 20% of Australia's landmass, extending beneath the arid and semi-arid regions of Queensland, New South Wales, South Australia and the Northern Territory (Figure 1.6). The Great Artesian Basin stretches from the Great Dividing Range to the Lake Eyre depression (a total area of over 1,711,000 square kilometres) and stores an estimated 8,700 million ML of water (Hillier, 1996). The rate at which water flows varies between one and five metres per year. Recharge occurs mainly along the northeastern margin of the basin and natural discharge occurs mainly from springs in the south-western area (Habermahl, 1980). Water temperatures vary from 30ºC in the shallower areas to over 100ºC in the deeper areas. Around 5000 bores access the basin. Individual bore depths vary up to 2000 metres with the average being 500 metres. Many of these bores empty into open drainage systems for agricultural irrigation and stock watering purposes. Nearly 33,000km of bore drains are currently in use in Queensland and New South Wales, and individual drains may be in excess of 100km long (Hillier, 1996). As the water flows through these open drain systems, the temperature slowly decreases to ambient temperatures.

The Great Artesian Basin waters are dominated by a sodium-bicarbonate-chloride hydrochemistry, with a sodium-sulphate-chloride hydrochemistry appearing at the western margins (Habermahl, 1980). A wide variety of physiological groups of bacteria including sulfate reducers, carbohydrate fermenters, strict aerobes and strict anaerobes have been isolated from the Artesian Basin environment (Andrews & Patel, 1996; Denman *et al*., 1991; Love *et al*., 1992; Redburn & Patel, 1994; Wynter *et al*., 1996).

Figure 1.6: Map showing the Great Artesian Basin of Australia

The map of Eastern Australia depicting the aspects of the Great Artesian Basin was taken from Mudd (2000).

1.6.1.2d Man-made Environments

Thermophilic prokaryotes have also been isolated from a wide variety of man-made environments including compost systems (Beffa *et al*., 1996; Blanc *et al*., 1997) and hot water systems (Kristjánsson *et al*., 1994). As these environments are usually temporary, the dominant species are usually thermophilic sporeformers from the *Bacterial* domain (Blanc *et al*., 1997).

1.7 STUDIES ON PROKARYOTIC COMMUNITIES

1.7.1 THE NEED FOR MOLECULAR METHODS

The traditional microbiological approach to study prokaryote ecology using techniques such as cultivation and isolation has a number of limitations. These methods are intrinsically biased towards microorganisms that are favoured by the growth conditions employed e.g. temperature, media, or pH (Dunbar *et al*., 1997; Santegoeds *et al*., 1996; Saul *et al*., 1999; Ward *et al*., 1997). Previous studies show that the culturability of prokaryotes from the environment is low (Table 1.2).

Table 1.2: Culturability of Different Environments

Environment	Culturability (%)	References
Seawater	0.001-0.1	Fergusan *et al*. (1984); Kogure *et al*. (1979); Kogure *et al*. (1980)
Freshwater	0.25	Jones (1977)
Mesotrophic lakes	0.1-1	Staley & Konopka (1985)
Unpolluted esturine waters	0.1-3	Fergusan *et al*. (1984)
Activated sludge	1-15	Wagner *et al*. (1993); Wagner *et al*. (1994b)
Sediments	0.25	Jones (1977)
Soil	0.3	Torsvik *et al*. (1990)

The table was taken from Amann *et al*. (1995)

Denaturing gradient gel electrophoresis studies have shown that enrichment and isolation studies sometimes do not favour the dominant phylotype in the environment (Saul *et al*., 1999; Ward *et al*., 1997)

Characterisation of even simple microbial communities has posed many problems to the traditional microbiologist. Molecular methods enable the identification of community members without the need for cultivation. The use of these methods on a number of environments has expanded the current view of the natural microbial diversity.

Investigations of activated sludge microbial communities has revealed that many more species are present and involved in the wastewater treatment process than previously isolated and characterised (Bond *et al*., 1995; Hornsby & Horan, 1994; Kämpfer *et al*., 1996; Schade & Lemmer, 1994; Wagner *et al*., 1994a). Many studies on marine environments (sediments and benthic) have also revealed numerous taxa as yet not isolated (Britschgi & Giovannoni, 1991; Fuhrman *et al*., 1994; Giovannoni *et al*., 1996; Gray & Herwig, 1996; McCaig *et al*., 1994; Wise *et al*., 1997).

Soil, an environment to previously thought be well characterised, has also been shown to contain many novel bacterial species (Bintrim *et al*., 1997; Dunbar *et al*., 1999; O'Donnell & Gorres, 1999; Zarda *et al*., 1997). Thermophilic mat communities in Yellowstone National Park, USA, have been the most informative in expanding our knowledge of phylogenetic diversity, especially concerning thermophiles and those species considered more ancestral (Ferris *et al*., 1996a; Hugenholtz *et al*., 1998b; Reysenbach *et al*., 1994; Risatti *et al*., 1994; Ward *et al*., 1998).

1.8 Methods to Study Prokaryote Ecology

There are a number of methods in which to identify phylogenetic diversity within a community without the need for cultivation of pure isolates. These methods increasingly involve the extraction or identification of 16S rRNA genes from a community (Figure 1.7).

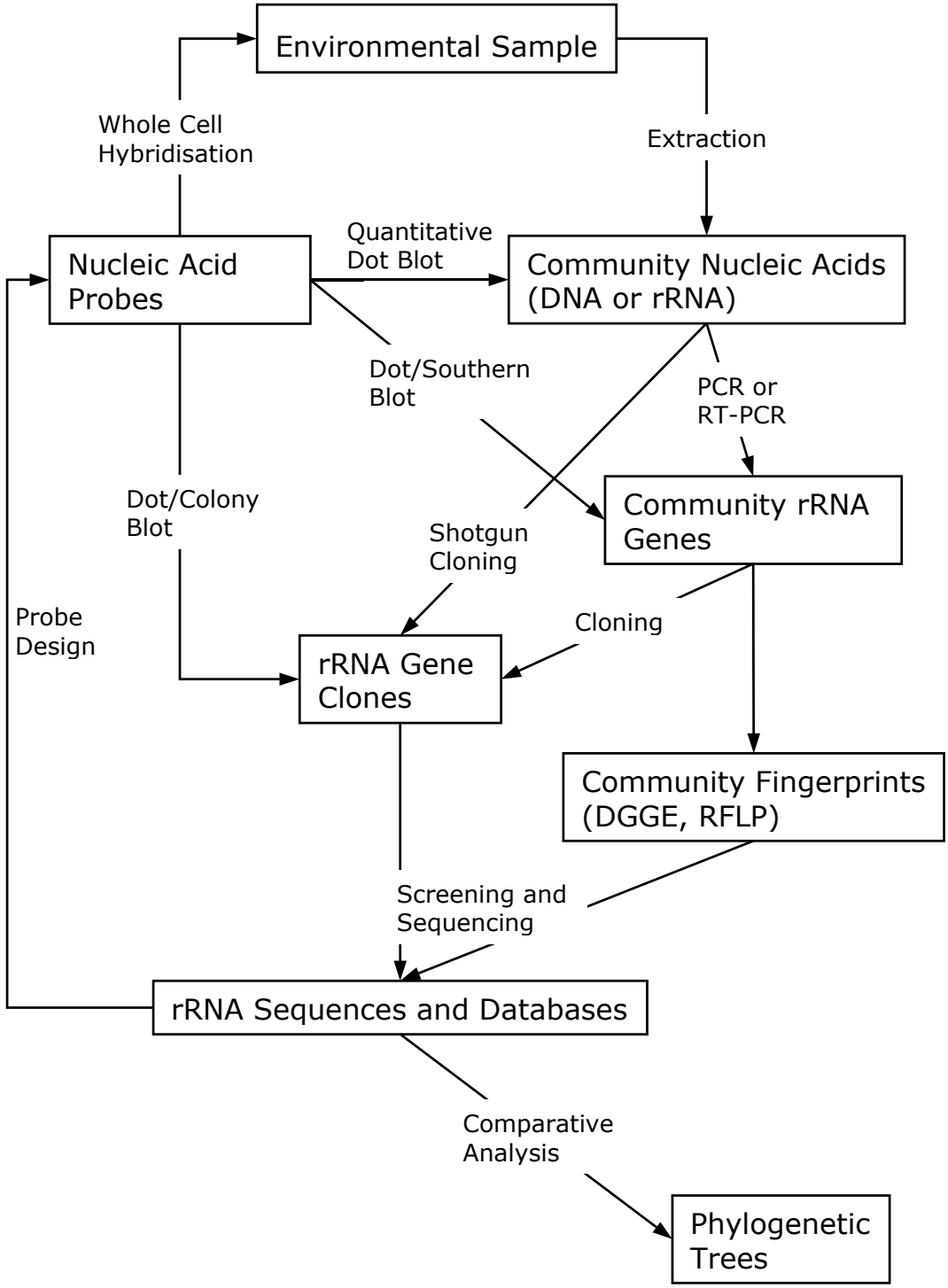


Figure 1.7: 16S rRNA gene-based strategies to characterise prokaryotic communities

Redrawn from Hugenholtz & Pace (1996).

1.8.1 IDENTIFYING PHYLOTYPES

1.8.1.1 NUCLEIC ACID EXTRACTION

To identify different phylotypes present within a community, the communities' 16S rRNA genes need to be extracted. There are a wide variety of methods available that enable this. Initially, the nucleic acids must be extracted from the sample. These methods are usually a combination of physical and chemical means to disrupt the cells while minimising damage caused to nucleic acids (Miller *et al*., 1999).

1.8.1.2 CLONING OF PHYLOGENETIC MARKERS

After nucleic acid purification, individual phylotypes must be separated from within the community. The most commonly used technique involves the specific PCR amplification of 16S rRNA genes followed by cloning (Bond *et al*., 1995; Dunbar *et al*., 1999; Schmidt *et al*., 1991). Shotgun cloning of community genomic fragments (Schmidt *et al*., 1991) and RT-PCR amplification of rRNA molecules (Felske *et al*., 1996) are other methods that can also provide phylogenetically useful information. Once cloned, the 16S rRNA genes can then be screened by hybridisation or restriction fragment length polymorphism (RFLP) and sequenced.

1.8.1.3 DENATURING GRADIENT GEL ELECTROPHORESIS

Denaturing gradient gel electrophoresis (DGGE) involves the separation of PCR-amplified 16S rRNA gene segments through a denaturant concentration gradient contained within an acrylamide gel matrix. The separation is based on differences in melting characteristics of the double-stranded DNA segments, which in turn is dependent on sequence differences. The result is detection of a profile of 16S rRNA gene segment bands that can then be re-amplified and sequenced (Muyzer, 1999).

This approach has been applied to many environments including Yellowstone hot spring communities (Heuer *et al*., 1997; Kowalchuk *et al*., 1997; Muyzer *et al*., 1993; Ward *et al*., 1997). Its greatest potential is in

the relatively easy comparison of different communities, or identifying the effect environmental changes have on the community structure (Ferris & Ward, 1997; Saul *et al*., 1999; Ward *et al*., 1997).

1.8.1.4 HYBRIDISATION TECHNIQUES

Once 16S rRNA genes have been identified from the environment, hybridisation probes can be designed. These probes are then used to describe, either qualitatively or quantitatively, the community structure. The design of the probes can enable identification of groups up to domain level through to species level identification. Visualisation of whole cells is also possible using fluorescent *in situ* hybridisation and microscopy (Amann, 1995; Harmsen *et al*., 1997a; Kämpfer *et al*., 1996; Mobarry *et al*., 1996). Newer techniques involve the use of hybridisation probes in real-time PCR to detect groups of prokaryotes and individual species (Brandt *et al*., 1998; Woo *et al*., 1998).

1.8.1.5 REAL-TIME PCR

The LightCycler™ is a microvolume, multisample rapid air thermal cycler with a built-in fluorometer that allows the real-time detection of amplification products (Wittwer *et al*., 1997). The PCR increases the levels of dsDNA present within the sample, which in turn can be indiscriminately and simply detected by the use of a fluorogenic dsDNA specific dye (e.g. SYBR® Green I). In addition, several complex techniques currently in use enable the identification and quantification of specific PCR-products by fluorescence resonance energy transfer (FRET) between two fluorophores.

1.8.1.5a Adjacent Hybridisation Probes Technique

In the adjacent hybridisation probe methods, two oligonucleotide probes bind to target sites separated by a single base pair. The 3'-end of the upstream probe is labeled with a donor fluorophore while the 5'-end of the downstream probe is labeled with an acceptor fluorophore. The specific hybridisation of both probes to a single amplicon results in FRET from the donor fluorophore to the acceptor fluorophore, decreasing the detected

levels of donor fluorophore fluorescence, and increasing the detected levels of acceptor fluorophore fluorescence (Figure 1.8) (Wittwer *et al*., 1997).

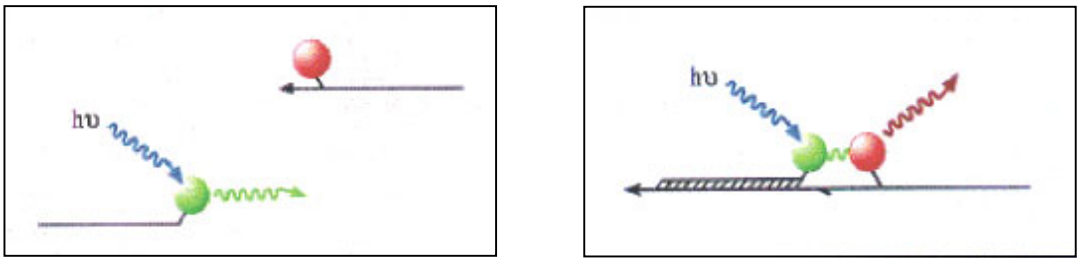


Figure 1.8: Increase in FRET by the acceptor fluorophore after hybridisation during PCR.

Adopted from Wittwer *et al.* (1997).

1.8.1.5b Hydrolysis Probe Technique

A single oligonucleotide probes is designed for use with the hydrolysis technique. This probe has both donor and acceptor (quencher) fluorophores. During PCR, the 5'→3' exonuclease activity of the *Taq* DNA polymerase hydrolyses the probe, separating the two fluorophores (Figure 1.9). The donor fluorophore is no longer subjected to the quenching activity of the acceptor fluorophore and results in an increase in detected fluorescence from the donor fluorophore, but a decrease in detected fluorescence of the acceptor fluorophore (Wittwer *et al*., 1997).

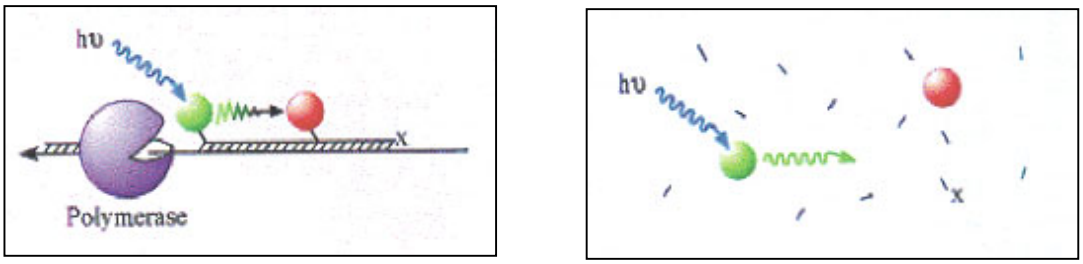


Figure 1.9: Decrease in FRET from the donor fluorophore after hydrolysis of the probe during PCR.

Adopted from Wittwer *et al.* (1997).

1.8.2 DIFFICULTIES IN MOLECULAR CHARACTERISATIONS

Molecular techniques surpass the traditional methods of enrichment and isolation in describing prokaryotic communities. These molecular techniques, however, do introduce biases and errors. The following describes some of the areas in which bias and errors can be introduced when using molecular techniques to characterise prokaryotic communities.

1.8.2.1 DNA EXTRACTION

To ensure that all genomes are extracted from the environment, harsh methods, both physical and chemical are employed. These methods can damage the nucleic acids e.g. fragmentation, causing problems downstream. Techniques less likely to damage the nucleic acids will not lyse all cells in the community and will create a community structure that will favour less hardy cells or species. Purity of the nucleic acids obtained may also pose problems for PCR and enzyme activity (Leff *et al*., 1995; Miller *et al*., 1999).

1.8.2.2 PCR AMPLIFICATION

1.8.2.2a DNA Polymerase Error Rates

Several problems arise when applying PCR to amplify genes from environmental communities. Humic substance coextracted with environmental DNA is commonly known to inhibit PCR amplification (Jackson *et al*., 1997; Tsai & Olson, 1992). Biased amplification is caused by differences in primer-template accessibility, primer-template formation, and extension efficiency for different templates (Suzuki & Giovannoni, 1996). Genome size and rRNA gene copy number also causes differential amplification in mixtures of genomic DNA (Farrelly *et al*., 1995; Polz & Cavanaugh, 1998).

Enzymes used in PCR are known to have error rates. *Taq* DNA polymerase has an error rate of 8.0×10^{-6} and *Pfu* DNA Polymerase has an error rate of 1.3×10^{-6} (Cline *et al*., 1996). These low rates will not have a significant

effect on phylogenetic analysis. However if multiple PCRs or reverse transcriptase-PCR (RT-PCR) were carried out, this misincorporation of bases may lead to higher error rates that may be significant.

1.8.2.2b Chimera Formation

Since the majority of prokaryotic species have not yet been cultured, sequence data used to provide phylogenetic information is obtained using methods such as 16S rRNA gene amplification and cloning. This method has been used to study a wide variety of natural populations (Bond *et al*., 1995; Britschgi & Giovannoni, 1991; Hugenholtz *et al*., 1998b; Moffett *et al*., 2000; Ward *et al*., 1990). The major flaw associated with PCR-based analysis of mixed populations is the creation of chimeric PCR products. A study on the RDP data set has shown that up 20% of its sequences may be chimeric (Robison-Cox *et al*., 1995).

Chimeras are formed between two DNA molecules with regions of high sequence similarity. Incomplete strand synthesis during the PCR process, subsequent annealing to a different template and complete extension forms chimeras. In a study involving barophilic bacteria, Liesack *et al*. (1991) found that chimeras were formed from low molecular weight DNA extracted from a mixed culture containing closely related species. Additionally, if the template DNA is damaged during the lysis procedure (e.g. sonication) the PCR produces recombinant products. Since harsh lysis conditions are required to extract DNA from environmental samples, this is likely to facilitate the creation of chimeras that can be seen in a number of studies (Byers *et al*., 1998; Kopczynski *et al*., 1994; Moyer *et al*., 1995; Schmidt *et al*., 1991).

There are a variety of methods to detect chimeric sequence data available including covariation analysis and analysis of predicted secondary structure. The most widely used methods rely on nearest neighbour analysis (e.g. Chimera Detection at RDP II). A sequence is split into two parts, and if the affiliation of the two parts differ to that of the whole, a chimera is suspected. If the sequence has no close relatives in the database, it is

likely that no method will be able to detect the likelihood of it being chimeric (Kopczynski *et al*., 1994; Robison-Cox *et al*., 1995).

1.8.2.2c 16S rRNA Gene Heterogeneity

Hybridisation studies have shown the presence of a number of copies of 16S rRNA genes within a single genome (Farrelly *et al*., 1995). Amann *et al*. (2000) has shown that *Haloarcula marismortui* has two 16S rRNA that are 5% dissimilar. Analysis of 16S rRNA genes amplified from a community may not reflect the true phenotypic heterogeneity that may be present as it is not clear that a single sequence represents a distinct organism, or one of many genes from one organism.

1.8.2.3 Sequence Data Analysis

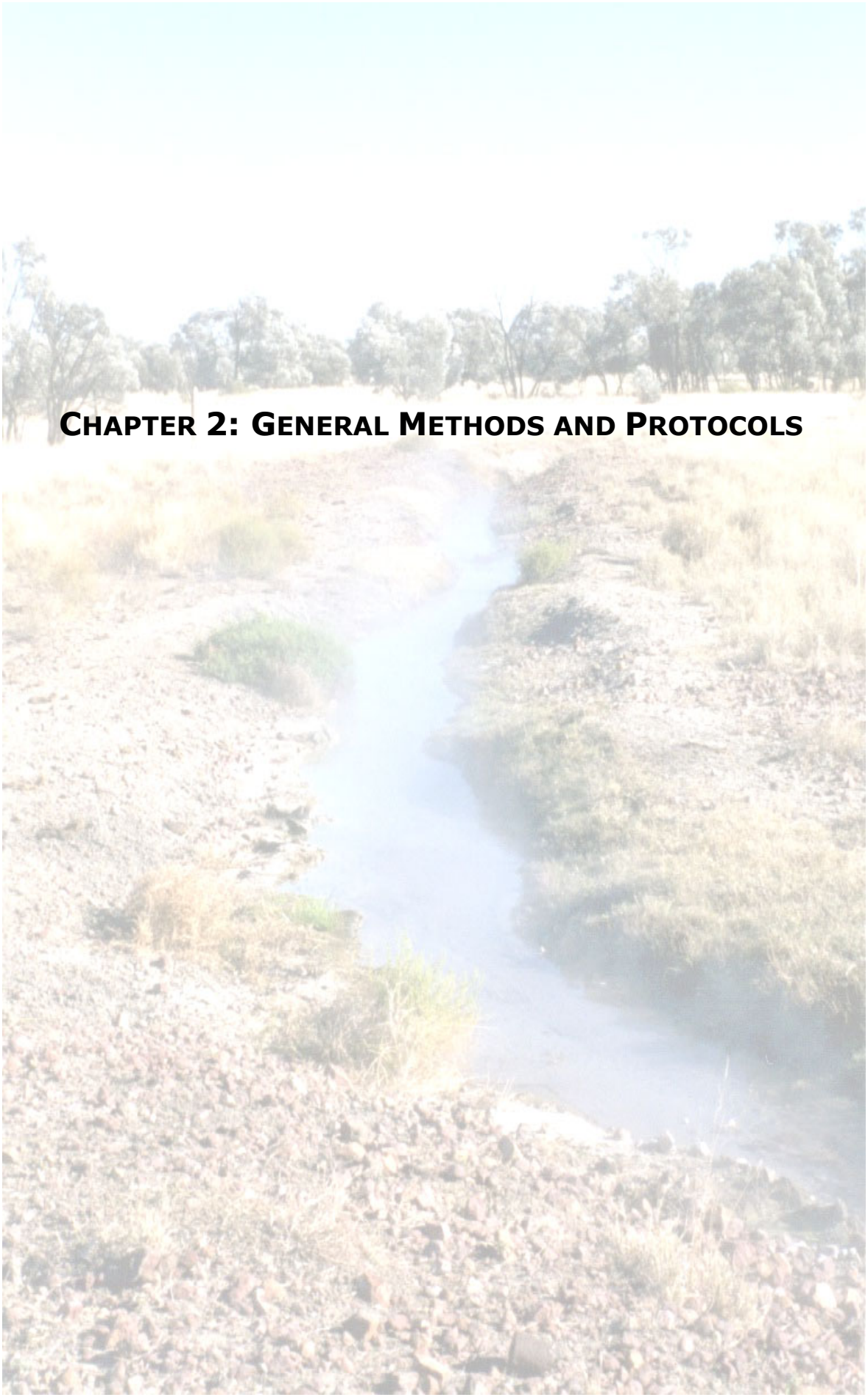
The number of sequences contained within the dataset limits the comparative sequence analysis. The RDP contains over 19,833 aligned SSU rRNA gene sequences (Maidak *et al*., 2001) but this is only a fraction of the total estimated prokaryotic diversity. Novel sequences obtained from environmental samples may not exhibit high similarity to sequences within the database. This leads to the question whether the sequences correspond to novel uncultured prokaryotes or represent phylogenetic taxa that have poor sequence data.

1.8.3 Validation of Phylogenetic Information

The best possible substantiation for sequence data obtained from the environment is the subsequent enrichment and pure isolation of the prokaryote. At present only a minority of prokaryotes can be isolated *ex situ*, and only a few studies are able to direct culturing techniques to isolate detected, yet novel prokaryotes (Huber *et al*., 1998; Hugenholtz, 2000).

Fluorescent *in situ* hybridisation is another technique that enables the clarification that sequence data obtained do belong to cells in the environment and are not artefacts of PCR. It also allows the determination

of cellular activity, as metabolically active cells will have a higher content of rRNA molecules that can bind SSU rRNA probes (Amann *et al*., 1995).



CHAPTER 2: GENERAL METHODS AND PROTOCOLS

2.1 Reagents and Chemicals

Unless otherwise specified, all reagents and chemicals used are molecular biology grade.

2.2 Buffers

TE buffer	10mM Tris-Cl (pH 7.4), 1mM EDTA (pH 8.0).
TAE buffer	40mM Tris-acetate, 2mM EDTA.
10x PCR buffer	50mM Tris-Cl (pH 8.3), 20mM $MgCl_2$, 2.5mg/mL bovine serum albumin (BSA).
6x loading buffer	0.25% bromophenol blue, 40% sucrose. Store at 4ºC.

2.3 Media

2.3.1 Luria Bertani (LB) Media

LB media is prepared by adding 10g tryptone, 5g yeast extract and 5g NaCl to 1L of dH_2O. The pH of the media was adjusted to 7.0 with 10M NaOH prior to sterilisation by autoclaving at 121ºC for 15 minutes.

LB agar plates were produced by amending LB media with 1.5% agar and sterilising by autoclaving at 121ºC for 15 minutes. After cooling to approximately 50ºC, the plates are poured and stored at 4ºC.

If required, ampicillin is added to LB media and plates after sterilisation to a final concentration of 100μg/mL. Ampicillin amended media was stored at 4ºC for 1 month.

To allow the blue/white colour selection of plasmid-containing clones, 100μL of 100mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 20μL of 50mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was spread over the surface of LB agar plates containing 100μg/mL ampicillin. The solutions were allowed to absorb for 30 minutes at 37ºC prior to use.

2.3.2 SOC MEDIA

SOC media used to aid in the recovery of transformed *E. coli* XL-10 cells. SOC media is prepared by adding 2.0g tryptone, 0.5g yeast extract, 1mL 1M NaCl, and 0.25mL 1M KCl to 90mL of ddH_2O. After sterilisation by autoclaving at 121ºC for 15 minutes, 1mL of filter-sterilised 2M Mg^{2+} solution (1M $MgCl_2.6H_2O$/1M $MgSO_4.7H_2O$) and 1mL of 2M glucose solution was added. Sterile ddH_2O was added to adjust the final volume to 100mL and the media again filter-sterilised (0.2μm filter, Sarstedt Australia Pty Ltd).

2.3.3 MEDIA D

2.3.3.1 MEDIA D

(Brock & Freeze, 1969; Castenholz, 1969)

Media D is used in the enrichment and isolation of *Thermus* and *Meiothermus*. It is prepared by adding 50mL of 20x Media D Stock Solution (Section 2.3.3.2), 1g tryptone, and 1g yeast extract to 1L of dH_2O. The pH was adjusted to 8-8.2 with NaOH prior to dispensation and sterilisation by autoclaving at 121ºC for 15 minutes.

2.3.3.2 MEDIA D 20X STOCK SOLUTION

(Brock & Freeze, 1969; Castenholz, 1969)

The 20x stock solution of Media D is prepared by adding 2g nitrilotriacetic acid, 1.2g $CaSO_4.2H_2O$, 2μg $MgSO_4.7H_20$, 0.16μg NaCl, 2.06g KNO_3, 13.78g $NaNO_3$, 2.22g Na_2HPO_4, 1 mL 0.3% $FeCl_3$, and 10 mL Nitch's Micronutrient Solution (Section 2.3.3.3) to a final volume of 1L dH_2O. To prevent the growth of contaminating organisms, the pH was adjusted to 3 with HCl and the media was stored at 4ºC.

2.3.3.3 NITCH'S MICRONUTRIENT SOLUTION

(Brock & Freeze, 1969; Castenholz, 1969)

Nitch's Micronutrient solution is prepared by adding 0.5mL H_2SO_4, 2.28g $MnSO_4.H_2O$, 0.5g $ZnSO_4.7H_2O$, 0.5g H_3BO_3, 0.016g $CuSO_4$, 0.25g $Na_2MoO_4.2H_2O$, and 0.46g of $CoCl_2.6H_2O$ in 1L of dH_2O. The solution was sterilised by autoclaving at 121ºC for 15 minutes.

2.3.4 TRYPTONE YEAST EXTRACT GLUCOSE (TYEG) MEDIA

2.3.4.1 TRYPTONE YEAST EXTRACT GLUCOSE (TYEG) MEDIA

(Patel *et al*., 1985)

TYEG media is used in the routine isolation of chemoheterotrophic anaerboic prokaryotes, especially members of the order *Clostridiales*. TYEG is prepared by adding 0.2g $MgCl_2$, 0.9g NH_4Cl, 0.75g KH_2PO_4, 1.5g K_2HPO_4, 9mL Zeikus' Trace Element Solution (Section 2.3.4.2), 5μL 10% $FeSO_4$, 1mL 0.2% resazurin, 5mL Wolin's Vitamin Solution (Section 2.3.4.3), 10g tryptone, 3g yeast extract, and 5g glucose to a final volume of 1L of dH_2O. The pH was adjusted to 7 with 5M KOH prior to dispensation and sterilisation. The media was prepared as described previously (Balch *et al*., 1979; Hungate, 1969; Macy *et al*., 1972) under a stream of oxygen-free nitrogen, and dispensed in 20mL volumes into serum bottles or 5 or 10mL volumes into Hungate tubes and autoclaved at 121°C for 20 minutes. Prior to inoculation, $Na_2S.9H_20$ (2%) and $NaHCO_3$ (10%) were injected from anaerobic sterile stock solutions to obtain final concentrations of 0.04% and 0.2% respectively.

2.3.4.2 ZEIKUS' TRACE ELEMENT SOLUTION

(Zeikus *et al*., 1979)

Zeikus' trace element solution is prepared by adding 0.2g $FeCl_3.4H_2O$, 0.1g $MnCl_2.4H_2O$, 17mg $CoCl_2.6H_2O$, 0.1g $CaCl_2.2H_2O$, 0.1g $ZnCl_2$, 0.2g $CuCl_2$, 0.01g H_3BO_3, 0.01g $NaMoO_4.2H_2O$, 1.0g NaCl, 0.02g Na_2SeO_3, and 12.5g

nitrilotriacetic acid to 1L of dH_2O. The pH of the solution was adjusted to 6.5 prior to sterilisation at 121ºC for 15 minutes. The sterile trace element solution was stored at 4ºC.

2.3.4.3 WOLIN'S VITAMIN SOLUTION

(Wolin *et al*., 1963)

Wolin's vitamin solution is prepared by adding 2mg biotin, 2mg folic acid, 10mg pyridoxine-HCl, 5mg riboflavin, 5mg thiamine, 5mg nicotinic acid, 5mg pantothenic acid, 0.1mg vitamin B12, 5mg para-amino benzoic acid, and 5mg thiotic acid to 1L of sterile ddH_2O. The solution was filter-sterilised (0.2μM, Sarstedt Australia Pty Ltd, Australia) and stored at 4ºC.

2.4 SAMPLE COLLECTION

2.4.1 SAMPLE SITE: THE NEW LORNE BORE

The New Lorne bore (registered bore number 17263) is situated near Blackall in central Queensland (24º 54' 48''S, 145º 08' 18''E) (Figure 2.1), some 1000 km northwest of Brisbane, Queensland, Australia. It was drilled on October 29th, 1966 and has a depth of 1613m. The bore water has an outflow temperature of 89ºC and a flow rate of 7.56L/s. Results of a chemical analysis of the water are shown in Table 2.1. The water from the New Lorne bore has a sodium-bicarbonate-chloride hydrochemistry that is typical of bore waters found in central Queensland (Habermahl, 1996). The bore outlet empties into a small pool approximately 1m^3 in volume that has a temperature of 88ºC (Figure 2.2). This pool then flows into an open drainage system allowing the water temperature to decrease to ambient levels (Figure 2.3). The New Lorne bore was chosen for this study due to the high outlet temperature and the open drainage system that provided a unique temperature gradient.

Figure 2.1: Map of Queensland showing the New Lorne Bore site

Map produced by SunMap. Obtained from http://www.queensland-holidays.com.au. Arrow indicates approximate position of the New Lorne Bore (registered bore number 17263).

Table 2.1: Chemical analysis of the water from the New Lorne Bore

Conductivity	540μS/cm
Temperature	89ºC
pH	8.5
Si	60mg/L
Total Ions	430.22mg/L
Total Solids	360.60mg/L
Hardness	5
Alkalinity	217
Sodium Absorption Ratio	22.4
RAH	4.22
Na	3.7mg/L
Ca	2.0mg/L
Mg	0.1mg/L
Mn	0.01mg/L
HCO_3	255mg/L
Fe	0.01mg/L
CO_3	4.5mg/L
Cl	38mg/L
F	2.1mg/L
NO_3	0.5mg/L
SO_4	4.3mg/L

Data obtained from the Department of Natural Resources, Queensland, Australia. Chemical analysis was carried out in 1991.

2.4.2 COLLECTION OF SAMPLES

All samples were stored on ice in the field and in transit. In the laboratory, the samples were stored at 4ºC.

Water samples collected from the bore outlet pipe and pool (Figure 2.2) were each taken in sterilised 2L Schott bottles. Sediment samples from the bottom of the pool (Figure 2.2) were collected in sterilised 250mL Schott bottles.

A number of filamentous mat communities were selected for analysis. These were a grey coloured community at 75ºC (Figure 2.4), a red coloured community at 66ºC (Figure 2.5), a green coloured community at 57ºC (Figure 2.6), and a brown coloured community at 52ºC (Figure 2.7). Each community had approximately 5 to 10g of filamentous growth (wet weight) collected and stored in 50mL screw cap tubes (Sarstedt Australia Pty Ltd) with either sterile TE buffer (pH 7.4) or water taken from the drain near the community. Sediment slurry samples near each community were also taken and stored in 100mL sterilised Schott bottles.

2.5 Light and Electron Microscopy

Cell morphology was observed using a Nikon Optiphot microscope equipped with a Nikon UFX-IIa camera attachment and a FX-35WA camera. Examination slides were prepared using either liquid culture or emulsifying a small amount of colony grown on solid media. Photographs were taken using Kodak 135/36 T400CN black and white film.

Electron microscopy was performed as previously described (Andrews & Patel, 1996). Gram reaction was performed as previously described (Collee *et al*., 1996).

Figure 2.2: Bore outlet pipe and pool of the New Lorne Bore

The temperatures at the outlet and in the pool are 89ºC and 88ºC respectively.

Figure 2.3: Open drainage system present at the New Lorne bore

The open drain allows a temperature gradient from 89ºC to ambient temperatures to form.

Figure 2.4: Grey coloured filamentous growth

The grey coloured community was present at 75ºC in the New Lorne bore open drainage system.

Figure 2.5: Red coloured filamentous growth

The red coloured community was present at 66ºC in the New Lorne bore open drainage system.

Figure 2.6: Green coloured filamentous growth

The green coloured community was present at 57ºC in the New Lorne bore open drainage system.

Figure 2.7: Brown coloured filamentous growth

The brown coloured community was present at 52ºC in the New Lorne bore open drainage system.

2.6 DNA EXTRACTION AND PURIFICATION

2.6.1 DNA EXTRACTION FROM PURE CULTURES FOR PCR

DNA was extracted from pure cultures and enrichments following a modification of Marmur's method (Marmur, 1961). Cells from 10-30mL of a late log phase culture (24-48hr old) were harvested by centrifuging at 5400rpm for 5 minutes (Sigma 4K15, Quantum Scientific Pty Ltd, Australia). The pellet was resuspended in 487μL of TE buffer (pH 7.4), 8μL of lysozyme (50mg/mL), 40μL of achromopeptidase (10mg/mL) and incubated for 1 hour at 37ºC. 30μL of 10% SDS and 3μL of Proteinase K (20mg/mL) was added and the solution was incubated at 50ºC for 1 hour. The cell lysis procedure was checked by phase-contrast microscopy. 5M NaCl (100μL) and 80μL of a solution containing 10% CTAB/0.7M NaCl were added. The mixture was vortexed and incubated at 65ºC for 10 minutes. DNA was purified from the suspension by extracting with equal volumes of chloroform:iso-amyl-alcohol (24:1), then phenol:chloroform:iso-amyl-alcohol (25:24:1). Chromosomal DNA was recovered by adding 450μL of isopropanol and spinning at 14000rpm for 15 minutes in a microcentrifuge (Sigma 1-15, Quantum Scientific Pty Ltd, Australia). The chromosomal DNA pellet was then washed with 250μL of 70% ethanol, dried and resuspended in 100μL TE buffer (pH 7.4). RNase A was added to a final concentration of 200μg/mL before incubating for 30 minutes at room temperature. Chromosomal DNA was checked by agarose gel electrophoresis (Section 2.7).

2.6.2 DNA EXTRACTION FROM FILAMENTOUS GROWTH FOR PCR

Approximately 2g (wet weight) of the filamentous material was added to 5mL of TE buffer (pH 7.4). Filamentous growth was disrupted by sonication for 5 seconds and chromosomal DNA was extracted from 1mL of this suspension as described above (Section 2.6.1).

2.6.3 DNA Extraction from Sediments for PCR

DNA was extracted from sediments following a modification of the procedure described by Porteous *et al*. (1994). Approximately 100mg of sediment was resuspended in 350μL of Solution A (250mM NaCl; 100mM EDTA, pH 8.0). 10μL of lysozyme (50mg/mL) and 40μL of achromopeptidase (10mg/mL) were added and the solution incubated at 37ºC for 1 to 3 hours. 350μL of Solution B (250mM NaCl; 250mM EDTA, pH 8.0; 4% SDS) and 50μL of 5M guanidine thiocyanate were added. The solution was vortexed and incubated at 65ºC for 1-3 hours. The solution was centrifuged at 14000rpm for 15 minutes at 4ºC and the supernatant was transferred to a sterile 1.5mL microcentrifuge tube. The DNA was precipitated following the addition of 500μL cold isopropanol, incubation for 30 minutes at -20ºC, and centrifugation at 14000rpm for 15 minutes at 4ºC (Sigma 1-15, Quantum Scientific Pty Ltd, Australia). The resulting pellet was washed with 500μL of 70% ethanol and centrifuged at 14000rpm for 15 minutes (Sigma 1-15, Quantum Scientific Pty Ltd, Australia). The ethanol was removed and the pellet allowed to air dry. Once dry, the DNA was resuspended in an appropriate volume (20 to 50μL) of TE buffer (pH 7.4). RNase A was added to a final concentration of 200μg/mL mL before incubating for 30 minutes at room temperature. Chromosomal DNA was checked by agarose gel electrophoresis (Section 2.7).

2.6.4 DNA Extraction from Pure Cultures for DNA-DNA Hybridisation and G+C mol% Calculation

Cells from a 1L liquid culture in late log phase were pelleted by centrifugation at 3000g for 10 minutes. The pellet was resuspended in 10mL in TE buffer (pH 7.4) and lysozyme and achromopeptidase were added to final concentrations of 1mg/mL each. The suspension was incubated at 37ºC overnight. SDS and proteinase K is added to final concentrations of 1% and 0.1mg/mL respectively and incubated at 60ºC for 3 hours. DNA was purified from the suspension by successive extractions with equal volumes of chloroform:iso-amyl-alcohol (24:1) and phenol:chloroform:iso-amyl-alcohol (25:24:1).

2 volumes of cold 100% ethanol was added to the tube and mixed by gentle inversion. High molecular weight DNA from the ethanol-aqueous interface was recovered by spooling and subsequently dissolved in TE buffer (pH 7.4). RNase A was added to a final concentration of 200μg/mL before incubating for 30 minutes at room temperature. Chromosomal DNA was assessed by agarose gel electrophoresis (Section 2.7) and quantitated as described in Section 2.12.

The RNase A was removed by successive extractions with equal volumes of chloroform:iso-amyl-alcohol (24:1) and phenol:chloroform:iso-amyl-alcohol (25:24:1). Ethanol precipitation and centrifugation at 14000rpm for 15 minutes recovered the high-molecular weight DNA. The chromosomal DNA pellet was then washed with 500μL of 70% ethanol and air-dried.

2.7 AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis was performed as previously described (Sambrook *et al*., 1989). Agarose gel solutions of 0.8% to 1.0% are prepared by dissolving DNA grade agarose powder in 1x TAE buffer (Section 2.2) in a microwave oven. The solution was cooled to approximately 50ºC before ethidium bromide was added to a final concentration of 0.1μg/mL. The molten gel was poured into a casting tray and allowed to set. After setting, the gel was placed in a horizontal electrophoresis unit and 1x TAE buffer added until the gel was submerged. The samples were prepared by adding a 0.2 volume of a 6X loading buffer (Section 2.2) prior to loading. To standardise gels, 500ng of λ DNA/*Hin*d III marker or 500ng of a Low Mass Ladder was run parallel with the samples. Electrophoresis occurred at 5V/cm (80V for 50mL gels and 120V for 100mL gels) until the dye front reached an appropriate distance from the well (usually one half to three-quarters of the gel length). DNA bands were visualised by long wavelength ultraviolet radiation. Digital images of the ethidium bromide stained gels were captured using a UVP GDAS 1200 Gel Documentation Analysis System (Pathtech Pty Ltd, Australia) and edited using a simple image editor (e.g. PaintShop Pro v4.12).

2.8 OLIGONUCLEOTIDES USED IN PCR AND SEQUENCING

Amplification and sequencing oligonucleotides used have been described previously (Redburn & Patel, 1993) and are listed in Table 2.2. Primers were resuspended to a stock concentration of 200μM in TE buffer (pH 7.4). Amplification and sequencing primers were diluted in 10mM Tris-Cl (pH 8.5) to 50μM and 3.2μM respectively. All primers were stored at –20ºC.

Table 2.2: Oligonucleotides used in PCR and sequencing

Primer	***E. coli* position**	**Sequence 5'→ 3'**
Amplification		
Fd1	8-27	AGA GTT TGA TCC TGG CTC AG
Rd1	1542-1526	AAG GAG GTG ATC CAG CC
Sequencing		
F1	339-357	CTC CTA CGG GAG GCA GCA G
F1.1	519-536	CAG CAG CCG CGG TAA TAC
F2	785-805	CAG GAT TAG ATA CCC TGG TAG
F3	907-926	AAA CTC AAA GGA ATT GAC GG
F4	1391-1406	TGT ACA CAC CGC CCG T
R1	357-342	CTG CTG CCT CCC GTA G
R2	536-519	GTA TTA CCG CGG CTG CTG
R3	802-785	CCA GGG TAT CTA ATC CTG
R4	926-907	CCG TCA ATT CCT TTG AGT TT
R5	1115-1100	GGG GTT GCG CTC GTT G
R6	1513-1494	TAC GGT TAC CTT GTT ACG AC

Primer list is adapted from Redburn & Patel (1993).

2.9 PCR AMPLIFICATION OF 16S RRNA GENES

The polymerase chain reaction (PCR) was used to amplify the 16S rRNA genes from chromosomal DNA. Reactions were prepared on ice in sterile 0.2mL thin-wall tubes (Quantum Scientific Products Pty Ltd, Australia). Chromosomal DNA concentrations were titrated to optimise the PCR amplification for a given reaction. Concentrations ranged from neat to 10^{-2}, serially diluted in sterile TE buffer (pH 7.4).

Reactions consisted of 5μL of 10x PCR buffer, 0.5μL of 20mM dNTPs (5mM dATP, 5mM dGTP, 5mM dCTP, and 5mM dTTP), 1μL of 50μM Fd1 primer (see Table 2.2), 1μL of 50μM Rd1 primer (see Table 2.2), 0.2μL of 5U/μL of *Taq* DNA Polymerase (Promega Corp.), 2μL of chromosomal DNA, and 40.3μL of sterile ddH_2O.

The PCR was carried out in a RapidCycler (Idaho Technology Inc., USA) with the following parameters: 1 cycle of 94ºC for 2 minutes; and 30 cycles of 94ºC for 1 minute, 50ºC for 1 minute, 74ºC for 1 minute and 30 seconds with a slope of 9.9. No mineral oil is added to the PCR. Each PCR run contained a negative control (2μL sterile dH_2O instead of template DNA) and a positive control (2μL of known amplifiable DNA instead of template DNA). A 5μL aliquot of each PCR was checked by agarose gel electrophoresis.

2.10 PURIFICATION OF PCR PRODUCTS

PCR products from three reactions were pooled and purified using QiaQuick® PCR Purification Spin Columns as per manufacturer's instructions (Qiagen Pty Ltd, Australia).

If gel purification was required, the PCR amplification reactions were pooled and precipitated to reduce the sample volume loaded onto the gel. Adding 2 volumes of chilled 100% ethanol to the PCR products and centrifuging at 14000rpm (Sigma 1-15, Quantum Scientific Pty Ltd, Australia) carried out the precipitation. The supernatant was removed and the pellet allowed to dry before resuspending in 10-20μL of TE buffer (pH 7.4).

Agarose gels (0.8%) were prepared and the entire PCR products were electrophoresed. A gel slice containing the desired DNA was excised using sterile scalpel blades and placed in a sterile pre-weighed microcentrifuge tube. The PCR-amplified DNA was purified using QiaQuick® Gel Purification Spin Columns as per manufacturer's instructions (Qiagen Pty Ltd, Australia) and eluted in 30μL of 10mM Tris-CL (pH 8.5). The elutant was assessed by agarose gel electrophoresis and stored at –20ºC.

2.11 PLASMID EXTRACTION AND PURIFICATION

Plasmid DNA was extracted from 1.5mL LB cultures containing 100μg/mL ampicillin following overnight incubation at 37ºC with shaking. The plasmids were extracted and purified using Qiagen's QIAprep® Miniprep columns following the manufacturer's instructions. The purified plasmid extracts were checked by agarose gel electrophoresis.

2.12 NUCLEIC ACID QUANTITATION

DNA concentration was measured spectrophotometrically using conversion factors of A_{260} 1.0 = 50μg/mL for double stranded DNA (Sambrook *et al*., 1989).

2.13 CREATION OF 16S RRNA GENE CLONE LIBRARIES

2.13.1 PREPARATION OF COMPETANT *ESCHERICHIA COLI* XL-10 CELLS

N.B. All resuspension of cell pellets were carried out by gentle agitation, not vortexing.

Stock cultures were prepared by inoculating 25mL of LB broth (Section 2.3.1) with a single *E. coli* XL-10 colony grown on LB agar plates at 37ºC. The 25mL culture was incubated at 37ºC with shaking overnight. This was then used to inoculate 500mL of pre-warmed LB broth. This culture was grown at 37ºC with shaking until the OD_{600} reached 0.4 (3 to 4 hours). The culture was then chilled on ice for 30 minutes before the cells were harvested by centrifugation at 3000g for 10 minutes at 4ºC. The cells were twice washed with decreasing amounts of cold sterile ddH_2O (250mL then 40mL) and harvested by centrifuging at 3000g for 10 minutes at 4ºC. Once washed, the cells were resuspended in 10mL of cold sterile 20% glycerol before pelleting at 5400rpm for 10 minutes at 4ºC (Sigma 4K-15, Quantum Scientific Pty Ltd, Australia). Once pelleted, the supernatant was removed and the cells resuspended in 2mL of 10% glycerol. Aliquots of 50μL were

placed into sterile 1.5mL microcentrifuge tubes, snap-frozen by dropping in liquid nitrogen, and stored at –80ºC.

For electroporation, one aliquot of 50μL of frozen *E. coli* XL-10 cells was used to inoculate 5mL of LB broth. This was grown at 37ºC for 3 to 4 hours (with shaking). Centrifuging at 5400rpm at 4ºC for 10 minutes pelleted the cells. The cells were successively washed with 2mL of sterile cold ddH_2O three times. The final suspension volume of cells was 50μL of sterile cold ddH_2O.

2.13.2 LIGATION AND TRANSFORMATION

Purified PCR product was ligated using a TA cloning strategy (Zhou *et al*., 1995) into the pGEM®-T Easy Vector according to the manufacturer's instructions (Promega Corporation, USA). 1μl of the ligation mix was electroporated into a 50μL volume of freshly prepared competent *E. coli* XL-10 cells. Electroporation as described by Dower *et al*. (1988) took place in Gene Pulser® II (Bio-Rad Laboratories Australia Pty Ltd) in Gene Pulser® cuvettes (Bio-Rad Laboratories Australia Pty Ltd) with an electrode gap of 0.2cm. Settings used were a voltage of 1.75kV; 25μF capacitance; and 200Ω resistance.

After electroporation, the cells were immediately suspended in 950μL of SOC media (Section 2.3.2) and incubated for 90 minutes at 37ºC with shaking. 100μL of the transformation culture were then plated on LB agar plates supplemented with 100μg/mL ampicillin, IPTG, and X-Gal and incubated overnight (Section 2.3.1). Positive clones were picked using the blue/white colour selection capacity and replated.

2.14 AUTOMATED DYE TERMINATOR CYCLE SEQUENCING

Sequence reactions were prepared on ice in sterile 0.6mL tubes. Reactions consisted of 20ng purified PCR product or 300-500ng purified plasmid preparation, 1μL of 3.2μM primer (Table 2.2), 4μL of ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Mix (Applied Biosystems, Australia), and sterile ddH_2O to a final volume of 20μL. An overlay of 40μL

of sterile mineral oil was added. Thermal cycling was carried out in a Corbett Research Thermal Sequencer (FTS-1) following the ABI recommended cycling program of 25 cycles of 96ºC for 30 seconds, 50ºC for 15 seconds, 60ºC for 4 minutes.

The sequence products were purified using ABI's recommended ethanol precipitation for BigDye™ Terminators by adding the 20μL sequencing reaction volume to 80μL of 80% ethanol. The solution remained at room temperature for a minimum of 15 minutes, but less than 24 hours before centrifuging at 14000rpm in a Sigma Microcentrifuge (1-15) for 20 minutes. The supernatant was carefully removed by aspiration. Adding 250μL of 70% ethanol and centrifuging for 10 minutes washed the pellet. Again the supernatant was removed by aspiration, and the pellet dried by heating to 95ºC for 1 minute in a Thermoline heating block (DB-1).

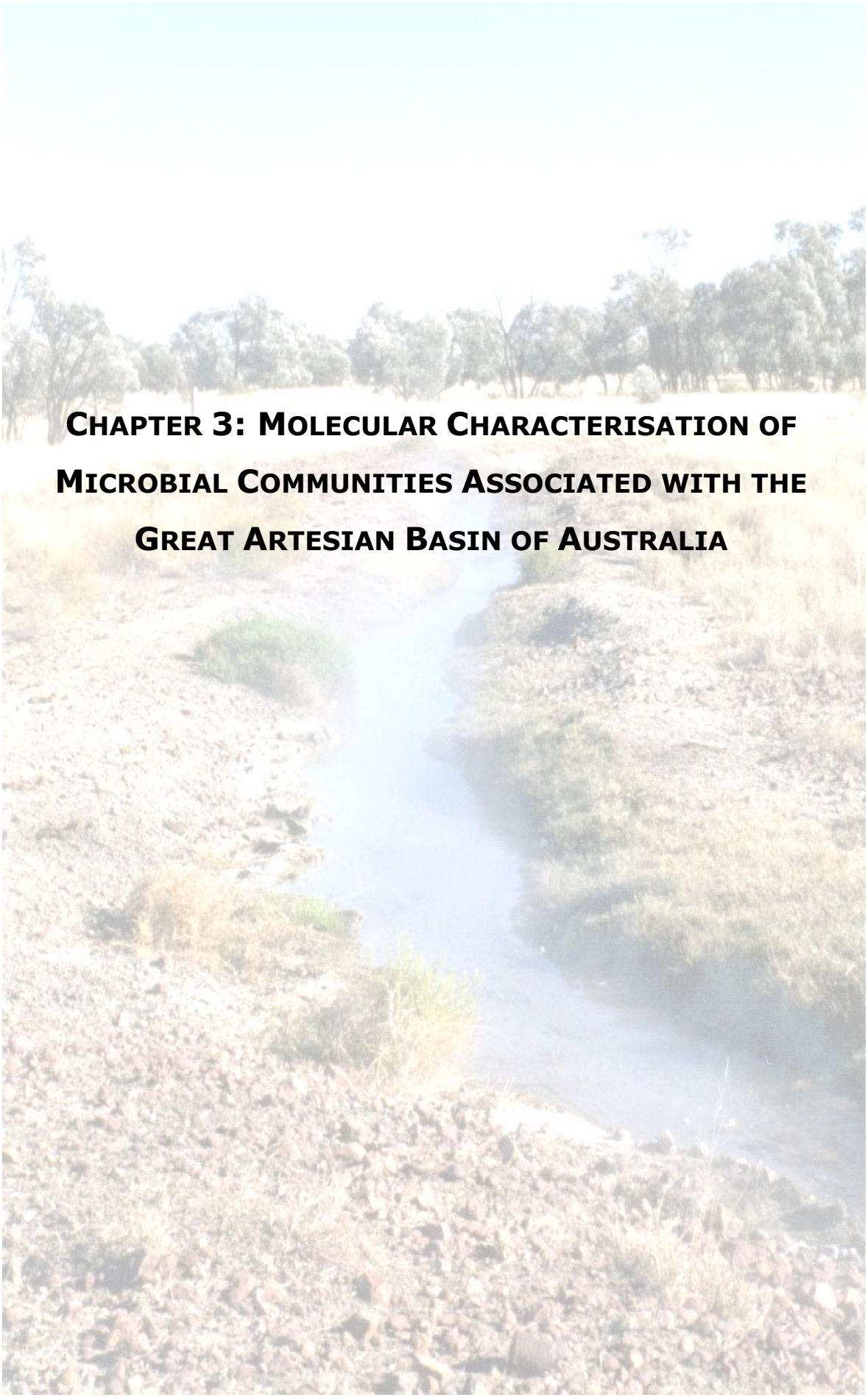
Sequence reaction products were stored in the dark at 4ºC prior to electrophoresis on a 4.8% denaturing polyacrylamide gel. Electrophoresis was performed on an Applied Biosystems 377 DNA sequencer with 96-lane upgrade (Applied Biosystems, Australia) with a 0.2mm thick comb, on 36cm well-to-read plates for 7 hours at 1.68kV (Molecular Biology Facility, Griffith University, Brisbane, Qld, Australia).

2.15 PHYLOGENETIC ANALYSIS

Sequence data was imported into the sequence editor BioEdit v5.0.1 (Hall, 1999) and the base calling examined. A contiguous consensus sequence was obtained for each phylotype or isolate. The full sequence was aligned using the RDP Sequence Aligner program (Maidak *et al*., 2001). The consensus sequence was then manually adjusted to conform to the 16S rRNA secondary structure model (Winker & Woese, 1991).

A non-redundant *blastn* search of the full sequence through GenBank (Altshul *et al*., 2001; Benson *et al*., 1999) identified its closest relative. Sequences used in the phylogenetic analysis were obtained from the RDP (Maidak *et al*., 2001) and GenBank (Benson *et al*., 1999). Positions of sequence and alignment ambiguity were omitted. Comparative

phylogenetic analysis was carried out using the TreeCon program (Van de Peer & De Wachter, 1994). Pair-wise evolutionary distances were calculated using the method of Jukes and Cantor (Jukes & Cantor, 1969). Dendrograms were constructed using the neighbor-joining method (Saitou & Nei, 1987). Confidence in the tree topology was determined by using 100 bootstrapped trees (Felsenstein, 1985) and expressed as a precentage near the branching point.



CHAPTER 3: MOLECULAR CHARACTERISATION OF MICROBIAL COMMUNITIES ASSOCIATED WITH THE GREAT ARTESIAN BASIN OF AUSTRALIA

3.1 INTRODUCTION

Naturally occurring thermal environments exist in many parts of the world. Many of these are volcanically heated (Reysenbach *et al*., 2000; Saul *et al*., 1999), marine (Harmsen *et al*., 1997b; Marteinsson *et al*., 1997; Moyer *et al*., 1995), or oil field environments (Beeder *et al*., 1994; Nilsen *et al*., 1996). The Great Artesian Basin of Australia is dissimilar to other well-studied environments and provides a unique niche for prokaryotic communities to develop.

The Great Artesian Basin of Australia is a deep subsurface geothermally heated freshwater aquifer. It underlies arid and semi-arid regions of Australia and lies beneath approximately 20% of Australia's landmass (Habermahl, 1980). Its waters are chemically distinctive from volcanic thermal environments (Mazor, 1995). The accessed water is mainly distributed through open drain runoff channels for uses in stock watering and irrigation (Habermahl, 1980).

The temperature at the sources of these bores can be as high as 100ºC with temperatures in runoff channels cooling to ambient thereby producing unique temperature gradients in which distinct microbial communities develop. These prokaryotic communities are separated spatially and can be distinguished on general characteristics such as colour, morphology, and temperature at which the community is found.

Characterising microbial communities using traditional enrichment and isolation techniques results in limited success (Amann *et al*., 1995; Saul *et al*., 1999; Ward *et al*., 1997). To further understand natural microbial communities, molecular methods have been introduced. Many of these approaches are based on the use of the 16S rRNA gene as a phylogenetic and evolutionary marker (Ferris *et al*., 1996a; Heuer *et al*., 1997; Muyzer & Ramsing, 1995; Risatti *et al*., 1994; Weisburg *et al*., 1991). The comprehensive use of the 16S rRNA gene in microbial community studies has resulted in large databases, such as the RDP (Maidak *et al*., 2001), which provides ribosome related data and services.

Here the culture-independent method of 16S rRNA gene amplification, cloning and sequencing to phylogenetically characterise the microbial communities found in the bore pool sediment and the run-off channel of a Great Artesian Basin bore is detailed.

3.2 MATERIALS AND METHODS

3.2.1 SITE AND SAMPLE COLLECTION

Sediment and filamentous mat samples were collected from the run-off channel of the New Lorne Bore situated near Blackall, Central Queensland, as described in Section 2.4.1. The bore outlet pool sediment and four filamentous mat communities were taken for analysis. These included the bore pool sediment at 88ºC, a grey mat community at 75ºC, a red mat community at 66ºC, a green mat community at 57ºC, and a brown mat community at 52ºC.

3.2.2 DNA EXTRACTION AND 16S RRNA GENE AMPLIFICATION

Chromosomal DNA was extracted from the bore pool sediment as specified in Section 2.6.3. Chromosomal DNA was extracted from the filamentous mat communities as described in Section 2.6.2. Chromosomal DNA was checked by agarose gel electrophoresis as detailed in Section 2.7.

The communal 16S rRNA genes are amplified from the chromosomal DNA by PCR as described in Section 2.9. PCR amplification and purity was checked by agarose gel electrophoresis as detailed in Section 2.7. The PCR products from three reactions were pooled and purified using QiaQuick® PCR Purification Spin Columns as per manufacturer's instructions (Qiagen Pty Ltd, Australia).

3.2.3 CLONING AND SEQUENCING

The purified PCR product was ligated, electrotransformed into competent *E. coli* cells and positive transformants selected as detailed in Section 2.13.2.

The extraction of plasmids is detailed in Section 2.11 and inserts were detected by agarose gel electrophoresis as specified in Section 2.7.

Sequencing of plasmid inserts is carried out as described in Section 2.14.

3.2.4 SEQUENCE EDITING AND PHYLOGENETIC ANALYSIS

The sequence data was corrected, collated and phylogenetically analysed as described in Section 2.15. A maximum limit of 98% similarity between sequences and tree topology was used to select the phylotypes. A clone from each phylotype was then fully sequenced as described previously. Chimeras were identified using the Chimera Check program at RDP (Maidak *et al*., 2001) and the T7 and Sp6 generated sequence data. For cases of chimera-ambiguity, topology of separate 3'- and 5'- dendrograms was examined.

3.3 RESULTS

3.3.1 PCR-AMPLIFIED 16S RRNA GENE CLONE LIBRARY ANALYSIS

Total chromosomal DNA was extracted from the bore pool sediment and four filamentous mat communities and the 16S rRNA genes from each amplified and cloned. From the bore pool sediment library, 64 transformants were selected for further analysis. From each filamentous mat community library 96 positive transformants were selected for further analysis. Of these, agarose gel electrophoresis analysis reduced the number of plasmids containing an appropriately sized insert to 46 (bore pool sediment library), 68 (grey mat library), 72 (red mat library), 88 (green mat library) and 92 (brown mat library).

Partial sequencing of each insert using the plasmid-specific primers T7 and Sp6 produced an average sequence length of 519 bp and 505 bp respectively. Chimera analysis of the partial sequences removed a further 41 clones (0, sediment library; 2, grey mat library; 14, red mat library; 19, green mat library; and 20, brown mat library). Phylogenetic analysis of the T7- and Sp6-generated sequences identified 1 phylotype from the bore pool

sediment library, 10 phylotypes from the grey mat library, 12 phylotypes from the red mat library, 16 phylotypes from the green mat library and 19 phylotypes from the brown mat library. Full sequences of each phylotype were produced.

3.3.2 PHYLOGENETIC AND *BLASTN* ANALYSIS

Table 3.1 shows the *blastn* results of the full-length phylotype sequences obtained from the bore pool sediment, grey, red, green and brown mat clone libraries respectively. *Blastn* matches ranged from 88% to 99% and were spread throughout the *Bacterial* domain. Table 3.2 shows a comparison of community members across all clone libraries. Due to the number and diversity of phylogenetic groups, the analysis was divided into phyla or classes as defined by Boone & Castenholz (2001). Figures 3.1 to 3.15 are dendrograms indicating the phylogenetic placement of the phylotypes obtained from the analysis of all clone libraries. Reference sequences used in the phylogenetic analysis were selected on the basis of the 16S rRNA gene sequence length and diversity present within the division examined.

The output from the Chimera Check program at the RDP (Maidak *et al*., 2001) detected a number of chimeric inserts. Several ambiguous results, however, necessitated the creation and analysis of separate 5' and 3' phylogenetic dendrograms to elucidate the sequence's chimeric status.

The 16S rRNA gene sequence data produced has been deposited to GenBank and the accession numbers are listed in Appendix II.

Table 3.1: Blastn results of the phylotypes obtained

Clone	***Blastn* Result**	Accession Number	Similarity
Sed01	*Hydrogenobacter subterranea*	AB026268	1474/1499 (98%)
Y03	*Thermus scotoductus* strain ITI-252T	Y18410	1448/1466 (98%)
Y04	*Hydrogenobacter subterranea*	AB026268	1494/1498 (99%)
Y10	*Thermus igniterrae* strain GE-2	Y18408	1463/1472 (99%)
Y27	Unidentified bacterium clone DA040	AJ000985	1417/1508 (93%)
Y30	*Rhodothermus marinus* 16S rRNA	X80994	1210/1328 (91%)
Y36	*Clostridium litorale* (DSM 5388)	X77845	1218/1384 (88%)
Y63	Unidentified *Cytophagales* OPB88	AF027006	1308/1406 (93%)
Y71	*Thermus igniterrae* strain GE-2	Y18408	1452/1472 (98%)
Y88	*Thermus igniterrae* strain GE-2	Y18408	1451/1472 (98%)
Y90	*Thermus scotoductus* strain ITI-252T	Y18410	1440/1466 (98%)
R03	*Meiothermus ruber* strain 16106	Y13597	1434/1436 (99%)
R08	*Chloroflexus aurantiacus*	D38365	1357/1406 (96%)
R10	Clone BSV20	AJ229185	1356/1408 (96%)
R15	Unidentified *Cytophagales* OPB88	AF027006	1307/1406 (93%)
R16	*Clostridium* sp. partial strain RPec1	Y15985	1374/1433 (95%)
R27	*Clostridium* sp.	X95274	1316/1394 (94%)
R35	*Clostridium aceticum* DSM 1496	Y18183	1187/1251 (95%)

R38	Uncultured eubacterium env.OPS 3	AF018188	1433/1449 (98%)
R57	*Hydrogenobacter subterranea*	AB026268	1486/1499 (99%)
R58	*Thermus scotoductus* strain ITI-252T	Y18410	1448/1466 (98%)
R75	*Thermus igniterrae* strain GE-2	Y18408	1452/1472 (98%)
R82	*Tindallia magadii*	Y15626	1213/1322 (92%)
G01	*Fischerella muscicola*	AF132788	1365/1397 (98%)
G06	Uncultured bacterium MS8	AF232922	1347/1418 (94%)
G07	Uncultured bacterium SJA-143	AJ009494	1422/1521 (93%)
G10	Uncultured bacterium SJA-149	AJ009495	1411/1493 (94%)
G13	Anaerobic thermophile IC-BH	U40230	1162/1324 (88%)
G18	Uncultured bacterium #0319-7F4	AF234144	788/862 (91%)
G19	*Nitrospira moscoviensis*	X82558	1476/1533 (96%)
G21	*Meiothermus ruber* strain 16106	Y13597	1430/1436 (99%)
G24	*Thermus oshimai* strain SPS-17T	Y18416	1457/1463 (99%)
G32	*Chloroflexus aurantiacus*	D38365	1356/1406 (96%)
G34	*Meiothermus cerbereus* strain GY-5	Y13595	1415/1436 (98%)
G55	Unidentified delta proteobacterium OPB78	AF026989	1027/1173 (88%)
G58	Uncultured eubacterium WCHA1-89	AF050556	745/835 (89%)
G62	Bacterium str. 96446	AF227855	1339/1434 (93%)
G73	*Xylella fastidiosa* strain PP4-5	AF159580	1310/1459 (90%)
G94	Unidentified beta proteobacterium OPB37	AF026985	1178/1298 (91%)

B01	*Meiothermus cerbereus* strain GY-5	Y13595	1424/1436 (99%)
B10	*Porphyrobacter* sp. KK348	AB033325	1450/1477 (98%)
B11	Uncultured bacterium SJA-149	AJ009495	1422/1493 (95%)
B13	*Roseococcus thiosulatophilus*	X72908	1316/1402 (94%)
B15	Unidentified eubacterium clone DA122	Y12598	1371/1451 (94%)
B16	Uncultured eubacterium env.OPS 3	AF018188	1219/1325 (92%)
B25	Uncultured Antarctic bacterium LB3-100	AF173817	1135/1282 (88%)
B27	Uncultivated soil bacterium clone C002	AF013515	1347/1462 (92%)
B35	Bacterium str. 96446	AF227855	1340/1432 (94%)
B37	*Sandaracinobacter sibiricus*	Y10678	1352/1404 (96%)
B44	Uncultured bacterium MS8	AF232922	1348/1418 (95%)
B53	*Azospirillum brasilense* (NCIMB 11860)	Z29617	1398/1453 (96%)
B55	*Chloroflexus aurantiacus*	D38365	1354/1405 (96%)
B63	Iron-oxidizing lithotroph ES-1	AF012541	1367/1481 (92%)
B66	Uncultivated soil bacterium clone C019	AF013522	12781399 (91%)
B83	*Planctomycete* str. 567	AJ231172	878/985 (89%)
B86	Uncultured bacterium #0319-7F4	AF234144	786/861 (91%)
B90	Uncultured bacterium SJA-149	AJ009495	1365/1466 (93%)
B95	*Fischerella muscicola*	AF132788	1374/1412 (97%)

Table 3.2: Comparison of members for each prokaryote community

Phylum	**Sediment (88°C)**		**Grey (75°C)**		**Red (66°C)**		**Green (57°C)**		**Brown (52°C)**	
Species	**Clones**	**%**	**Clones**	**%**	**Clones**	**%**	**Clones**	**%**	**Clones**	**%**
Aquificae										
H. subterranea	Sed01	100	Y04	6	R57	3				
Deinococci-Thermus										
T. scotoductus			Y03, Y90	60	R58	4				
T. igniterrae			Y10, Y71, Y88	12	R75	3				
T. oshimai							G24	1		
M. ruber					R03	47	G21	4		
M. cerberus							G34	2	B01	34
Chloroflexi										
C. auranticus					R08	4	G32	10	B55	3
Nitrospira										
N. muscoviensis							G19	4		
Verrucomicrobia										
Unaffiliated									B66	1
Cyanobacteria										
F. musicola							G01	36	B95	1
Planctomycetes										
Unaffiliated					R38	1	G18	2	B16, B83, B86	5

Acidobacteria										
Unaffiliated							G10, G58	2	B11, B27, B90	8
Bacteroidetes										
Unaffiliated			Y30, Y63	10	R15	6				
α-*Proteobacteria*										
Unaffiliated							G62	1	B10, B13, B15, B35, B37, B53, B79	20
β-*Proteobacteria*										
Unaffiliated							G06, G94	13	B44	2
γ-*Proteobacteria*										
Unaffiliated							G73	1	B63	1
δ-*Proteobacteria*										
Unaffiliated							G13, G55	2		
Firmicutes										
Unaffiliated			Y27, Y36	9	R10, R16, R27, R35, R82	12	G07	1		
Unaffiliated									B25	4
Chimeras				3		20		21		21

Figure 3.1 shows a distinct relationship between clones obtained from the bore pool sediment, grey mat and red mat communities and members of the phylum *Aquificae*. There is a high similarity (99%) of 16S rRNA gene sequences between the three GAB clones and *Hydrogenobacter subterranea*. Only one phylotype was identified from the sediment clone library.

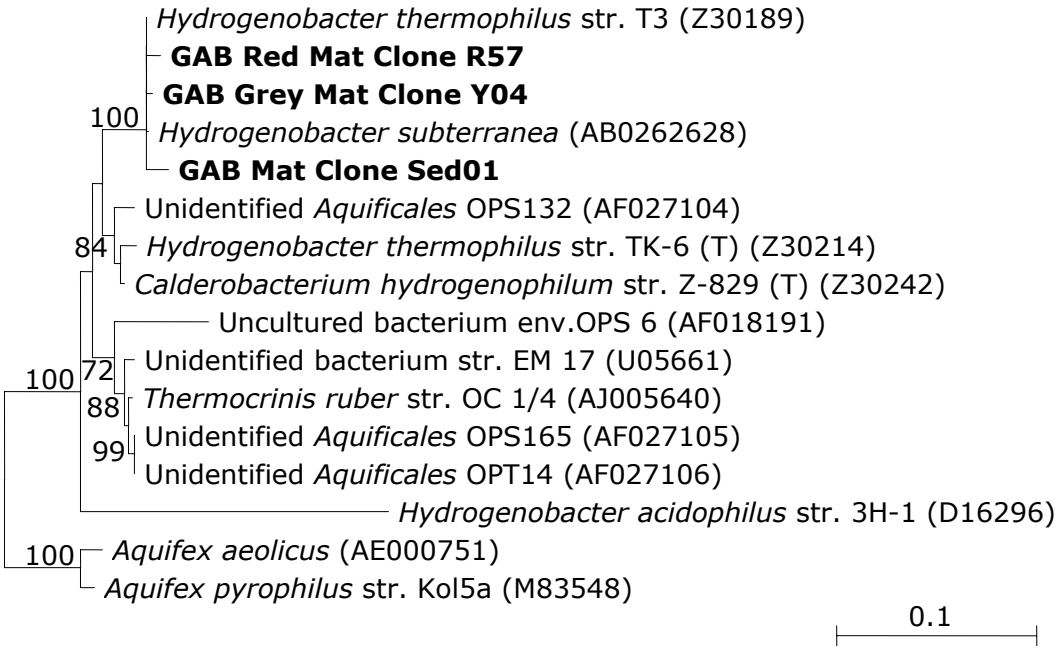


Figure 3.1: The phylogenetic analysis of the phylotypes within the phylum *Aquificae*

The dendrogram was created using a total of 16 sequences comprising of 3 GAB clone library sequences and 13 reference sequences. GenBank accession numbers are presented in brackets. The masked data set included 702 non-ambiguous bases. The phylogenetic analysis was carried out as described in Section 2.15.

The phylum *Deinococci-Thermus* segregates the aerobic chemoorganotrophic filamentous rod-shaped prokaryotes into the high-temperature *Thermus* and the lower-temperature *Meiothermus* species. Members from the *Thermus* group were identified from the grey, red, and green mat communities. The *Meiothermus* groups had representative in the red, green and brown mat communities. There appears to be a natural micro-diversity of *Thermus* and *Meiothermus* present within each filamentous mat as shown by the spread of the clones in the phylogenetic analysis (Figure 3.2). This phylogenetic diversity is discussed further in

Chapter 4. Clones from this phylum dominated the grey (72%), red (54%) and brown (34%) mat clone libraries.

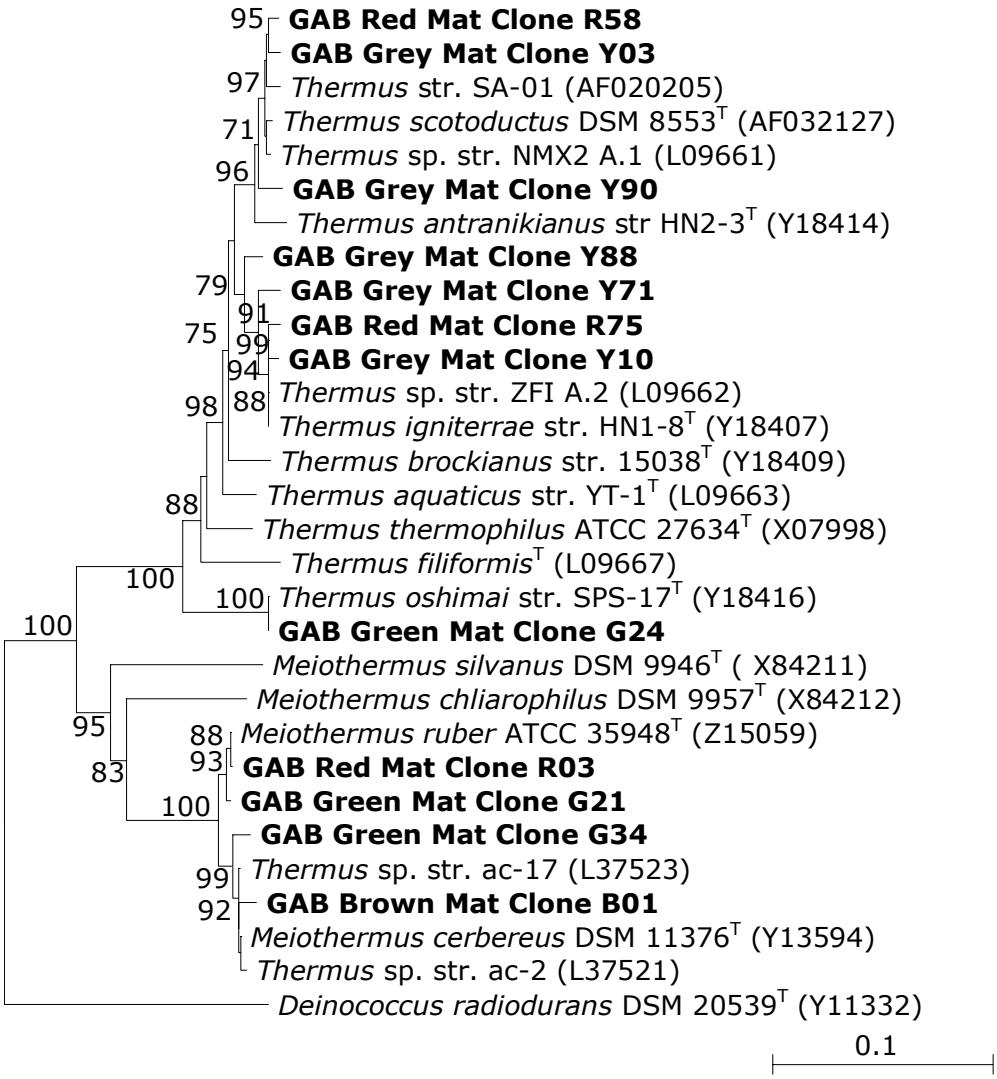


Figure 3.2: The phylogenetic analysis of the phylotypes within the phylum *Deinococci-Thermus*

The dendrogram was created using a total of 30 sequences comprising of 12 GAB clone library sequences and 18 reference sequences. GenBank accession numbers are presented in brackets. The masked data set included 1280 non-ambiguous bases. The phylogenetic analysis was carried out as described in Section 2.15.

The red mat, green mat and brown mat had clones that grouped closely within the phylum *Chloroflexi* (Figure 3.3). Each had a high similarity of 96% to *Chloroflexus auranticus*. This photosynthetic prokaryote was not identified in the higher temperature grey mat or sediment community.

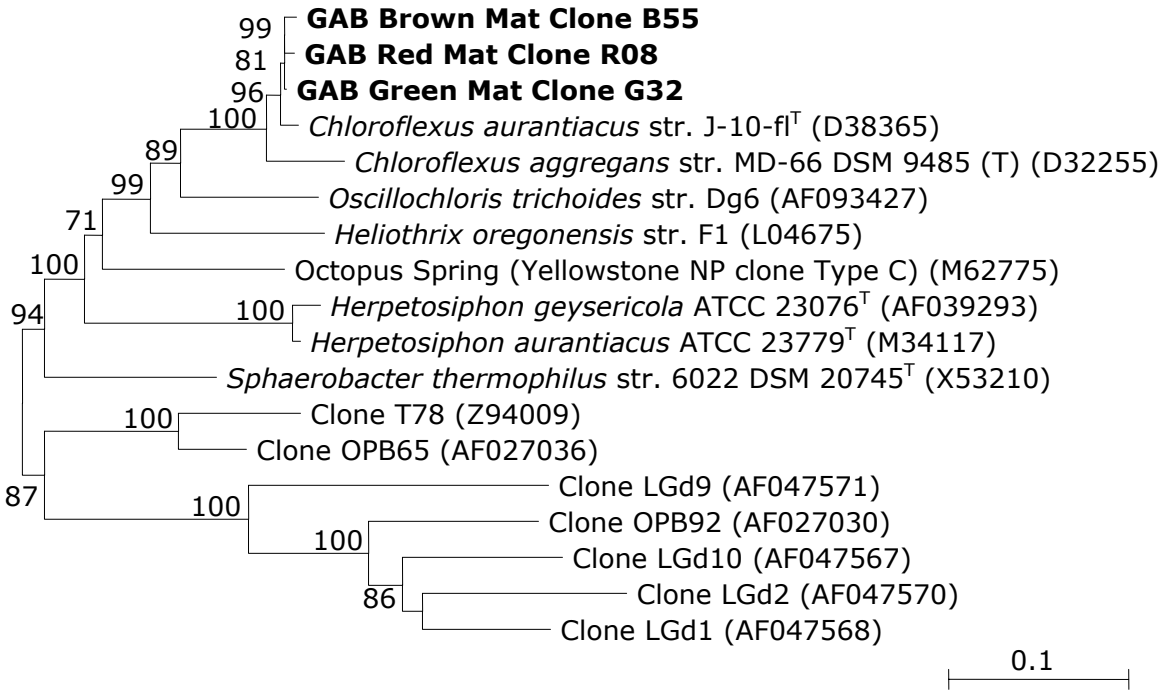


Figure 3.3: The phylogenetic analysis of the phylotypes within the phylum *Chloroflexi*

The dendrogram was created using a total of 18 sequences comprising of 3 GAB clone library sequences and 15 reference sequences. GenBank accession numbers are presented in brackets. The masked data set included 589 non-ambiguous bases. The phylogenetic analysis was carried out as described in Section 2.15.

One clone from the green mat community grouped closely to *Nitrospira* species within the phylum *Nitrospira* (Figure 3.4). *Nitrospira* have only been found in marine (Ehrich *et al*., 1995) and wastewater environments (Burrell *et al*., 1998), and the identification of *Nitrospira* species in the GAB extends its known ecology.

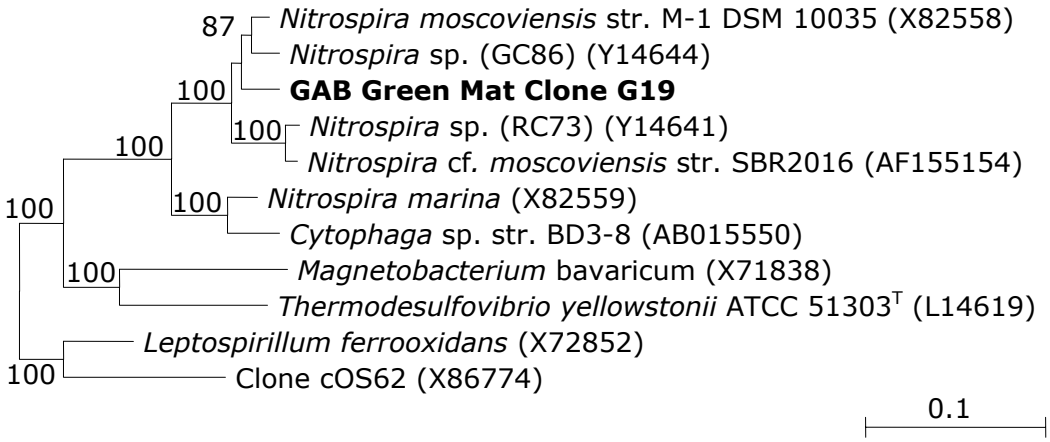


Figure 3.4: The phylogenetic analysis of the phylotype within the phylum *Nitrospira*

The dendrogram was created using a total of 11 sequences comprising of 1 GAB clone library sequence and 10 reference sequences. GenBank accession numbers are presented in brackets. The masked data set included 995 non-ambiguous bases. The phylogenetic analysis was carried out as described in Section 2.15.

One member from the brown mat community grouped with environmental clones from the phylum *Verrucomicrobia* (Figure 3.5).

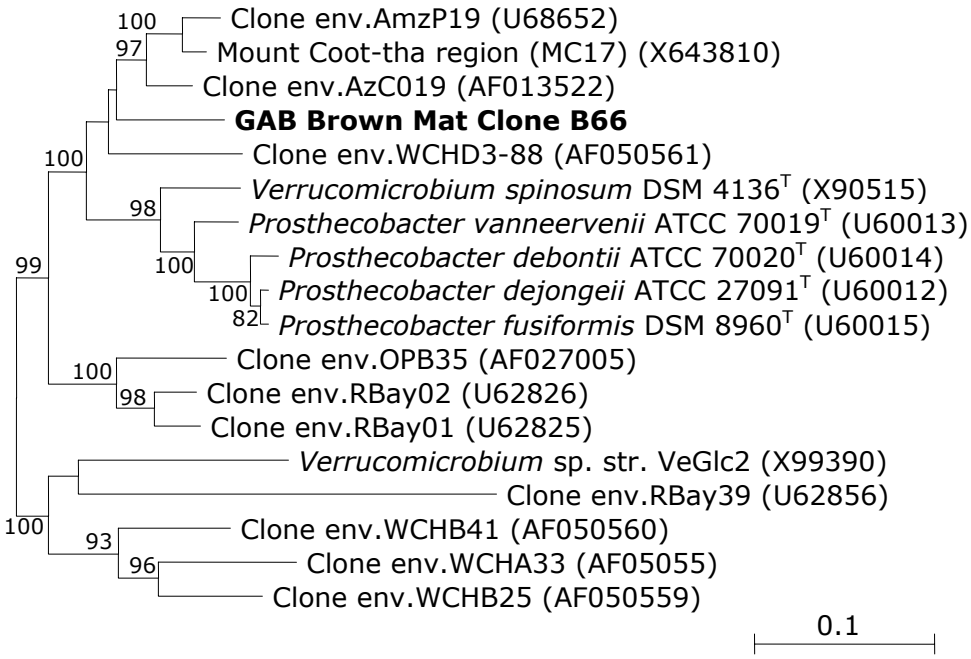


Figure 3.5: The phylogenetic analysis of the phylotype within the phylum *Verrucomicrobia*

The dendrogram was created using a total of 18 sequences comprising of 1 GAB clone library sequence and 17 reference sequences. GenBank accession numbers are presented in brackets. The masked data set included 499 non-ambiguous bases. The phylogenetic analysis was carried out as described in Section 2.15.

The phylum *Cyanobacteria* had clones from the green mat and brown mat communities (Figure 3.6). Both had high similarity to each other (99%) but did not have high similarity to any characterised species within this group. The green mat clone library was dominated by this phylotype (36%).

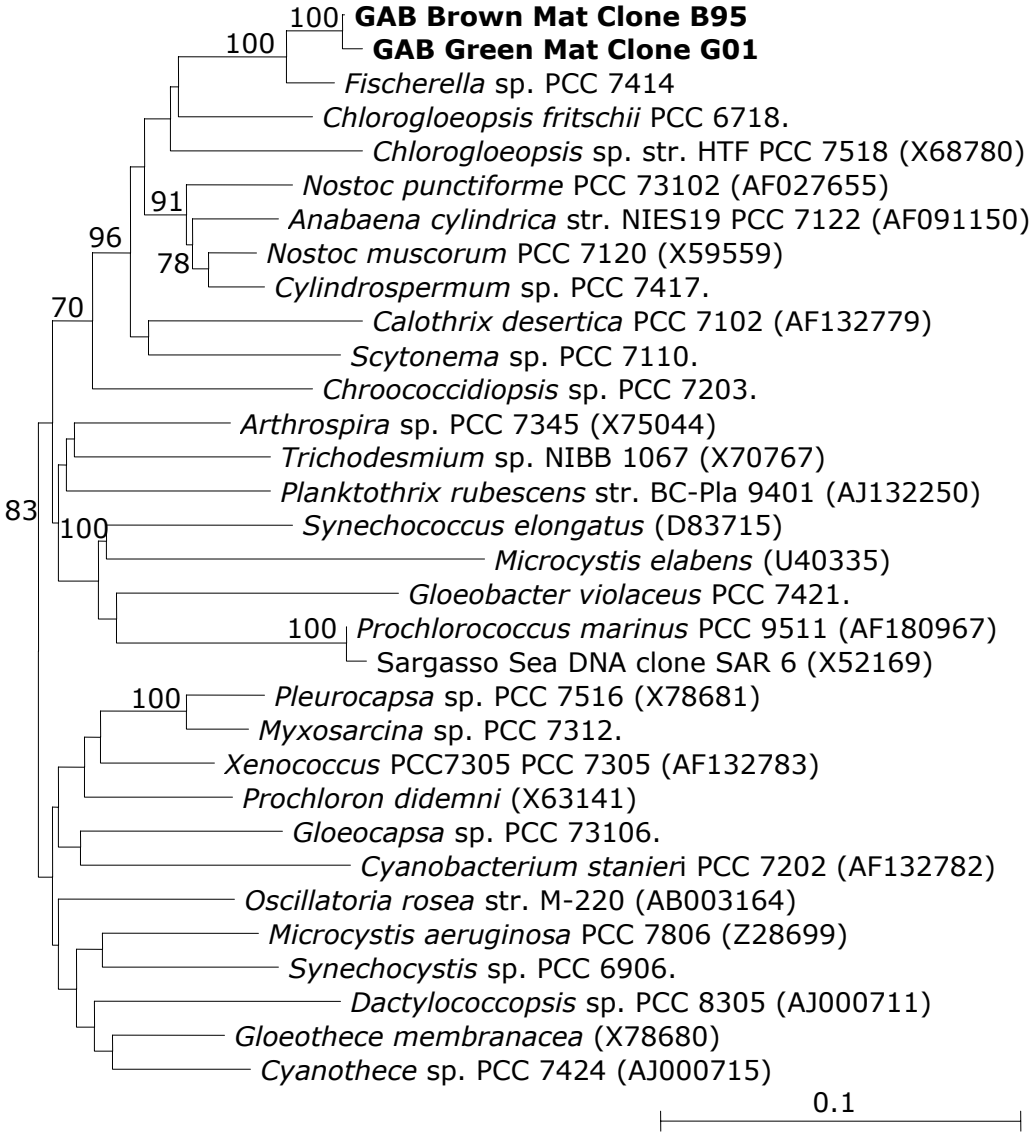


Figure 3.6: The phylogenetic analysis of the phylotypes within the phylum *Cyanobacteria*

The dendrogram was created using a total of 32 sequences comprising of 2 GAB clone library sequences and 30 reference sequences. GenBank accession numbers are presented in brackets. The masked data set included 776 non-ambiguous bases. The phylogenetic analysis was carried out as described in Section 2.15.

The GAB Red mat clone R38 has high similarity to the uncharacterised isolate 2BP-58 and a clone from thermophilic environment at Yellowstone National Park, USA. The remaining GAB clones from the green and brown mats were deep branching within the phylum *Planctomycetes* and clustered with environmental clones from a variety of ecosystems (Figure 3.7).

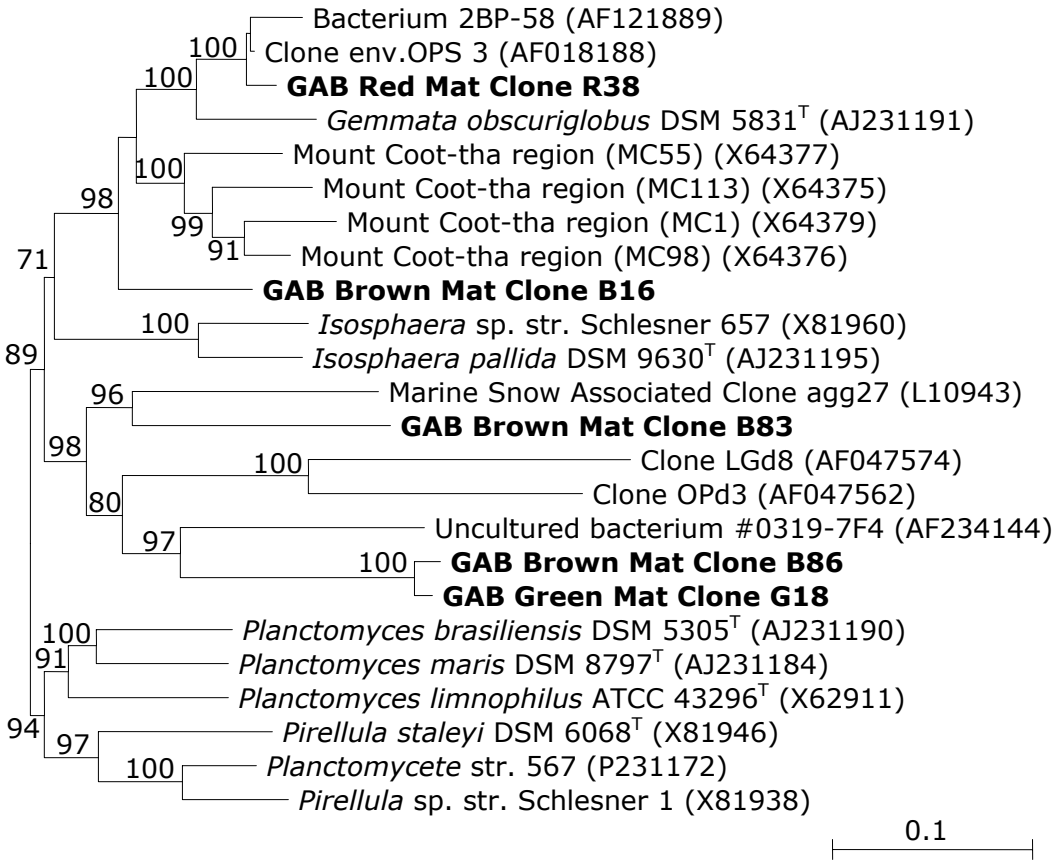


Figure 3.7: The phylogenetic analysis of the phylotypes within the phylum *Planctomycetes*

The dendrogram was created using a total of 24 sequences comprising of 5 GAB clone library sequences and 17 reference sequences. GenBank accession numbers are presented in brackets. The masked data set included 849 non-ambiguous bases. The phylogenetic analysis was carried out as described in Section 2.15.

The phylum *Acidobacteria* is poorly defined by characterised isolates. Their presence in soil communities is known (Hiraishi *et al*., 1995). Clones detected from the green and brown mats phylogenetically grouped within this phylum (Figure 3.8). GAB green mat clone G10 and GAB brown mat clone B11 had a high similarity of 97% to each other. The remaining clones were deep branching and associated with environmental sequences.

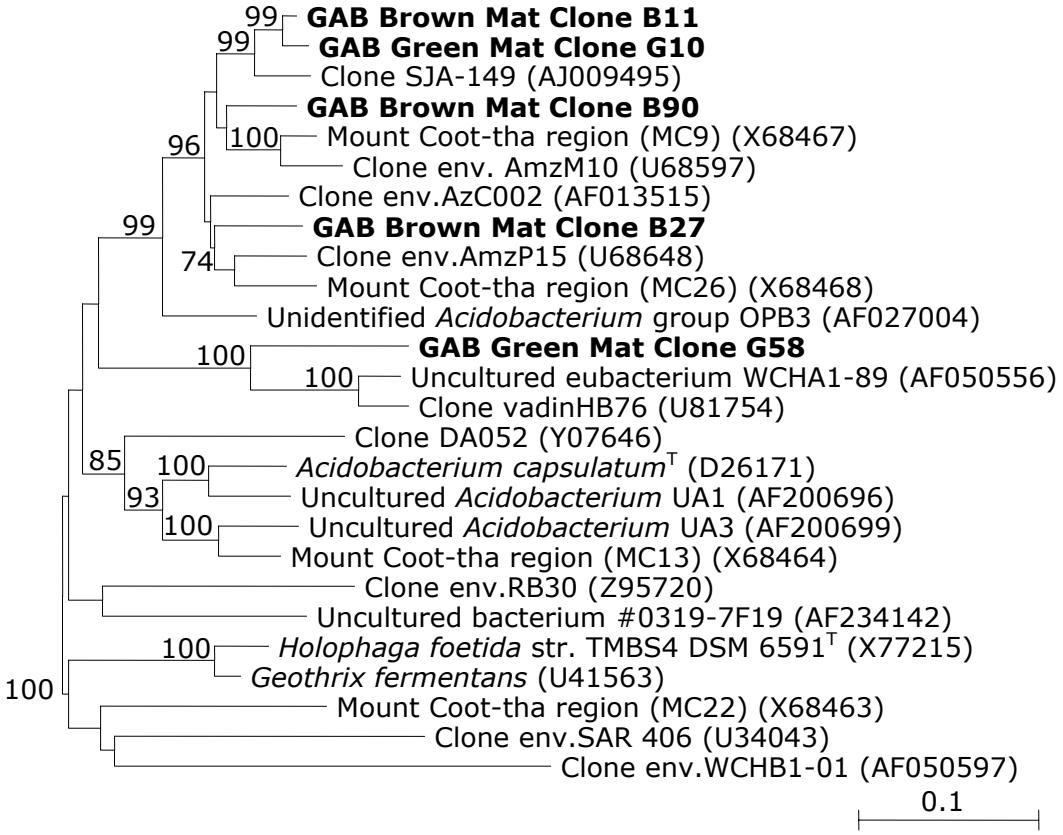


Figure 3.8: The phylogenetic analysis of the phylotypes within the phylum *Acidobacteria*

The dendrogram was created using a total of 26 sequences comprising of 5 GAB clone library sequences and 21 refernce sequences. GenBank accession numbers are presented in brackets. The masked data set included 482 non-ambiguous bases. The phylogenetic analysis was carried out as described in Section 2.15.

The phylogenetic analysis of clones associating with the phylum *Bacteroidetes* reveal a high coherence with clones obtained from the Yellowstone National Park, USA (Figure 3.9). Two clones obtained from the GAB (grey mat clone Y63 and red mat clone R15) had 99.5% similarity to each other. Members from this phylum were limited to the grey mat and red mat communities.

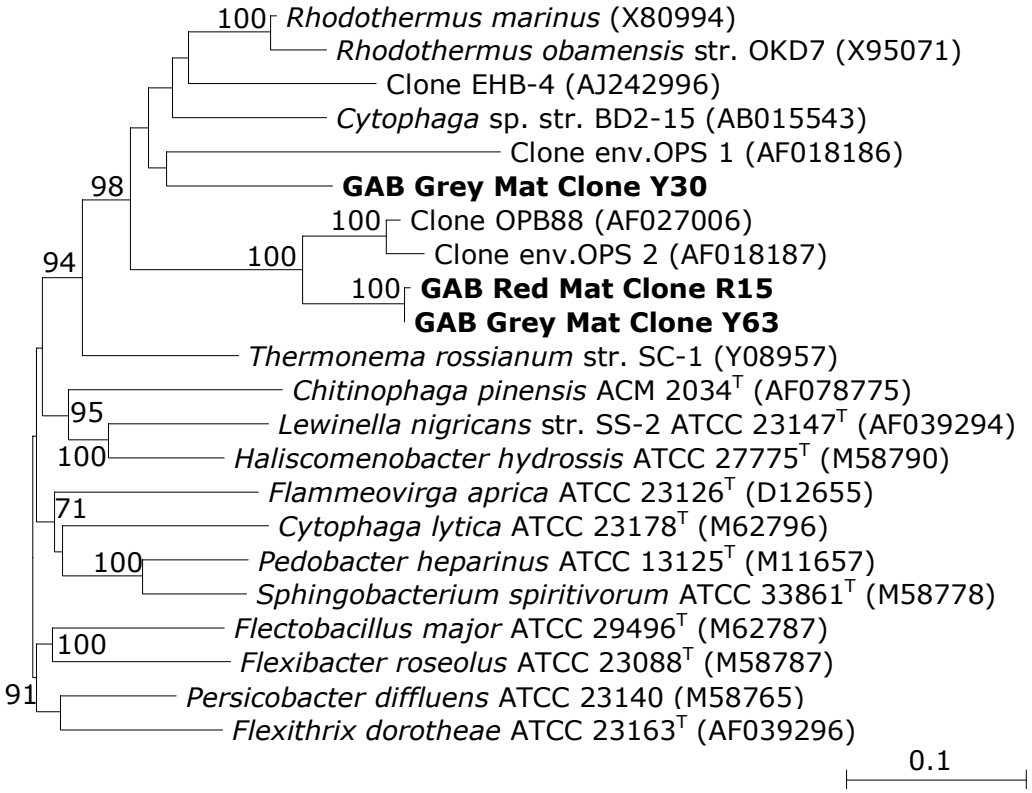


Figure 3.9: The phylogenetic analysis of the phylotypes within the phylum *Bacteroidetes*

The dendrogram was created using a total of 22 sequences comprising of 3 GAB clone library sequences and 19 reference sequences. GenBank accession numbers are presented in brackets. The masked data set included 1095 non-ambiguous bases. The phylogenetic analysis was carried out as described in Section 2.15.

Members from the phylum *Proteobacteria* were identified only from the green and brown mat communities. Members from the brown mat dominated the representatives from the α-class of the *Proteobacterial* phylum (Figure 3.10). One clone from the green mat library (G62) had a high similarity to a member from the brown mat library (B35). Of the α-class *Proteobacterial* clones, only two grouped clearly with well-characterised genera (GAB brown mat clone B10 with *Porphyobacter*, and GAB brown mat clone B53 with *Azospirllum*). The remaining are associated with environmental clones or genera with few representatives species.

The β- and γ-classes of *Proteobacteria* (Figures 3.11 and 3.12 respectively) included clones from both the green and brown mat communities. All were deep branching with a relatively low similarity to recognised genera. The δ-class of the *Proteobacteria* contained phylotypes only from the green mat

community (Figure 3.13). They were both very deep branching and did not correlate with any recognised genera.

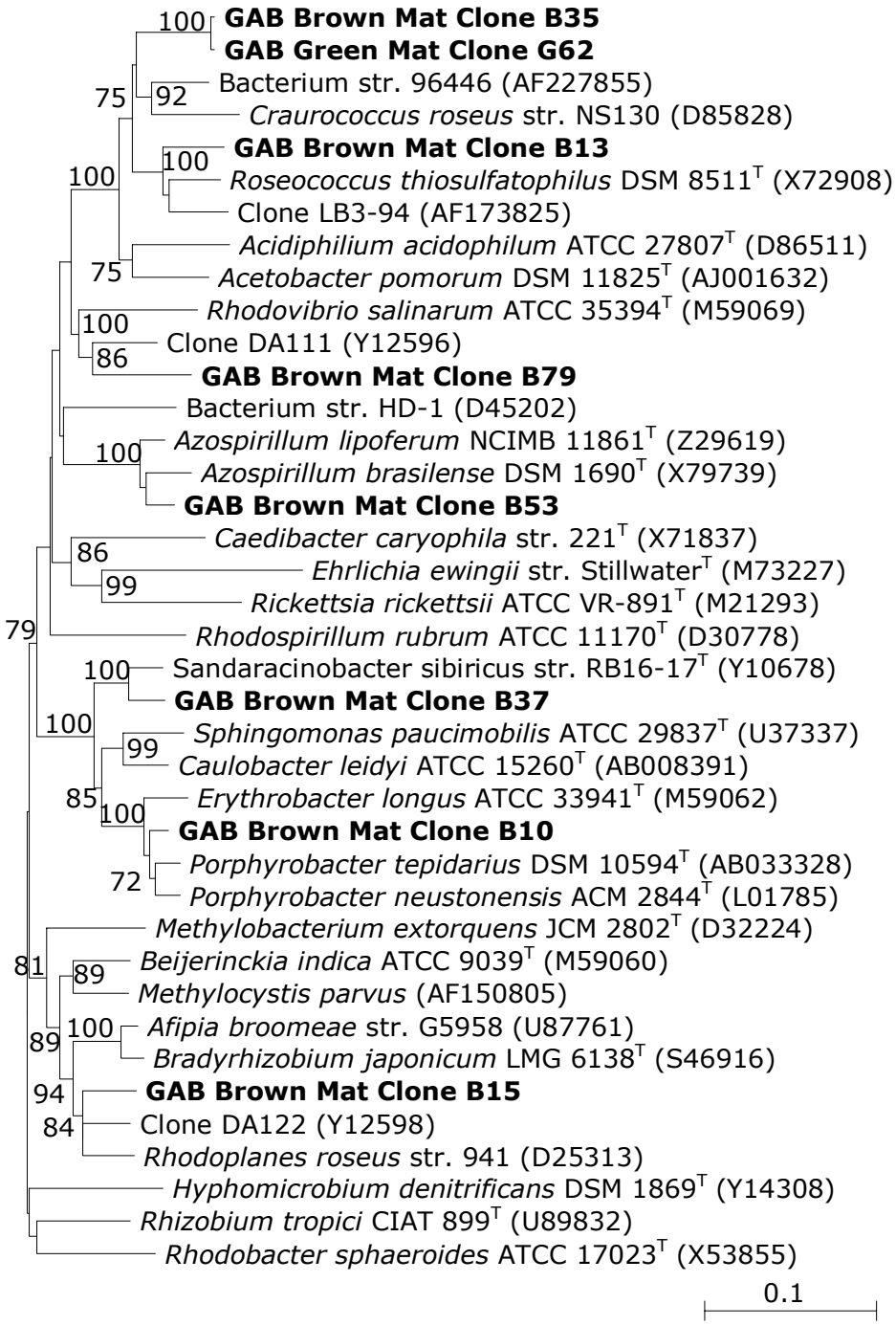


Figure 3.10: The phylogenetic analysis of the phylotypes within the α- class of the phylum *Proteobacteria*

The dendrogram was created using a total of 39 sequences comprising of 8 GAB clone library sequences and 31 reference sequences. GenBank accession numbers are presented in brackets. The masked data set included 1161 non-ambiguous bases. The phylogenetic analysis was carried out as described in Section 2.15.

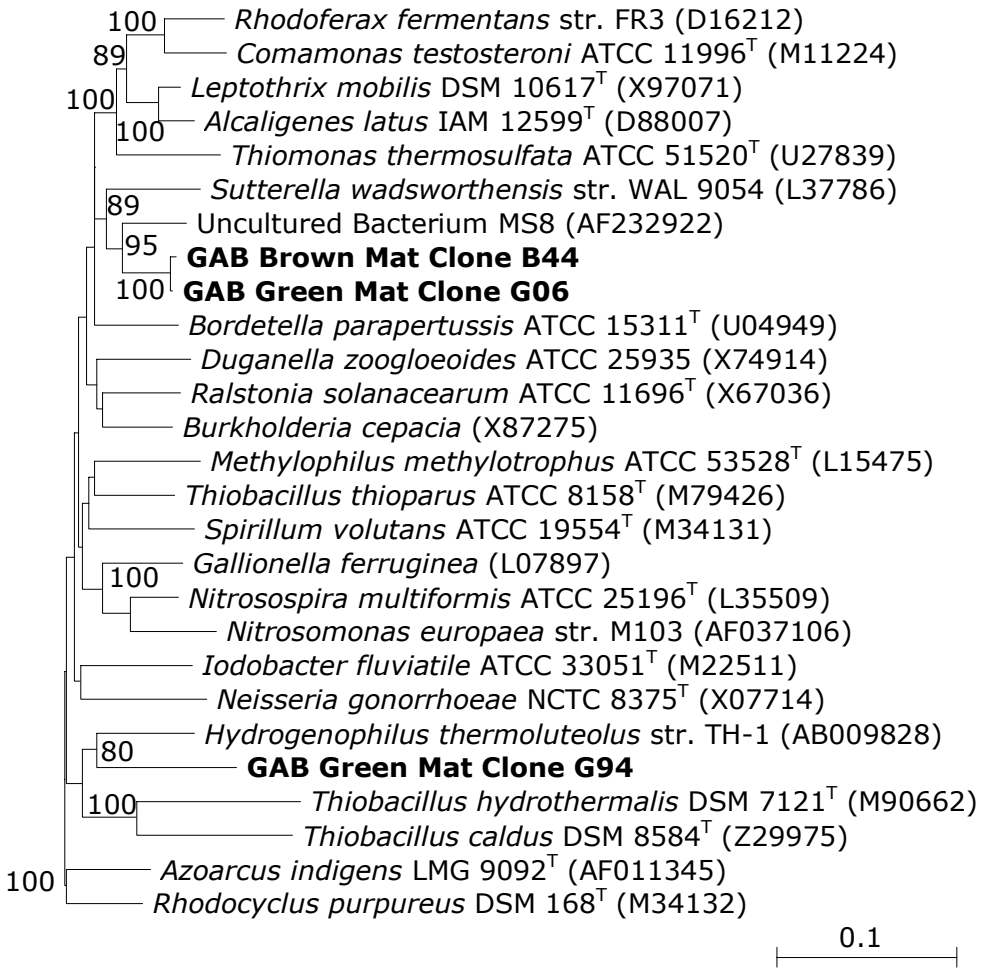


Figure 3.11: The phylogenetic analysis of the phylotypes within the β-class of the phylum *Proteobacteria*

The dendrogram was created using a total of 27 sequences comprising of 3 GAB clone library sequences and 24 reference sequences. GenBank accession numbers are presented in brackets. The masked data set included 1092 non-ambiguous bases. The phylogenetic analysis was carried out as described in Section 2.15.

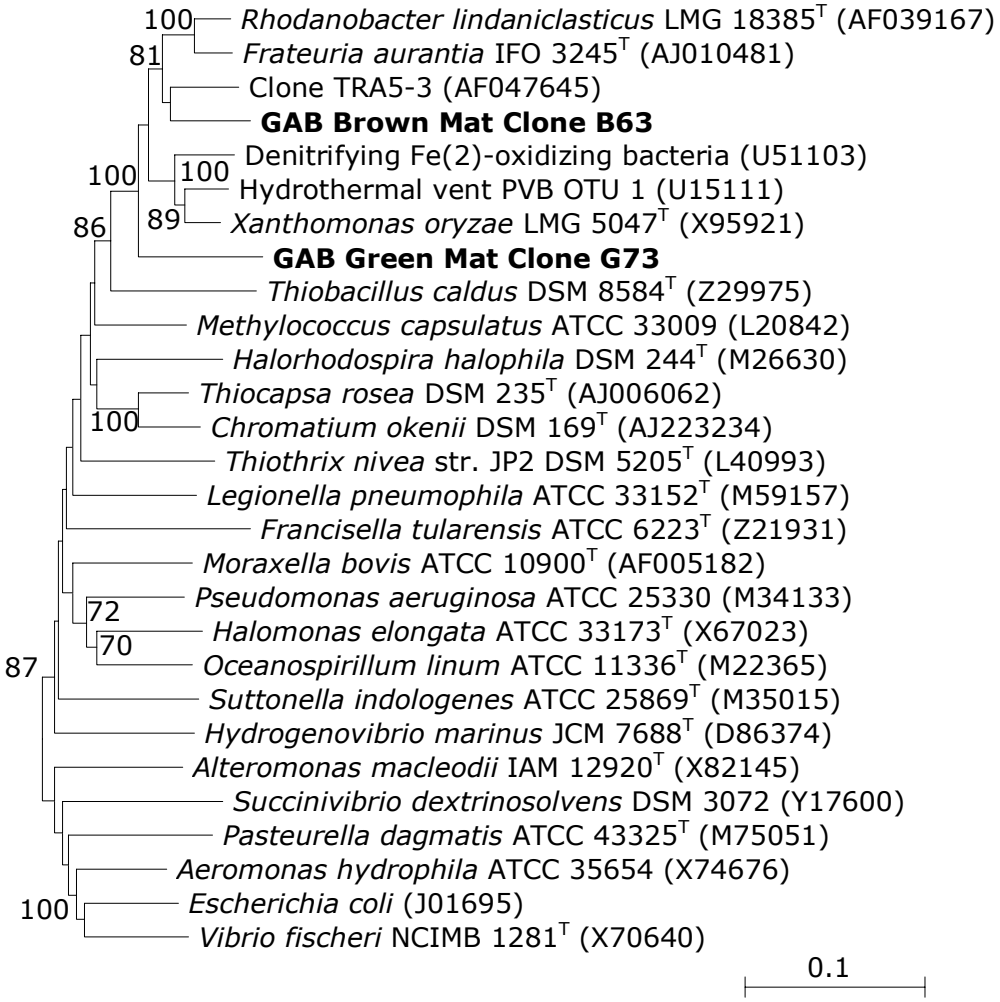


Figure 3.12: The phylogenetic analysis of the phylotypes within the γ-class of the phylum *Proteobacteria*

The dendrogram was created using a total of 28 sequences comprising of 2 GAB clone library sequences and 26 reference sequences. GenBank accession numbers are presented in brackets. The masked data set included 1183 non-ambiguous bases. The phylogenetic analysis was carried out as described in Section 2.15.

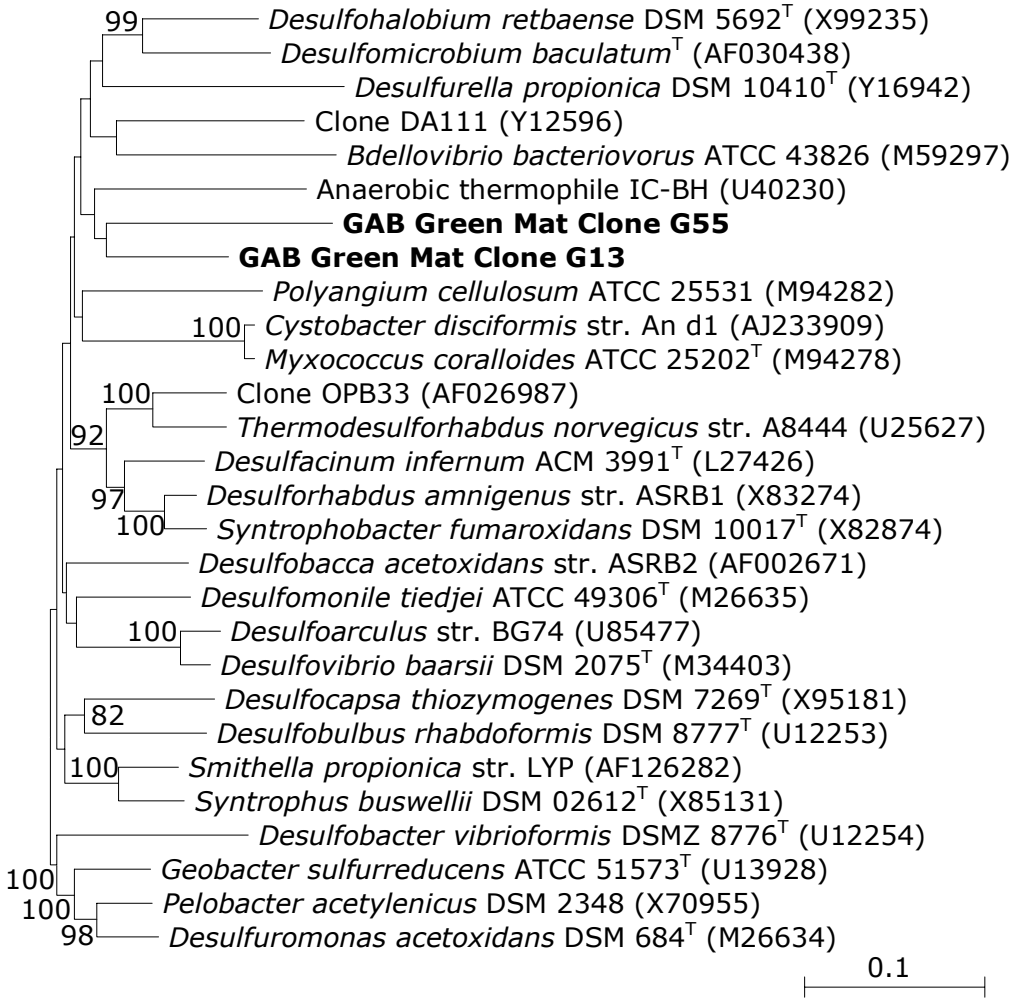


Figure 3.13: The phylogenetic analysis of the phylotypes within the δ-class of the phylum *Proteobacteria*

The dendrogram was created using a total of 28 sequences comprising of 2 GAB clone library sequences and 26 reference sequences. GenBank accession numbers are presented in brackets. The masked data set included 1040 non-ambiguous bases. The phylogenetic analysis was carried out as described in Section 2.15.

Representatives from the grey, red and green mat communities were identified as belonging to the *Firmicutes* phylum (Figure 3.14). Clones from the grey and red mats associated with the genus *Clostridia* and the green mat phylotype associated with the genus *Sporomusa*. One phylotype from the grey mat clone library (Y27) affiliated with the genus *Paenibacillus*, within the class *Bacilli*.

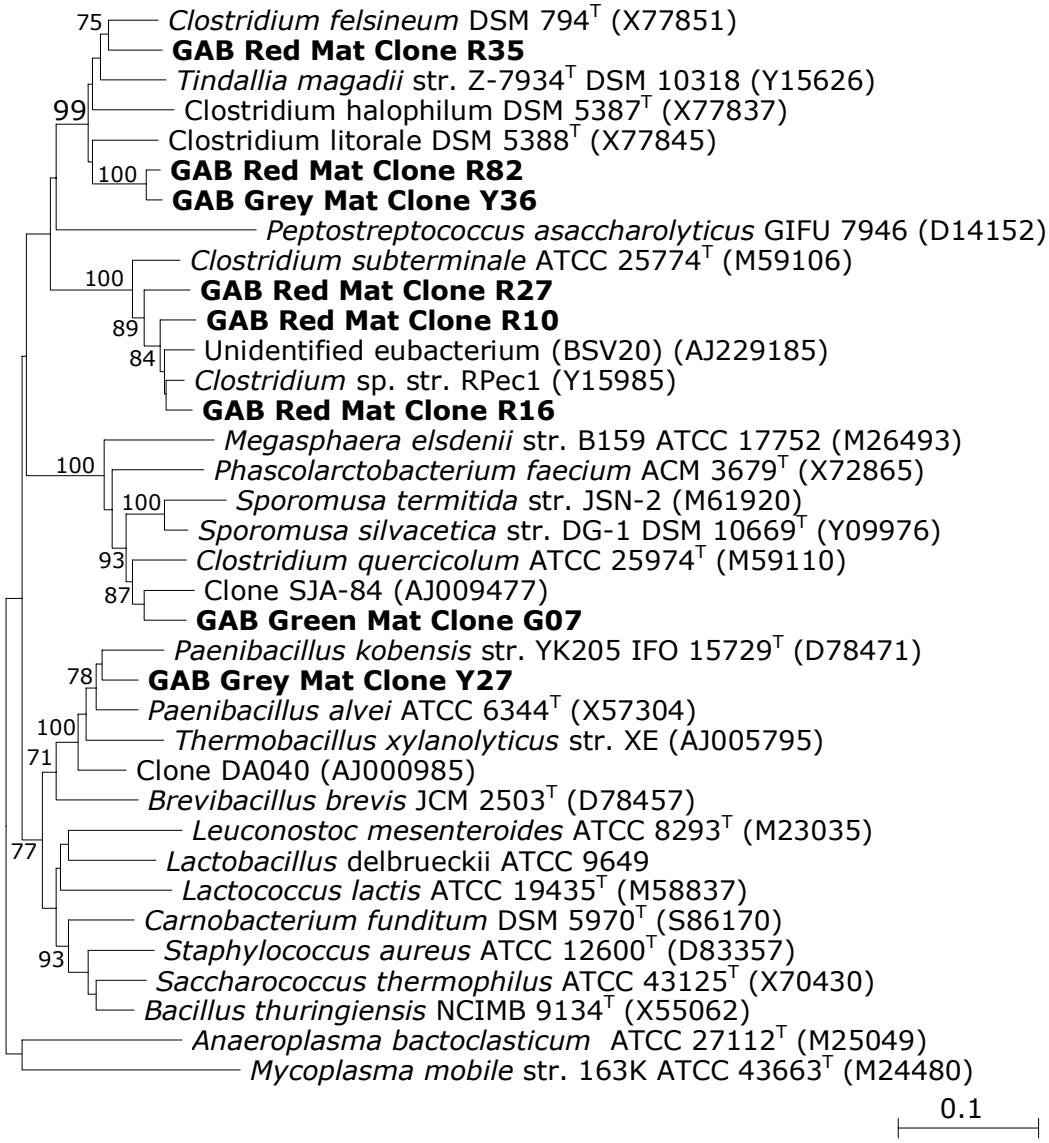


Figure 3.14: The phylogenetic analysis of the phylotypes within the phylum *Firmicutes*

The dendrogram is created using a total of 15 sequences comprising of 7 clone library sequences and 31 reference sequences. GenBank accession numbers are presented in brackets. The masked data set included 982 non-ambiguous bases. The phylogenetic analysis was carried out as described in Section 2.15.

A clone from the brown mat community correlated with a group of environmental clones that have no characterised relative (Figure 3.15). These clones come from a wide variety of environments such as the Antarctic, aquifers, marine environments and soils.

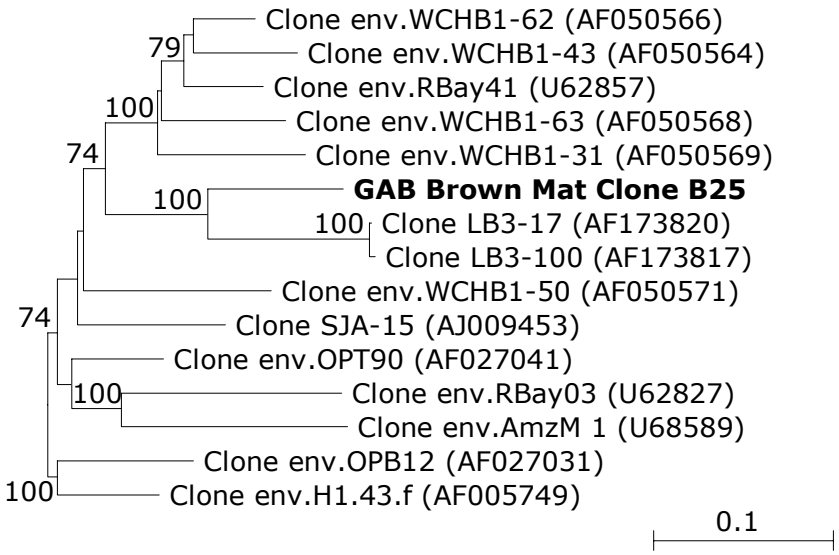


Figure 3.15: The phylogenetic analysis of the phylotype within a cluster of environmental clones

The dendrogram was created using a total of 15 sequences comprising of 1 GAB clone library sequence and 14 reference sequences. GenBank accession numbers are presented in brackets. The masked data set included 781 non-ambiguous bases. The phylogenetic analysis was carried out as described in Section 2.15.

3.4 DISCUSSION

3.4.1 PCR AND CLONE LIBRARY CONSTRUCTION

A PCR-mediated approach is used to investigate the microbial communities associated with the New Lorne bore of the Great Artesian Basin. Methods based on culturing to study microbial communities are well known to have intrinsic limitations that do not provide a comprehensive view of the populations present (Dunbar *et al*., 1999; Hugenholtz *et al*., 1998b; Kämpfer *et al*., 1996; Saul *et al*., 1999; Ward *et al*., 1997). Culture independent methods, such as PCR and cloning, do have a number of drawbacks. DNA extraction, amplification bias, gene copy number bias as well as cloning and sequencing artefacts are known to have an effect on the phylotypes identified (Ekendahl *et al*., 1994; Farrelly *et al*., 1995; Frostegård *et al*., 1999; Liesack *et al*., 1991; Wintzingerode *et al*., 1997).

To ensure the DNA extraction and purification is comprehensive, rigorous chemical and enzymatic methods were applied. The microscopic examination of lysed extracts revealed a low number of intact cells ensuring

that a loss of phylotypes due to incomplete lysis was minimal. Chromosomal DNA was titrated in the PCR in an effort to optimise amplification prior to ligation. To maximise the ligation of correctly sized inserts, the PCR products were gel purified. Transformation by electroporation of the ligated plasmids into competant *Escherichi coli* XL-10 cells was chosen as electroporation produces a much higher rate of transformation than heat-shock transformation (Dower *et al*., 1988).

Clones with inappropriately sized inserts (as shown by agarose gel electrophoresis) were considered negative. All 16S rRNA gene clones sequenced were approximately 1500bp in length. A phylogenetic analysis of partial sequences identified phylotypes from each clone library and full sequences for these were obtained. Chimeric inserts were identified utilising the Chimera Check at RDP (Maidak *et al*., 2001), and in cases of ambiguity, separate 5' and 3' trees elucidated the status of inserts. The Chimera Check program is most useful if the parent sequences (or close relatives of the parent sequences) of the chimera are available in the database for matching, and in some cases there was a need for further clarification.

Some of the commonly occurring chimeric artefacts found in the clone libraries were derivatives closely related to *Thermus* sp. str. NMX2 A.1, *Thermus* sp. str ZFI A.2, *Meiothermus ruber*, *Meiothermus cerberus*, and *Chloroflexus auranticus*. The chimeras from the 16S rRNA gene clone library from the red coloured mat were dominated by these derivatives, but they were not exclusive to his mat. The chimeras from the 16S rRNA gene clone libraries of the Green and Brown coloured mats possessed a much greater diversity in their chimeric artefacts, including sequences closely related to *Thiobacillus hydrothermalis* str. R3 DSM 7121, *Stenotrophomonas maltophilia* str. N4-15, *Nitrospira moscoviensis* str. M-1 DSM 10035, *Acidiphilium acidophilum*, *Ralstonia* sp. str. TFD41, *Hyphomicrobium* M3 ATCC 202122, *Planctomyces brasiliensis* DSM 5305, *Thermomicrobium roseum* ATCC 27502, *Roseococcus thiosulfatophilus* str. RB-3 DSM 8511, and other groups with only cloned 16S rRNA gene sequence data available. Of the chimeras that were fully sequences the most probable point of

chimera formation ranged from 690bp to 1110bp (*E. coli* numbering). Of the parent sequences, the majority was detected in the clone libraries.

3.4.2 *BLASTN* ANALYSIS

Blastn analysis of the full sequences of the phylotypes was undertaken to identify their closest relatives. Most *blastn* results from 16S rRNA gene clones that identified high similarity (>98%) were identified with isolated and characterised strains. Low *blastn* matches were not limited to clone sequences, with some of the closest relatives of the GAB clones belonging to phylogenetic groups with low numbers of characterised members.

Interestingly, one clone (GAB Red Mat Clone R38) had 98% identity with a clone sequence obtained from the Obsidian Pool, Yellowstone Park, USA (Barns *et al*., 1994). This is unusual, as the Obsidian Pool is chemically dissimilar to the GAB. The Obsidian Pool (75-95ºC) is rich in reduced iron, sulfide, CO_2, and hydrogen. The New Lorne bore in the GAB is relatively lower in reduced iron and sulfide, and the presence of near identical clones from both environments may be linked to the turnover of some other nutrient (e.g. hydrogen).

The *blastn* analysis of the clone libraries indicated that temperature has a critical effect on microbial community structure. The clone libraries from the environments with the highest temperatures had fewer phylogenetic members than those identified from environments at lower temperatures.

Some clone library members were found in more than one clone library. This may have been caused by the flow of the bore water through the drain. However, not one phylotype was identified from all clone libraries and this may imply that flow through of members and their subsequent detection in the clone libraries is inconsequential. The numbers of these identical clones from consecutive environments may indicate that they play an ecological role in each community.

3.4.3 PHYLOGENETIC ANALYSIS

The number of prokaryotes that can be cultivated using standard techniques is relatively low. It is a challenge to elucidate the roles that uncultivated microorganisms have in the environment based solely on molecular data. A rRNA sequence does little to provide a sense of the physiological properties of the prokaryote responsible for that sequence. If phylogenetic analysis, however, places that prokaryote within a group of prokaryotes that possess coherent characteristics, then it is likely that those characteristics occur in the uncultivated prokaryote. Many of the GAB clones clustered with collections of prokaryotes that exhibit consistent phenotypic properties, and it is possible to surmise the phenotypic properties of the uncultured prokaryote. Inferences about the metabolisms and nutrient cycles within the microbial communities may also be inferred from studying their constitutive phylogeny.

Members from the phylum *Aquificae* are known to inhabit high temperature aquatic environments. *Hydrogenobacter* species have been isolated and detected in hot springs, sulfur turfs and petroleum reservoirs (Kawasumi *et al*., 1984; Yamamoto *et al*., 1998). This is the first time members from the genus *Hydrogenobacter* have been identified from the GAB. As *Hydrogenobacter* are hydrogen-oxidisers and require microaerophilic conditions, it is not unexpected to detect their presence in this bore's waters. The high outflow temperature of 89ºC ensures that the solubility of oxygen is low, maintaining microaerophilic conditions, and it is within the range for growth of *Hydrogenobacter*. Clones with high similarity (98%) were also detected at 75ºC and 66ºC suggesting that *Hydrogenobacter* species play a role in these communities as well. At lower temperatures, they were absent in the green mat community at 57ºC and the brown mat community at 52ºC.

Members from the *Deinococci-Thermus* phylum were also detected from GAB. Each mat community had representatives from this phylum. Phylotypes from the grey mat (75ºC) and the red mat (66ºC) clustered around *T. scotoductus* and *T. igniterrae*. The green mat had one phylotype that grouped with *T. oshimai*. Phylotypes from the red mat (66ºC) and the

green mat (57ºC) had a high similarity (99%) and clustered with *Meiothermus ruber* while other related phylotypes (98% similarity) from the green mat (57ºC) and the brown mat (52ºC) communities were present within the genus of *Meiothermus*.

Obligately thermophilic and heterotrophic, *Thermus* and *Meiothermus* were first isolated from volcanic hot springs in the USA (Brock & Freeze, 1969) and Russia (Loginova *et al*., 1975) respectively. Members from these genera of are well known to inhabit a variety of thermal environments and their presence in the GAB is expected. Previous studies have shown that isolates related to *Thermus aquaticus* and *Meiothermus ruber* are easily cultured from this thermal aquatic environment (Byers *et al*., 1997; Denman *et al*., 1991). The different phylotypes obtained from the mat libraries indicates a high microdiversity present in this environment. Studies in New Zealand (Moreira *et al*., 1997; Saul *et al*., 1999) and the USA (Munster *et al*., 1986) have also indicated a wide diversity of *Thermus* genotypes in the natural environment.

The difference in phylotypes from the four mat communities reveals a definite change in the *Thermus-Meiothermus* populations from the highest temperature of 75ºC to the lowest temperature of 52ºC. At the higher temperatures *Thermus* phylotypes were present in the grey and red filamentous mats, while at the lower temperatures *Meiothermus* phylotypes were present in the green and brown mats. The red mat community had members from all and it is possible that it is a transitional environment for the dominant populations. The green mat had one phylotype closely related to *Thermus oshimai*, different to the populations detected in the grey and red mats.

The red, green and brown mats had phylotypes corresponding to *Chloroflexus auranticus*. With similarities higher than 98%, it signifies a high coherence in the *Chloroflexus* populations from 66ºC to 52ºC. *Chloroflexus* is another species that is detected in hot spring communities. Thermophilic and phototrophic, *Chloroflexus* is usually considered to be a descendent of the first phototroph as it is the most phylogenetically ancient of the anaerobic phototrophs (Madigan *et al*., 2000). Phototrophy is

considered to be limited to temperatures lower than 70ºC (Brock, 1967; Castenholz, 1969) and may be linked to the thermal stability of the photosynthetic apparatus. No photosynthetic members were identified in the grey mat at 75ºC. *Synechococcus*, a common thermophilic phototroph usually found in association with *Chloroflexus*, is easily detected in sulfur hot springs (Ferris *et al*., 1996b; Miller *et al*., 1998), and their absence in GAB is associated with the different hydrochemistry.

The phylum *Cyanobacteria* is thought to be recently evolved and possess an oxygenic phototrophic metabolism. The thermophilic limit on photosynthesis applies to this group and most are mesophilic and found in aquatic environments. *Cyanobacteria* are commonly detected in the volcanic hot spring environment (Nübel *et al*., 1999; Ruff-Roberts *et al*., 1994; Ward *et al*., 1998) and as they are oxygenic, are usually found in the top layers of mat communities. GAB clones from the green mat (57ºC) and brown mat (52ºC) communities were both identified as belonging to this group. A high correlation between the two (99%) indicates that a single dominant C*yanobacterial* phylotype is present.

The red mat phylotype R38 grouped closely with an uncharacterised bacterial isolate related to *Gemmalla obscuriglobus* in the phylum *Planctomycetes*. The related phylotypes from the brown mat and green mat branched deeply within the phylum and any definitive association with a genus in *Planctomycetes* is dubious. Within this phylum is the genus *Isosphaera*, a hot spring community member (Giovannoni *et al*., 1987; Ward *et al*., 1998), and the presence of deep-branching, but related, phylotypes in the GAB is probable.

A relatively new and uncharacterised phylogenetic group, *Acidobacteria*, is represented by phylotypes from the red, green and brown mat clone libraries. This phylogenetic group has constituents that are largely environmental soil clone sequences. The sole characterised isolate, *Acidobacterium capsulatum* (Hiraishi *et al*., 1995), is chemoorganotrophic and it may indicate that the deep branching related phylotypes possess a similar metabolism. The phylum is limited to temperatures lower than 66ºC. This study furthers the known ecology of this group.

The genus *Rhodothermus* within the phylum *Bacteroidetes* is represented by thermophilic prokaryotes that are isolated from hydrothermal vent ecosystems and submarine hot springs (Alfredsson *et al*., 1988; Sako *et al*., 1996). Affiliating phylogenetically with this genus is a number of 16S rRNA gene clones identified from volcanic hot springs in the USA (Hugenholtz *et al*., 1998b). The GAB clones belonging to the thermophilic grey mat (75ºC) and red mat (66ºC) communities are deep branching and group clearly with the volcanic hot spring clones. The presence of these clones only in the higher temperature mats indicates that these clones represent novel thermophilic prokaryotes.

Gram positive prokaryotes are represented in the grey mat (75ºC), red mat (66ºC) and green mat (57ºC) communities. The *Firmicutes* have a wide variety of thermophilic representatives including *Caloramator* (Chrisostomos *et al*., 1996), *Thermoanaerobacter* (Cayol *et al*., 1995), and *Clostridium* (Collins *et al*., 1994). The red mat had 5 phylotypes spread through the class *Clostridia*. One of these (R82) had a high similarity of 95% to the grey mat clone Y36 allowing that this phylotype may extend the upper limit to 75ºC for growth of a member of the class *Clostridia*. The green mat clone grouped with the *Sporomusa* genus.

The only representative from the class *Bacilli* is present in the 16S rRNA gene clone library from the grey mat at 75ºC. It grouped confidently within the *Paenibacillus* genus, a facultatively anaerobic and alkalophilic group (Ash *et al*., 1993; Shida *et al.*, 1997). The hydrochemistry of the bore water is favourable to the growth of this group. The absence of *Bacilli* in the communities is notable, as members from this group (notably *Bacillus*) are easily isolated from this and most other environments. Their easy isolation may be due to fact that they are spore-formers and can survive the high temperatures present in the GAB. Later enrichment studies have shown that their numbers are at least 100 times lower than *Thermus* and *Meiothermus* populations (see Chapter 4) and this may explain their noticeable absence in the clone libraries.

The phylum *Proteobacteria* has a wide diversity of phenotypes thought to evolve from a phototrophic ancestor. *Proteobacteria* were limited to the

green mat (57ºC) and brown mat (52ºC) communities. Members from the α-, β-, γ- and δ- classes of the phylum *Proteobacteria* were present. There was no close relationship between any clone and a characterised isolate. Most cloned rRNA genes were deep branching indicating that the GAB is a novel environment for the study of new and uncharacterised *Proteobacteria*. Isolates such as *Desulfovibrio* (Redburn & Patel, 1994) from the GAB belong to the δ-class of the *Proteobacteria* indicating the presence of sulfate-reducers in this ecosystem.

GAB mat clones also affiliated to the phyla *Nitrospira* and *Verrucomicrobium* were identified. One clone (GAB Brown Mat B25) affiliated with a group of clone sequences that had no isolated and cultured representative.

A member of the anaerobic, thermophilic, heterotrophic species *Fevidobacterium* has been isolated from the GAB (Andrews & Patel, 1996) and detected in a previous study (Byers *et al*., 1998) was not identified in this investigation. The absence of a clone with high similarity to *F. gondwanense* may be explained by the differing hydrochemistry between different bores in the GAB. The flow of bore water into the pool and drain may have maintained a microaerobic environment, ensuring the numbers of this species remain low.

3.4.4 COMPOSITIONAL ANALYSIS OF THE CLONE LIBRARIES

The composition of each library varied. The clone library created from the bore pool sediment (88ºC) consisted of a single phylotype, Sed01, which closely matched *Hydrogenobacter subterranea* (98% similarity). A limited number of *Bacterial* species grow at this temperature, and it is possible that the *Hydrogenobacter* species are the dominant species at the higher temperatures. There were no observed chimeras in this library, a fact that points to a low diversity of phylotypes (Wang & Wang, 1997). The probability that *Hydrogenobacter subterranea* is the sole inhabitant of this environment is high and indicates that the ecosystem's temperature of 88ºC has a restrictive effect on the populations that grow. Reysenbach *et al*. (2000) has shown that at 83ºC in Yellowstone National Park, USA, a

member of the phylum *Aquificae* dominated the community present with few members of *Korarchaeota* being detected.

The grey coloured mat community clone library was dominated by species of *Thermus* that accounted for over 70% of the clones analysed. The relatively high temperature of 75ºC of this environment also plays a significant role in limiting the diversity of prokaryotic populations that are maintained as representatives from only 5 phyla were detected.

At 66ºC in the red coloured mat community, clones related to *Meiothermus* accounted for almost 47% of the clones analysed, with clones related to *Thermus* accounting for a further 7%. The lower temperature allowed a greater diversity of species to survive, with members from the phyla *Chloroflexi* (8%), *Bacteroidetes* (6%) and *Firmicutes* (4%) detected.

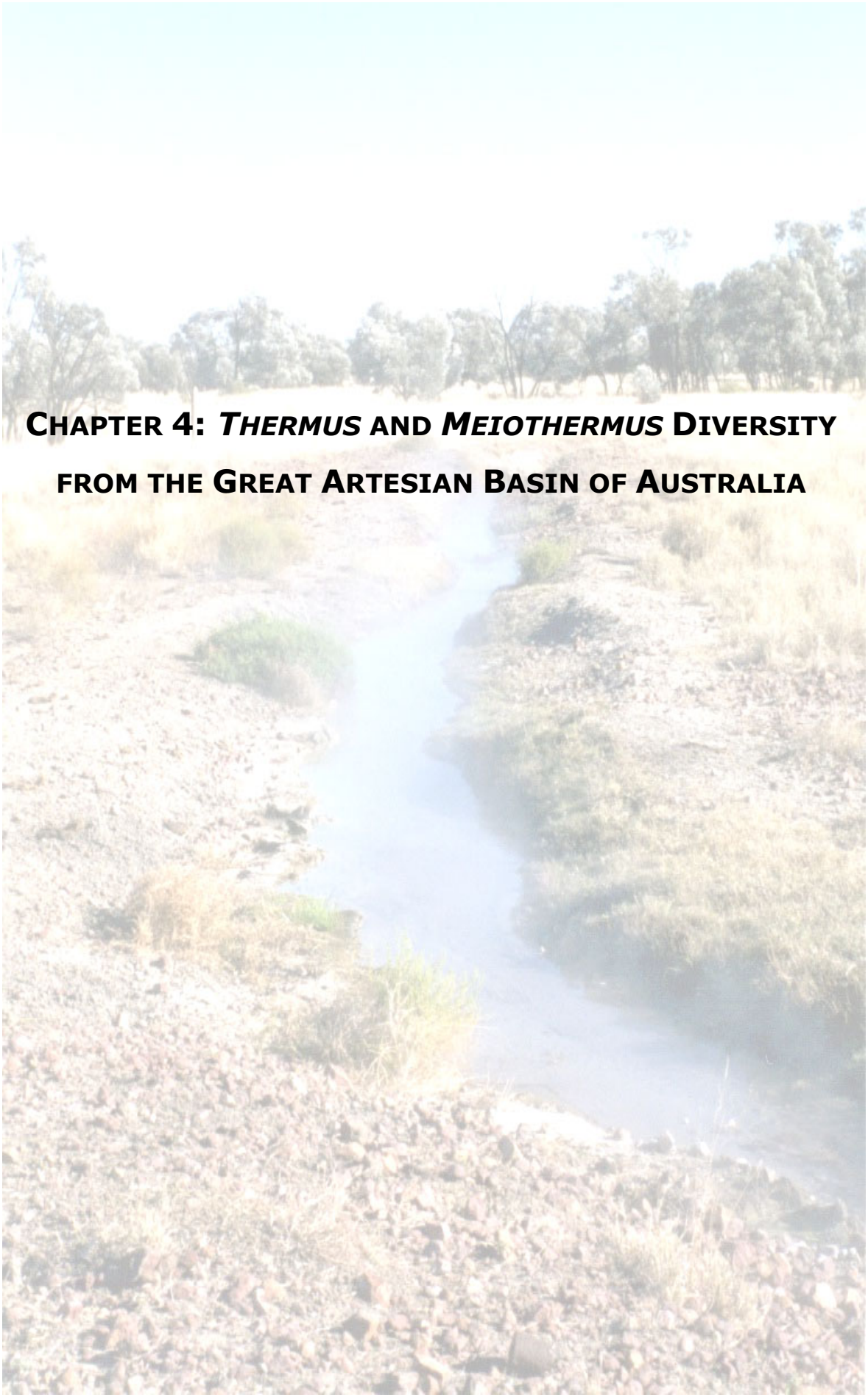
Representatives of the phylum *Cyanobacteria* dominated the green coloured community clone library at 57ºC (36% of the library). The temperature of 57ºC is ideal for the proliferation of photosynthetic bacteria. *Cyanobacteria* are known to dominate in some thermal, aquatic ecosystems where the temperature is around 50-60ºC (Ruff-Roberts *et al*., 1994; Ward *et al*., 1998). β-*Proteobacteria* and *Chloroflexi* comprised 13% and 10% of the green community clone library respectively. Members of these phyla have also been identified in hot spring communities from the USA at similar temperatures (Santegoeds *et al*., 1996; Ward *et al*., 1998). 7% of this library consisted of members from the phylum *Dienococci-Thermus*.

The brown filamentous mat community clone library was dominated by members from the phylum *Deinococci-Thermus* (34%) and α-*Proteobacteria* (20%). The phylum *Deinococci-Thermus* consisted of a sole phylotype (B01) that was closely related to *Meiothermus cerberus* (99% similarity). The α-*Proteobacteria* was comprised of 7 different phylotypes, showing a great increase in phylogenetic diversity at the lower temperatures.

In total, 24 phylotypes from 6 phyla had a similarity of 96% or more to cultured isolates and comprised 73% of all clones analysed. 34 phylotypes from 11 phyla had less than 96% similarity to cultured isolates, or were related to cloned 16S rRNA gene sequences, and comprised 27% of the

clones analysed. These results lead to the conclusion that the prokaryotic ecology of the Great Artesian Basin environment includes a diverse range of many uncultured, novel species.

The 16S rRNA gene amplification and cloning approach used here to phylogenetically describe five communities present in a bore drain associated with the Great Artesian Basin of Australia indicated a broad spectrum of prokaryotes throughout the *Bacterial* domain.



CHAPTER 4: *THERMUS* AND *MEIOTHERMUS* DIVERSITY FROM THE GREAT ARTESIAN BASIN OF AUSTRALIA

4.1 INTRODUCTION

Thermus and *Meiothermus* are strictly aerobic, thermophilic heterotrophs. Members of the genus *Thermus* are non-pigmented to pale or very brightly yellow pigmented. Most isolates grow with an optimum temperature of between 65ºC and 75ºC. Some *Thermus* species display halotolerance, with marine isolates being more halotolerant than their terrestrial relatives (Kristjánsson *et al*., 1986; Manaia & da Costa, 1991; Sharp & Williams, 1988). In general, however, there is very little inter- and intra- phenotypic variation amongst *Thermus* isolates. There are eight distinct species described on the basis of DNA-DNA homology studies (Boone & Castenholz, 2001), namely *T. aquaticus*, *T. brockianus*, *T. oshimai*, *T. filiformis*, *T. thermophilus*, *T. scotoductus*, *T. igniterrae* and *T. antranikianus*. *Meiothermus* species generally possess pale red to bright red pigmentation, with the exception of *M. chilarophilus* that has a pale yellow pigmentation, and grow optimally at temperatures between 50ºC and 60ºC. Despite these differences, early chemotaxonomic and numerical studies placed members of *Meiothermus* in the genus *Thermus* (Loginova *et al*., 1984; Sharp & Williams, 1988). However, subsequent phylogenetic and DNA-DNA hybridisation showed that *Thermus* and *Meiothermus* are closely related but phylogenetically distinct (Chung *et al*., 1997; Tenreiro *et al*., 1995). The four species of *Meiothermus, M. chilarophilus*, *M. cerberus*, *M. silvanus* and *M. ruber*, can be distinguished on the basis of their physiology, fatty acid composition and DNA-DNA homology (Boone & Castenholz, 2001).

Thermus and *Meiothermus* were first isolated from neutral and alkaline hot springs in Yellowstone National Park, USA (Brock & Freeze, 1969). Strains have subsequently been isolated from terrestrial and shallow marine hot springs in Iceland (Chung *et al*., 2000; Kristjánssen & Alfredsson, 1983), New Mexica (Saul *et al*., 1993), deep sea hydrothermal vents (Marteinsson *et al*., 1995), New Zealand (Saul *et al*., 1999), Japan (Oshima & Imahori, 1971; Saiki *et al*., 1972), Russia (Loginova *et al*., 1975), shallow marine hot springs in Portugal (Manaia *et al*., 1994; Manaia & da Costa, 1991), and from the subterranean Great Artesian Basin of Australia (Byers *et al*., 1997; Denman *et al*., 1991). In addition to natural environments, *Thermus*

strains have been isolated from man-made environments such as composts (Beffa *et al*., 1996), and hot water systems (Kristjánsson *et al*., 1994).

Members of the genera *Thermus* and *Meiothermus* strains are generally found in natural aquatic environments that are neutral to slightly alkaline with temperature ranging from 50-85ºC. The presence of *Thermus* and *Meiothermus* in environments that fall outside these temperature and pH restrictions are thought to originate from areas where growth conditions are suitable. Their growth is inhibited by high concentrations of organic materials, and numerous studies have shown that *Thermus* and *Meiothermus* species are associated with photosynthetic and chemolithotrophic prokaryotes that provide continuous low concentrations of organic compounds (Nold & Ward, 1995; Ward *et al*., 1997).

Using 16S rRNA gene sequence data of *Thermus* and *Meiothermus* species obtained from Great Artesian Basin isolates and clone libraries, this chapter extends the current phylogenetic diversity of these genera in the Great Artesian Basin. It will also further the understanding of the geographical distribution and limitations of *Thermus* and *Meiothermus* species throughout the world.

4.2 METHODS

4.2.1 SITE AND SAMPLES

The site selected for study was the New Lorne bore (Section 2.4.1). Samples were taken from the bore as described in Section 2.4.2. Bore sediment, grey, red, green and brown filamentous mats were used as inocula for the enrichment and isolation of *Thermus* and *Meiothermus* isolates.

4.2.2 MEDIA, ENRICHMENT AND ISOLATION

Media D broth was prepared as detailed in Section 2.3.3. In the case of the filamentous mat communities, 5mL of each sample were sonicated to disrupt the filamentous nature prior to inoculation. 1mL of each sample was

used to inoculate 9mL of Media D broth. A ten-fold serial dilution of the enrichment to 10^{-9} was prepared. These were then incubated at 70ºC and 55ºC for up to 72 hours. 1mL of sediment slurry contained approximately 0.3g of sediment. 1mL of the grey, red, green and brown mat slurries contained approximately 0.01g, 0.04g, 0.12g, and 0.15g respectively of filamentous material. Growth was determined by microscopic examination of the cultures and positive enrichment cultures were subcultured under identical growth conditions. Pure isolates were obtained by streaking a few of the serially diluted positive enrichment cultures on Media D plates amended with 2% agar followed by incubation at the enrichment temperatures. Single well-separated distinct colonies were picked and restreaked. This procedure was repeated at least twice before the culture was considered pure. Pure cultures obtained were stored in a 50:50 Medium D-glycerol mix at -20ºC

4.2.3 IDENTIFICATION OF *THERMUS* AND *MEIOTHERMUS* ISOLATES

Identification of presumptive *Thermus* and *Meiothermus* isolates was based solely on colony and cell morphology. Cell morphology was examined by phase contrast microscopy as specified in Section 2.5.

4.2.4 DNA EXTRACTION FROM PURE CULTURES

The chromosomal DNA from pure isolates was extracted as detailed in Section 2.6.1.

4.2.5 16S rRNA GENE AMPLIFICATION AND SEQUENCING

The 16S rRNA gene was amplified using PCR as described in Section 2.9. The PCR product was purified and sequenced as stated in Sections 2.10 and 2.14 respectively.

4.2.6 Source of *Thermus* and *Meiothermus* 16S rRNA Gene Clone Sequences from the Great Artesian Basin

Thermus and *Meiothermus* 16S rRNA gene clone sequences were obtained as described in Section 3.2.

4.2.7 Phylogenetic Analysis of 16S rRNA Gene Sequence Data

The phylogenetic analysis of 16S rRNA gene sequences obtained from waters associated with the Great Artesian Basin was carried out according to Section 2.15. Reference sequences and their respective sites of isolation used in the phylogenetic analysis are shown in Table 4.1.

Table 4.1: *Thermus* and *Meiothermus* 16S rRNA gene sequences and site of isolation

Strain	Accession Number	Site of Isolation	Reference
T. aquaticus YT-1^T	L09663	Yellowstone National Park, USA	Saul *et al*. (1993)
T. brockianus str 15038^T	Y18409	Yellowstone National Park, USA	Chung *et al*. (2000)
Unidentified *Thermus* OPS15	AF027023	Yellowstone National Park, USA	Hugenholtz *et al*. (1998b)
Unidentified *Thermus* OPB31	AF027020	Yellowstone National Park, USA	Hugenholtz *et al*. (1998b)
Thermus str. YSPID A.1	L10070	Yellowstone National Park, USA	Saul *et al*. (1993)
Thermus str. ac-2	L37521	Yellowstone National Park, USA	Nold & Ward (1995)
Thermus str. ac-7	L37522	Yellowstone National Park, USA	Nold & Ward (1995)
Thermus str. ac-17	L37523	Yellowstone National Park, USA	Nold & Ward (1995)
Thermus str YS38	Z15062	Yellowstone National Park, USA	Munster *et al*. (1986)
T. filiformis str. WAI 33 A1 ATCC 43280^T	X58345	New Zealand	Bateson *et al*. (1990)
Thermus str. W28 A.1	L10068	New Zealand	Saul *et al*. (1993)
Thermus str. Rt4 1A	L09669	New Zealand	Saul *et al*. (1993)
Thermus str. HS A.1	L09670	New Zealand	Saul *et al*. (1993)
T. thermophilus str. HB-8 ATCC 27634^T	X07998	Japan	Murzina *et al*. (1988)
T. flavus AT-62 ATCC 33923	L09660	Japan	Saul *et al*. (1993)
Thermus str. T2	AB054646	Japan	Ishiguro *et al*.(*unpublished*)
Unidentified *Thermus* H21.73.f	AF005751	Subsurface	Chandler *et al*. (1998)

Thermus str. NTU-024	AF324062	Taiwan	Chen & Tsay (*unpublished*)
T. antranikianus str. HN3-7^T	Y18411	Iceland	Chung *et al*. (2000)
T. igniterrae str. RF-4^T	Y18406	Iceland	Chung *et al*., 2000)
T. scotoductus str. SE-1 DSM 8553^T	AF032127	Iceland (hot water system)	Kristjánsson *et al*. (1994)
Thermus str. ZFI A.2	L09662	Iceland	Saul *et al*. (1993)
Thermus str. SRI-248	AF255591	Iceland	Skirnisdottir *et al*. (*unpublished*)
Thermus str. ZHGI A.1	L09664	Iceland	Saul *et al*. (1993)
Thermus str. SRI-1E1	AF255592	Iceland	Skirnisdottir *et al*.(*unpublished*)
M. silvanus str. V1-R2 DSM 9946^T	X84211	Iceland	Tenreiro *et al*. (1995)
M. chliarophilus str. ALT-8 DSM 9957^T	X84212	Iceland	Tenreiro *et al*. (1995)
M. ruber str. 16105	Y13596	Iceland	Chung *et al*. (1997)
M. cerbereus str. GY-1 DSM 11376^T	Y13594	Iceland	Chung *et al*. (1997)
T. oshimai SPS-17^T	Y18416	Portugal	Chung *et al*. (1997)
Thermus str. ViI7	Z15061	Portugal	Embley *et al*. (1993)
Thermus str. Fiji 3A.1	L10067	Fiji	Saul *et al*. (1993)
T. thermophilus str. CT1	AJ251940	Switzerland (composts)	Beffa *et al*. (1996)
Thermus str. NMX2 A.1	L09661	New Mexico, USA	Saul *et al*. (1993)
Thermus str. SA-01	AF020205	South Africa (mine waste)	Kieft *et al*. (1999)
M. ruber str. Loginova 21 ATCC 35948^T	Z15059	Russia	Bateson *et al*. (1990)
M. rosaceus RH99-01	AF312766	China	Chen *et al*.(*unpublished*)

4.3 RESULTS

4.3.1 ENRICHMENT AND ISOLATION

Enrichment results for 70ºC and 55ºC are shown in Tables 4.2 and 4.3 respectively. Colony and cell morphology of isolates obtained are shown in Tables 4.4 and 4.5 for enrichments at 70ºC and 55ºC respectively.

Table 4.2: Enrichment Results at 70ºC

Sample	**10^{-0}**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**
Sediment										
Grey Mat	✓*	✓	✓	✓*	✓	✓*				
Red Mat	✓*	✓	✓*	✓	✓*	✓	✓*			
Green Mat	✓*	✓	✓	✓*	✓	✓*				
Brown Mat	✓*	✓	✓*	✓	✓*					

* - Samples used for isolations

Table 4.3: Enrichment Results at 55ºC

Sample	**10^{-0}**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**
Sediment										
Grey Mat	✓*	✓*	✓	✓*						
Red Mat	✓*	✓	✓*	✓	✓*	✓*				
Green Mat	✓*	✓	✓*	✓	✓*					
Brown Mat	✓*	✓	✓*	✓*						

* - Samples used for isolations

Table 4.4: Cell and Colony morphologies of isolates cultured at 70°C

Isolate	Enrichment	Colony Morphology	Cell Morphology
Y70-01	Grey mat, 10^{-0}	1-2mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
Y70-02	Grey mat, 10^{-0}	1-2mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
Y70-03	Grey mat, 10^{-0}	1-2mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
Y70-04	Grey mat, 10^{-3}	1-2mm ∅, white, circular, flat	Rods 1.5μm x 5μm
Y70-05	Grey mat, 10^{-3}	1-2mm ∅, yellow, circular, flat	Rods/filaments 1μm x >10μm
Y70-06	Grey mat, 10^{-5}	1-2mm ∅, yellow, circular, flat	Rods/filaments 1μm x >10μm
Y70-07	Grey mat, 10^{-5}	1-2mm ∅, yellow, circular, flat	Rods/filaments 1μm x >10μm
R70-01	Red mat, 10^{-0}	1-2mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
R70-02	Red mat, 10^{-0}	1-2mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
R70-03	Red mat, 10^{-0}	1-2mm ∅, white, circular, flat	Rods, 1μm x 5-10μm; terminal spores 2μm ∅
R70-04	Red mat, 10^{-2}	1-2mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
R70-05	Red mat, 10^{-2}	2-3mm ∅, white, circular, flat	Rods 1μm x 5-10μm; terminal spores 2μm ∅
R70-06	Red mat, 10^{-4}	1-2mm ∅, yellow, circular, flat	Rods/filaments 1μm x >10μm
R70-07	Red mat, 10^{-6}	1-2mm ∅, yellow, circular, flat	Rods/filaments 1μm x >10μm
G70-01	Green mat, 10^{-0}	1-2mm ∅, white, circular, flat	Rods 1μm x 5-10μm; terminal spores 2μm ∅
G70-02	Green mat, 10^{-0}	1-2mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
G70-03	Green mat, 10^{-0}	1mm ∅, white, circular, flat	Rods 1μm x 5-10μm; terminal spores 2μm ∅
G70-04	Green mat, 10^{-0}	1mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm

G70-05	Green mat, 10^{-3}	1-2mm ∅, yellow, circular, flat	Rods/filaments 1μm x >10μm
G70-06	Green mat, 10^{-3}	1-2mm ∅, yellow, circular, flat	Rods/filaments 1μm x >10μm
G70-07	Green mat, 10^{-5}	1-2mm ∅, yellow, circular, flat	Rods/filaments 1μm x >10μm
G70-08	Green mat, 10^{-5}	2-3mm ∅, yellow, circular, flat	Rods/filaments 1μm x >10μm
B70-01	Brown mat, 10^{-0}	2mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
B70-02	Brown mat, 10^{-0}	2mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
B70-03	Brown mat, 10^{-0}	<1mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
B70-04	Brown mat, 10^{-2}	2-3mm ∅, yellow, circular, flat	Rods/filaments 1μm x >10μm
B70-05	Brown mat, 10^{-4}	<1mm ∅, yellow, circular, flat	Rods/filaments 1μm x >10μm

Table 4.5: Cell and Colony morphologies of isolates cultured at 55ºC

Isolate	From Enrichment	Colony Morphology	Cell Morphology
Y55-01	Grey mat, 10^{-0}	3mm ∅, white, irregular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
Y55-02	Grey mat, 10^{-0}	2mm ∅, white, irregular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
Y55-03	Grey mat, 10^{-0}	2mm ∅, white, irregular, flat	Rods, 1.5μm x 5μm; terminal spores 1.5μm x 2μm
Y55-04	Grey mat, 10^{-1}	1-2mm ∅, white, irregular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
Y55-05	Grey mat, 10^{-1}	3mm ∅, white, irregular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
Y55-06	Grey mat, 10^{-1}	2mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
Y55-07	Grey mat, 10^{-3}	<1mm ∅, yellow, circular, flat	Rods/filaments 1μm x >10μm
Y55-08	Grey mat, 10^{-3}	<1mm ∅, yellow, circular, flat	Rods/filaments 1μm x >10μm
Y55-09	Grey mat, 10^{-3}	<1mm ∅, yellow, circular, flat	Rods/filaments 1μm x >10μm
R55-01	Red mat, 10^{-0}	>5mm ∅, white, swarming	Rods, 1μm x 3-4μm; terminal spores 1.5μm x 2μm
R55-02	Red mat, 10^{-0}	1-2mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
R55-03	Red mat, 10^{-0}	>5mm ∅, white, swarming	Rods, 1μm x 3-4μm; terminal spores 1.5μm x 2μm
R55-04	Red mat, 10^{-0}	1-2mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
R55-05	Red mat, 10^{-2}	2-3mm ∅, white, irregular, flat	Curved rods, 1.5μm x >10μm
R55-06	Red mat, 10^{-2}	2-3mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
R55-07	Red mat, 10^{-2}	2-3mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
R55-08	Red mat, 10^{-4}	2mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
R55-09	Red mat, 10^{-4}	2mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 3μm

R55-10	Red mat, 10^{-5}	1mm ∅, red, circular, flat	Rods/filaments 1μm x >10μm
R55-11	Red mat, 10^{-5}	1mm ∅, red, circular, flat	Rods/filaments 1μm x >10μm
G55-01	Green mat, 10^{-0}	2mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
G55-02	Green mat, 10^{-0}	3mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
G55-03	Green mat, 10^{-0}	2-3mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
G55-04	Green mat, 10^{-2}	2-3mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
G55-05	Green mat, 10^{-2}	2mm ∅, white, irregular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
G55-06	Green mat, 10^{-2}	2-3mm ∅, white, irregular, flat	Rods, 1μm x 6μm; terminal spores 1.5μm x 2μm
G55-07	Green mat, 10^{-4}	2-3mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
B55-01	Brown mat, 10^{-0}	2mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
B55-02	Brown mat, 10^{-0}	1-2mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
B55-03	Brown mat, 10^{-2}	2-3mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
B55-04	Brown mat, 10^{-2}	2-3mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm
B55-05	Brown mat, 10^{-3}	3mm ∅, white, circular, flat	Rods, 1μm x 5μm; terminal spores 1.5μm x 2μm

4.3.2 ISOLATION OF *THERMUS* AND *MEIOTHERMUS*

Fifty-nine isolates were selected from the serial dilutions of the 4 disrupted mats after 3 days of incubation. Fourteen isolates (Y70-05, Y70-06, Y70-07, R70-06, R70-07, G70-05, G70-06, G70-07, G70-08, B70-04, B70-05, Y55-07, Y55-08, Y55-09) were identified as *Thermus* based on the presence of yellow pigmentation. Two isolates (R55-10 and R5-11) were identified as *Meiothermus* based on red pigmented colonies. The presence of a distinctive cell morphology (rods, 3-100μm x 0.5μm) and the absence of spores from cells of these isolates contributed further support for their identification. The remaining 43 isolates had opaque white colonies with cells greater than 1μm in diameter that sporulated, were most likely to be members of the aerobic, heterotrophic genus *Bacillus* and hence were not studied any further.

The total numbers of *Thermus* and *Meiothermus* in the 5 environmental samples were estimated by checking for the presence of typical yellow and/or red colored colonies on agar plates that had been streaked from positive serial dilution enrichment cultures and the results of these studies are presented in table 4.6.

Table 4.6: Approximate Numbers of *Thermus* and *Meiothermus*

	Thermus		***Meiothermus***	
	70ºC	**55ºC**	**70ºC**	**55ºC**
Sediment (88ºC)	N.D.	N.D.	N.D.	N.D.
Grey Mat (75ºC)	1×10^7	1×10^5	N.D.	N.D.
Red Mat (66ºC)	2.5×10^7	N.D.	N.D.	2.5×10^6
Green Mat (57ºC)	8×10^5	N.D.	N.D.	N.D.
Brown Mat (52C)	7×10^4	N.D.	N.D.	N.D.

Numbers expressed as cfu/g (wet weight of sample); N.D.- none detected.

Representatives of *Thermus* or *Meiothermus* were not isolated from the pool sediment, but were isolated from the four mat samples. *Thermus* in the grey and red mats were approximately 100 to 1000 times the numbers observed in the green and brown mats. *Thermus* was also isolated from the grey mat, but not the red, green or brown mats when incubated at 55ºC, but the numbers were approximately 100 time less. Me*iothermus* was only isolated from the red mat sample incubated at 55ºC, but the numbers of

Meiothermus were approximately 10 times less than *Thermus* in the same mat. Isolates of *Thermus* or *Meiothermus* were not observed in the green and brown mats enriched at 55ºC.

4.3.3 DNA Extraction and 16S rRNA Gene Sequencing

All presumptive *Thermus* and *Meiothermus* isolates had their chromosomal DNA extracted and their 16S rRNA genes amplified via PCR and sequenced.

4.3.4 Cloned 16S rRNA Gene Sequences Obtained from the Great Artesian Basin

A total of 367 clones were sequenced from 5 samples which included 46 clones from the bore pool sediment, 72 clones from the grey, 68 clones from the red, 88 clones from the green and 93 clones from the brown mat clone libraries. After partial sequencing (≈1000 nucleotides), clones from each library with greater than 98% similarity were regarded as identical and classed as a phylotype. Each phylotype was then and fully sequenced. A significant fraction of the 367 clones were found to represent *Thermus* (17% of the total) or *Meiothermus* (22%) with the remaining related to other phyla or were chimeras (Table 4.7). Collectively, 4 distinct *Thermus* phylogroups were found to exist in the clone libraries of the grey, red, green mats with the phylogroups related to *T. scotoductus* being the most dominant (63%) followed by the *T. igniterrae* (7%), *Thermus* strain SRI-248 (2%) and *T. oshimai* (1%) phylogroups. When phylotypes of individual mat samples were compared, *Thermus* was found to dominate the grey mat (75% of the total library), followed by the red mat (7% of the library) and the green mat (1% of the library) but *Thermus* were absent from the brown mat and the sediment sample. *T. scotoductus* dominated over *T. igniterrae* in the grey, and, only marginally, red mat samples. Collectively, two distinct phylogroups of *Meiothermus*, namely *M. ruber* and *M. cerberus*, were present in the red (47% of the library), green (6% of the library) and brown mat samples (34% of the library). The red mat was exclusively dominated by the *M. ruber* phylogroup, while the brown mat was exclusively dominated by the *M. cerberus* phylogroup. The green mat contained phylotypes from both *M. ruber* and *M. cerberus* phylogroups. *Thermus* or *Meiothermus* was not detected in the bore pool sediment library.

Table 4.7: Culture dependent and culture independent *Thermus* and *Meiothermus* from four microbial mats of the New Lorne Bore runoff channel

Sample		Phylogroups represented by [a]							
		T. scotoductus	*T. igniterrae*	*T. oshima*	*Strain SRI-248*	*M. ruber*	*M. cerberus*	Other phyla[b]	Chimera
Grey mat (75ºC)	Isolates [c]	-	Y70-05 to -07 Y55-07 to –09	-	-	-	-		
	% clones	59	14	-	2	-	-	16	9
	Clones [d]	Y03	Y10, Y88, Y90	-	Y71	-	-		
Red mat (66º)	Isolates		R70-05, R70-06		-	R55-10 to -11			
	% clones	4	3	-	-	47	-	22	24
	Clones	R58	R75	-	-	R03	-		
Green mat (57ºC)	Isolates	-	G70-05 to –08	-	-	-	-		
	% clones	-	-	1	-	4	2	72	21
	Clones	-	-	G24	-	G21	G34		
Brown mat (52ºC)	Isolates	B70-05	B70-04	-	-	-	-		
	% clones	-	-	-	-	-	34	45	21
	Clones	-	-	-	-	-	B01		

[a] Isolates or clones related to the phylogroups represented by *T. brockianus*, *T. aquaticus*, *T. antranikianus*, *T. filiformis*, *T. thermophilus*, *M. silvanus and M. chliarophilus* were not found.

[b] Data includes all other phyla except the order *Thermales*.

[c] The scheme used for naming the isolates is as follows: The alphabet indicates the mat color code (Y = grey mat, R = red mat, G = green mat and B = brown mat), the temperature of incubation (70 or 55^{o}C) followed by the isolate number.

[d] The clones are designated by the mat color followed by the clone number.

- indicates zero clones or isolates identified.

4.3.5 ISOLATES CULTURED FROM THE GREAT ARTESIAN BASIN

Thirteen of the fourteen *Thermus* isolates had a similarity of 100% with *Thermus igniterrae* with the remaining *Thermus* isolate (B70-05) was closely related to *Thermus* sp. SRI-96 (*T. scotoductus* phylogroup) with a similarity of 99.1%. Both *Meiothermus* isolates (R55-10 and R55-11) matched closely with *Meiothermus ruber* (similarity of 100%). These results support the earlier tentative identification that was based on cellular and colony characteristics (Table 4.7). The 16S rRNA gene sequences from the isolates have been deposited to GenBank and their accession numbers are listed in Appendix II.

4.3.6 PHYLOGENETIC ANALYSIS OF 16S rRNA GENES FROM ISOLATES AND CLONES

The phylogenetic analysis was split into *Thermus*-related (Figure 4.1) and *Meiothermus*-related (Figure 4.2) sequences. Using a 16S rRNA gene threshold similarity value of 99%, a total of nine phylogroups are shown within the genus *Thermus* and four phylogroups within the genus *Meiothermus*. The similarity values of the masked 16S rRNA gene sequence data set are not shown.

Within the *Thermus* genus, the nine phylogroups are composed of: *T. igniterrae* cluster (A); *T. brockianus* cluster (B); *Thermus* str SRI-248 (C); *T. aquaticus* cluster (D); *T. scotoductus* cluster (E); *T. antranikianus* cluster (F); *T. thermophilus* cluster (G); *T. oshima* cluster (H); and *T. filiformis* cluster (I). Of these, isolates from the Great Artesian Basin were found to belong to the *T. igniterrae* and *T. scotoductus* clusters. 16S rRNA gene clones from the Great Artesian Basin were found in the *T. igniterrae, Thermus* str SRI-248, *T. scotoductus,* and *T. oshima* clusters.

The *Meiothermus* genus was divided into the following phylogroups: *M. ruber* cluster (A); *M. cerbereus* cluster (B); *M. chliarophilus* cluster (C); and *M. silvanus* cluster (D). *Meiothermus* isolates from the Great Artesian Basin were only found in the *M. ruber* cluster, while Great Artesian Basin 16S rRNA gene clones were identified in the *M. ruber* and *M.cerberus* clusters.

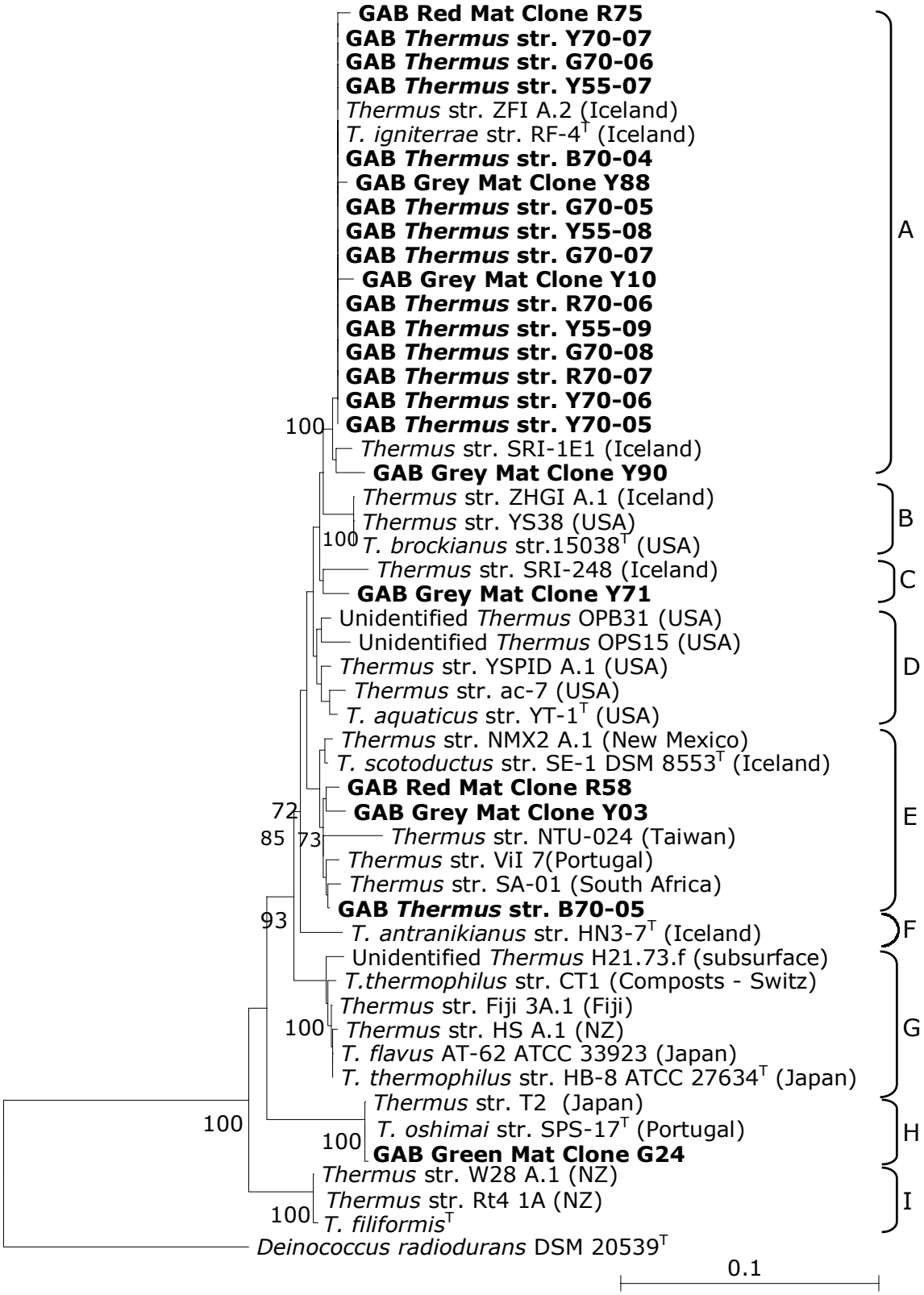


Figure 4.1: Phylogenetic analysis of *Thermus*-related prokaryotes from the Great Artesian Basin

The dendrogram was created using 30 database sequences, 2 GAB isolate sequences, and 8 GAB clone sequences. The unambiguous masked data set included 851 bp. GenBank accession numbers are listed in Table 4.1. Phylogenetic analysis is detailed in Section 2.15.

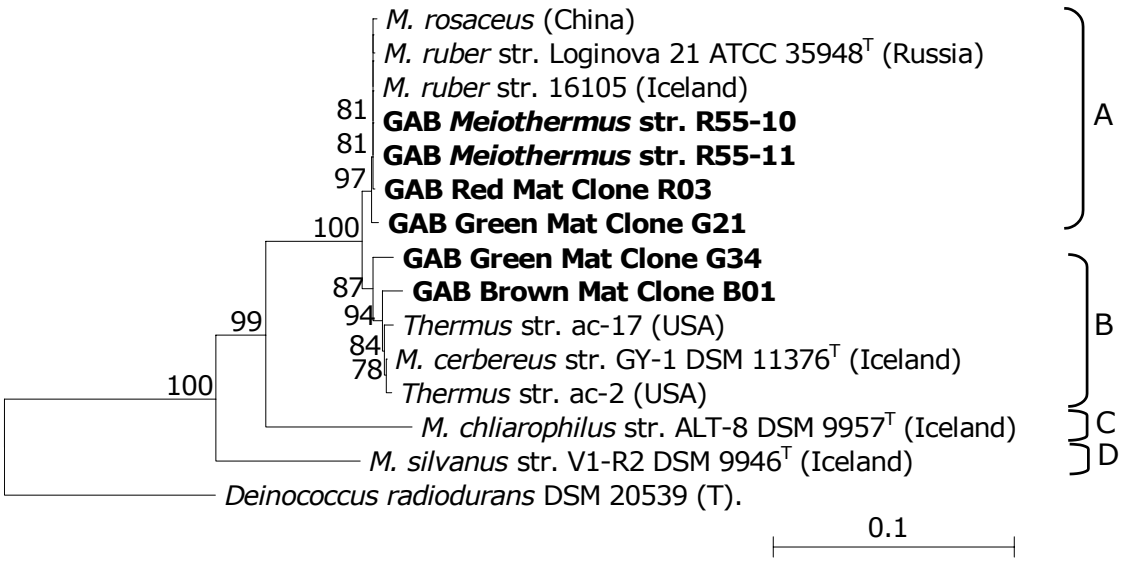


Figure 4.2: Phylogenetic analysis of *Meiothermus*-related prokaryotes from the Great Artesian Basin

The dendrogram was created using 9 database sequences, 2 GAB isolate sequences, and 4 GAB clone sequences. The unambiguous masked data set included 1381 bp. GenBank accession numbers are listed in Table 4.1. Phylogenetic analysis is detailed in Section 2.15.

4.4 DISCUSSION

4.4.1 ISOLATES OF *THERMUS* AND *MEIOTHERMUS*

The extensive presence of *Thermus* in the waters of the subsurface waters of the Great Artesian Basin has previously been reported (Denman *et al*., 1991). This research demonstrates that *Thermus* could not be isolated from samples taken directly below the bore source which had a temperature of 88ºC. Previous studies have shown that *Thermus* are only rarely isolated from environmental samples that have a temperature higher than 85ºC (Cometta *et al*., 1982; Hudson *et al*., 1987). This study therefore supports the idea that *Thermus* strains are ecologically limited to temperatures lower than 85ºC. *Thermus* was isolated from filamentous mat samples with temperatures ranging from 52ºC to 75ºC and these results concur with (Kristjánssen & Alfredsson, 1983) who showed that *Thermus* was most prevalent between 55ºC and 85ºC in hot springs in Iceland. This data also suggests that *Thermus* species are most dominant in the mats with temperatures between 66ºC and 75ºC, where the numbers are up to 100 and 1000 times higher than at 57ºC or 52ºC. These culture-independent

results also concur with the culture-independent results that show far higher numbers of *Thermus* clones in the grey and red mats than in the green or brown mats.

Previous studies have shown that *Meiothermus* can be isolated from volcanic hot springs with temperatures less than 70ºC and it has been suggested that they have adapted to a lower temperature mode of life than has *Thermus* (Boone & Castenholz, 2001). A previous report by Byers *et al*. (1997) has identified isolates from the Great Artesian Basin beloning to the species *Meiothermus ruber*. The isolation of R55-10 and R55-11 from the red coloured mat at 66ºC and the detection of *Meiothermus* in the 16S rRNA gene clone libraries from the red, green and brown mat communities provide further evidence for their ecological limitation to temperatures below 70ºC. The evidence presented here confirms that the limit for growth of *Thermus* and *Meiothermus* in thermally heated enviroments such as the subsurface derived waters of the Great Artesian Basin is temperature and is similar to that observed for volcanic hot springs.

4.4.2 PHYLOGENETIC CLUSTERING PATTERNS

Ecologically, thermal ecosystems can be considered as islands with a large dispersal barrier. The sporadic and discontinuous nature of thermal environments throughout the world provides a unique opportunity to study the speciation of thermophilic prokaryotes.

Plasmid restriction profiles have shown that *Thermus* isolates have a great amount of plasmid restriction polymorphism expected from their wide distribution and taxonomic diversity (Moreira *et al*., 1995). Pulsed-field gel electrophoretic studies of isolates belonging to the six species of *Thermus* have revealed a closely related organisation in isolates of the same species, especially if they were isolated from the same thermophilic environment (Moreira *et al*., 1997). The same study showed that isolates of *T. aquaticus* from Yellowstone National Park, USA, indicate the presence of several clones, whereas isolates of *T. brockianus* from the same environment indicate a sole clonal origin.

Hudson *et al*. (1989) showed that there was a distinct correlation between the pH and temperature of the environment and phylogenetic clusters observed. Studies by (Nold & Ward, 1995; Saul *et al*., 1993) have shown that *Thermus* phylogeny based on 16S rRNA gene sequence data also displays clustering of geographic isolates of *Thermus*.

The 16S rRNA sequence data obtained from the Great Artesian Basin had a very restrictive phylogenetic relationship. They were closely related to the *T. igniterrae* and *Thermus* strain SRI-248 phylogroups, which are solely represented by isolates from Iceland, and the *T. scotoductus* and *T. oshimai* phylogroups, which are resented by the more endemic *Thermus* species isolated from Japan, South Africa, New Mexico, Taiwan and Portugal. They were not closely related to, for example *T. brockianus* and *T. aquaticus,* which are exclusively found in Yellowstone National Park, or to the *T. filiformis* cluster, which is, so far, only represented by New Zealand isolates.

The clustering of a majority of *Thermus* clones to the Icelandic *Thermus* clusters represented by *T. igniterrae* and *Thermus* strain SRI-248 is not due to a bias due to lack of available data as other well studied thermal environments such as Yellowstone National Park and New Zealand thermal environments were also included in this studies. It suggests that this could be a result of the similar geochemical attributes of the Iceland and the Great Artesian Basin thermal environments which are highly alkaline and have very low sulfide concentrations (Chung *et al*., 2000; Kristjánssen & Alfredsson, 1983). Icelandic *Thermus* and *Meiothermus* strains have a much greater diversity than compared to their New Zealand or Yellowstone National Park counterparts. However, these studies have focused on a sole environmental site and may not represent fully the *Thermus* and *Meiothermus* diversity present in the Great Artesian Basin.

The Great Artesian Basin *Meiothermus* 16S rRNA gene sequences were predominantly related to *M. ruber* and *M. cerberus*. So far, the *M. ruber* cluster contains representatives from China, Russia and Iceland whereas the *M. cerbereus* cluster has so far only been restricted to members from Iceland and Yellowstone National Park. The other two species *of M. chliarophilus* and *M. silvanus* have only been reported from Iceland and

none of the Great Artesian Basin isolates are represented in these clusters. *Meiothermus* studies have not been exhaustively undertaken. A number of strains have been isolated but detailed studies have not been conducted (Byers *et al*., 1997). Given the lack of available data, no firm conclusions can be drawn.

Numerical classifications of *Thermus* and *Meiothermus* from globally distributed hot springs have shown that the phenotypic diversity may extend to over 20 clusters with some of these being represented by single isolates (Hudson *et al*., 1989). A comparison of restriction fragment length polymorphisms of the genomic DNA of fifty isolates of the six species of *Thermus*, however, revealed 38 different profiles. (Moreira *et al*., 1997). The same study also showed that isolates belonging to the same species have a closely related genomic organisation.

The phylogenetic clustering patterns observed in this study correlate with the patterns observed in a previous study by (Saul *et al*., 1993). The phylogenetic clustering observed with 16S rRNA gene sequence data does not precisely mirror that of previous numerical studies, however, this is not unexpected, as phenotypic markers do not necessarily provide phylogenetic conclusions. Most of the phylogenetic groups defined here have high bootstrap values indicating clear differences between the groups.

4.4.3 Loss of Diversity through Enrichment and Isolation

The phylogenetic diversity of culture-independent *Thermus* and *Meiothermus* members of the clone libraries was higher than that obtained through culture-based studies. Loss of prokaryotic diversity through enrichment is well known (Dunbar *et al*., 1999; Ferris *et al*., 1996b). More specifically, Saul *et al*. (1999) has shown that enrichment processes for *Thermus* yield a dominant phylotype. These results show that minor differences in 16S rRNA sequences can signify a phenotypic diversity in the natural environment that is not revealed during the enrichment process due to the selection of dominant strains.

In this study, although *T. scotoductus* phylotypes were detected in the grey and red mat clone libraries (59% and 4% of each respectively), the enrichment cultures were dominated by *T. igniterrae* isolates which accounted for 14% and 3% of the clones of each respective library. *T. igniterrae* was also isolated from the green and brown mats, but were not detected in their clone libraries. This also confirms the approximate cell count numbers, as the clone libraries compositions suggests that the populations of *T. igniterrae* is present at much higher concentrations in the grey and red mats than at lower temperature in the green and brown mat communities.

M. ruber phylotypes and *M. cerberus* phylotypes dominated the red mat clone library (47% of clones) and the brown mat clone library (34% of clones) respectively. However, only strains of *M. ruber* could be isolated from the red mat community. Although the majority of clones in the red mat clone library were *M. ruber*, cell numbers indicate that the population of *M. ruber* is 10 times less than the population of *T. igniterrae* in the same sample. This also confirms other reports of biases being introduced in both culture based, and culture-independent techniques when analysing environmental communities.

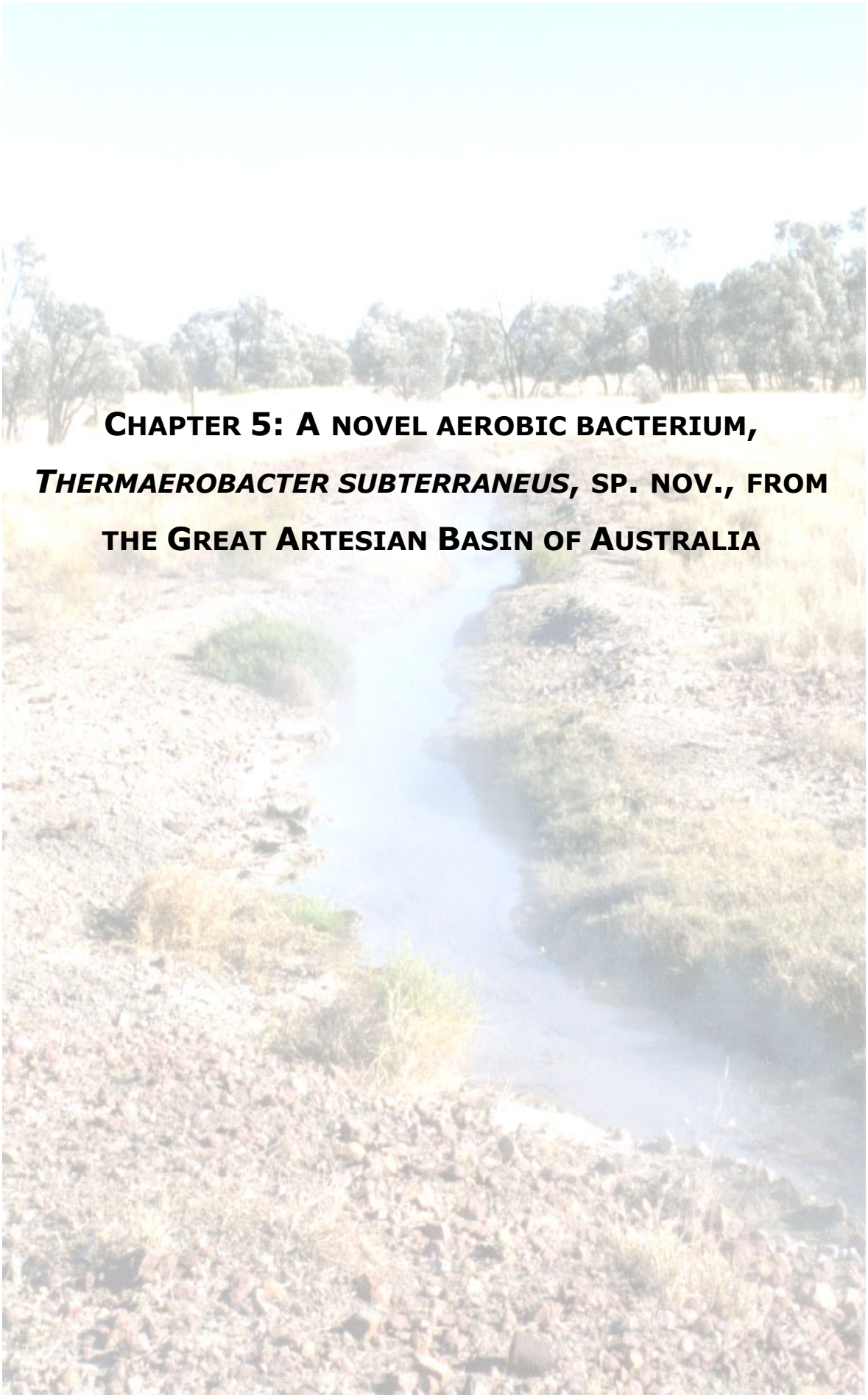
The ability to isolate all of the 4 phylogenetic representatives of the *Thermus* or *Meiothermus* present in the clone library failed and only a single most dominant phylotype was most represented. Although *Meiothermus* was detected in 3 of the 5 clone libraries, it was only isolated from the red mat. *Meiothermus* were enriched at 55ºC but the overgrowth by the *Bacillus*-like isolates made the detection of former very difficult and hence may be the cause of the failure.

Members of the family *Bacillaceae* are fast growing heterotrophs that can quickly outnumber slower growers like *Thermus* and *Meiothermus*. *Bacillus*-like isolates were only detected at much lower concentrations (up to 1000 times lower) than *Thermus* and *Meiothermus* which were detected at concentrations up to $2.5x10^7$cfu/g (wet weight). This explains their noticeable absence in the clone libraries created in Chapter 3 as the PCR generated clone libraries are known to bias more populous phylotypes.

4.4.5 BIOGEOGRAPHY OF *THERMUS* AND *MEIOTHERMUS*

Using 16S rRNA gene sequence data of *Thermus* and *Meiothermus* species obtained from Great Artesian Basin isolates and clone libraries, this chapter shows the diverse nature of these isolates. This study reveals that some of the *Thermus* species are in fact limited in their biogeography. *T. brockianus*, *T. filiformis, T. aquaticus*, and *T. antranikianus* have only been isolated or detected from a single geographical source. Other species such as *T. scotoductus* and *T. thermophilus* appear ubiquitous in their distribution. From limited studies on the genus *Meiothermus*, it can be seen that some of its' members (*M. ruber* and *M. cerberus*) are quite widespread as well. However, the limited phylogenetic information available does not enable further conclusions about its' biogeography.

In previous studies plasmid profiling (Denman *et al*., 1991) and DNA probe techniques (Byers *et al*., 1997) have revealed the diversity of *Thermus* and *Meiothermus* present in the thermal waters of the Great Artesian Basin of Australia. In this report these studies were extended and present evidence of the phylogenetic depth of these genera using culture dependent and culture independent studies.



CHAPTER 5: A NOVEL AEROBIC BACTERIUM, *THERMAEROBACTER SUBTERRANEUS*, SP. NOV., FROM THE GREAT ARTESIAN BASIN OF AUSTRALIA

5.1 INTRODUCTION

Though the deeper phyla of domain *Bacteria* such as *Aquificae* (Burggraff *et al*., 1992; Huber *et al*., 1992; Kawasumi *et al*., 1984) and *Thermotogae* (Fardeau *et al*., 1997) consist exclusively of thermophiles, other more recently evolved phyla such as *Firmicutes* are also represented by thermophilic members (Boone *et al*., 1995; Collins *et al*., 1994). Most of the thermophilic bacteria reported until recently were isolated mainly from volcanic hot springs and hydrothermal vents (Brock & Freeze, 1969; Harmsen *et al*., 1997b; Larson *et al*., 1997; Ward *et al*., 1998). Within the past few years, however, an increasing numbers of thermophilic microbes have also been isolated from subsurface non-volcanic thermal environments such as oil fields and aquifers (Andrews & Patel, 1996; Denman *et al*., 1991; Magot *et al*., 1997).

One such environment is the Great Artesian Basin of Australia. A wide variety of physiological groups of *Bacteria* including sulfate reducers, carbohydrate fermenters, strict aerobes and strict anaerobes have been isolated from the Artesian Basin environment (Andrews & Patel, 1996; Denman *et al*., 1991; Love *et al*., 1992; Redburn & Patel, 1994; Wynter *et al*., 1996).

This chapter describes the isolation of a novel spore-forming thermophilic strict aerobe isolated from the Great Artesian Basin of Australia. The Great Artesian Basin is a deep subsurface geothermal aquifer that underlies approximately 20% of Australia's landmass in mainly arid and semi-arid regions (Habermahl, 1980). The water is brought to the surface by approximately 5000 free-flowing bores and is distributed through open drain runoff channels for use as drinking water for domestic animals and irrigation. The temperature at the sources of these bores can be as high as 99ºC with temperatures in runoff channels cooling to ambient thereby producing unique temperature gradients in which distinct microbial mats develop.

5.2 METHODS

5.2.1 SOURCE OF CULTURES.

The environmental source was the New Lorne bore. The site and sample collection methodologies are described in Sections 2.4.1 and 2.4.2 respectively. The reference strain *Thermaerobacter marianensis*^{T} JCM 10246 (= DSMZ 12885) was purchased from DSMZ and cultured as described (Takai *et al*., 1999).

5.2.2 MEDIA, ENRICHMENT AND ISOLATION

0.5mL of the sediment slurry sample at 66ºC (red coloured filamentous mat, Figure 2.6) and 10-fold serial dilutions of the sample to 10^{-10} were inoculated into 10mL sterile Media D (Section 2.3.3) and incubated at 65ºC and 75ºC for up to 72 hours. Growth was determined microscopically and positive enrichment cultures were subcultured again under identical growth conditions. Pure cultures are isolated by streaking on to Media D plates amended with 2% agar, followed by incubation at the enrichment temperatures. Single well-separated distinct colonies were picked and grown. This procedure was repeated at least twice before the culture was considered pure and characterised further. The pure culture, designated isolate C21^T, was stored in a Medium D-glycerol (1:1) mix at -20ºC

5.2.3 CELL MORPHOLOGY AND CELL WALL ULTRASTRUCTURE

Cell morphology and cell wall ultrastructure were determined using light and electron microscopy as previously described (Section 2.5). Gram reaction and oxidase and catalase tests were performed as described by (Collee *et al*., 1996).

5.2.4 GROWTH CHARACTERISATION

All growth experiments were carried out in duplicate in liquid Media D at 70ºC unless otherwise stated. Growth was determined

spectrophotometrically at 660nm using a Novaspec LKB spectrophotometer (Pharmacia-Biotech Pty Ltd, Australia).

5.2.4.1 ANAEROBIC GROWTH

Anaerobic growth was determined by inoculating 10mL TYEG media (Section 2.3.4) with a 10% volume of a 48 hour culture and incubating at 70ºC for 72 hours.

5.2.4.2 BASAL MEDIA REQUIREMENTS

The effect of different concentrations of yeast extract (0%, 0.05%, 0.1% and 0.2%) and tryptone (0%, 0.05%, 0.1% and 0.2%) on the growth of isolate C21^T was determined. NaCl tolerance was determined by adding NaCl to Media D to a concentration of 1%, 2% or 3%.

5.2.4.3 SUBSTRATE UTILISATION

The nutritional spectrum of isolate C21^T was tested in Media D containing casamino acids, sucrose, cellobiose, glucose, dextrin, amylopectin, inositol, arabinose, mannose, fructose, gelatin, amylose, galactose, dextrose, xylose, lactic acid, pyruvic acid, maltose, L-sorbose, raffinose, benzoic acid, carboxymethylcellulose, cellulose, chitin, xylan, or starch at final concentrations of 0.1% and/or 0.5%. A 10% volume of overnight culture is used to inoculate 10mL of adjusted media and incubated at 70ºC overnight. Growth was recorded by measuring absorbency at 660nm and the change in pH.

5.2.4.4 ANTIBIOTIC SENSITIVITY

The effect of antibiotics (ampicillin, neomycin, penicillin, phosphomycin, polymixin B, streptomycin and tetracycline) on the growth of isolate C21^T was tested by adding filter-sterilised stock solutions of the antibiotics to 10mL of sterile Media D, to give final concentrations of 10μg/mL and 100μg/mL. Sodium azide was added to a final concentration of 250μg/mL and 500μg/mL. Media containing antibiotics were inoculated with 1mL of a

48 hour culture and incubated at 70ºC overnight. Growth was recorded by measuring absorbency at 660nm.

5.2.5 TEMPERATURE AND pH ANALYSIS

The temperature range and optima for growth was determined in 10mL volumes of Media D inoculated with 1mL of an overnight culture of isolate C21^T. Incubation occurred at a pH of 8.5 and temperatures ranging from 55ºC to 80ºC and growth was recorded by measuring absorbency at 660nm after 24 hours.

The pH growth range and pH optima for growth was determined by adjusting the pH of 50mL volumes of pre-sterilised Media D with HCl or NaOH. After autoclaving, the pH of a 5mL aliquot of media was recorded before the remainder was inoculated with a 10% volume of overnight culture. Incubation occurred at a temperature of 70ºC and growth was recorded by measuring absorbency at 660ηm after 24 hours.

5.2.6 GENERATION TIME

The generation time of *Thermaerobacter subterraneus* strain C21^T was determined by incubating 10mL of Media D with a 5% inoculum of an overnight culture using the optimum growth parameters (temperature of 70ºC and a pH of pH 8.5). Absorbency readings at 660nm were recorded every hour for the first 7 hours and a final reading was taken at 14 hours.

5.2.7 DNA EXTRACTION, 16S rRNA GENE AMPLIFICATION, SEQUENCING AND PHYLOGENY

Chromosomal DNA was extracted from isolate C21^T as described in Section 2.6.1. The 16S rRNA gene was amplified and purified as described in Sections 2.9 and 2.10 respectively. The sequence of the gene was determined and phylogenetically analysed as explained in Sections 2.14 and 2.15 respectively.

5.2.8 DNA EXTRACTION, DNA-DNA HYBRIDISATION AND DNA BASE COMPOSITION

High molecular weight DNA was extracted from isolate C21^T as described in Section 2.6.4. The DNA base composition (mol%G+C) of the chromosomal DNA was determined by DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). DNA-DNA hybridisation was performed at 55ºC using a colorimetric microplate hybridisation method (Ezaki *et al*., 1989) as modified by (Kusunoki *et al*., 1991) and (Maruyama *et al*., 2000). *Escherichia coli* (SMUM 344 = JCM 1649^T) genomic DNA was used as a negative reference.

5.3 RESULTS

5.3.1 ISOLATION AND COLONY MORPHOLOGY

Medium D enrichment cultures (10^{-0}, 10^{-1} and 10^{-2} dilutions) initiated from water-sediment slurries taken at 66ºC showed growth following incubation at 68ºC and 75ºC for 72hrs and could be successfully sub-cultured. Microscopic examination revealed similar rod shaped cells (2 – 10μm x 0.3μm) and similar colony morphologies (small, translucent and circular colonies) were observed from all the three enrichment cultures on Medium D agar plates after incubation for 48 hours at 65ºC. Several pure cultures were obtained by picking single well-isolated colonies and one of the cultures designated isolate C21^T, was characterised further.

5.3.2 CELL MORPHOLOGY AND CELL WALL ULTRASTRUCTURE

Isolate C21^T cells were non-motile, rod-shaped (2 – 10μm x 0.3μm) and occurred singly or in pairs. The cells stained Gram-negative but electron microscopic examination of thin sections revealed a distinct Gram-positive type cell wall (Figure 5.1). Cells of isolate C21^T produced terminal ellipsoidal spores that distended the cell (Figure 5.2).

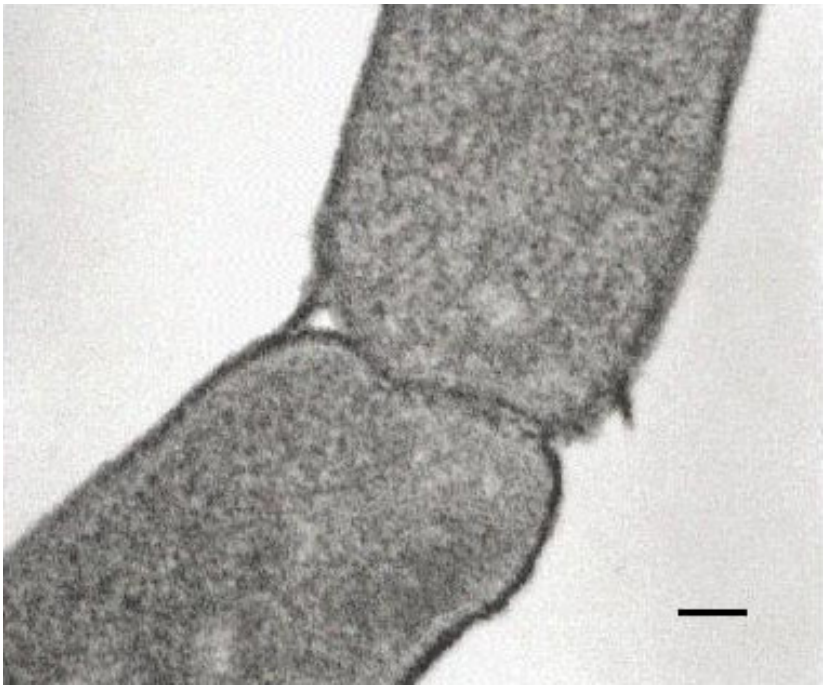


Figure 5.1: TEM of Isolate C21 showing Gram-positive cell wall

Bar represents 50nm

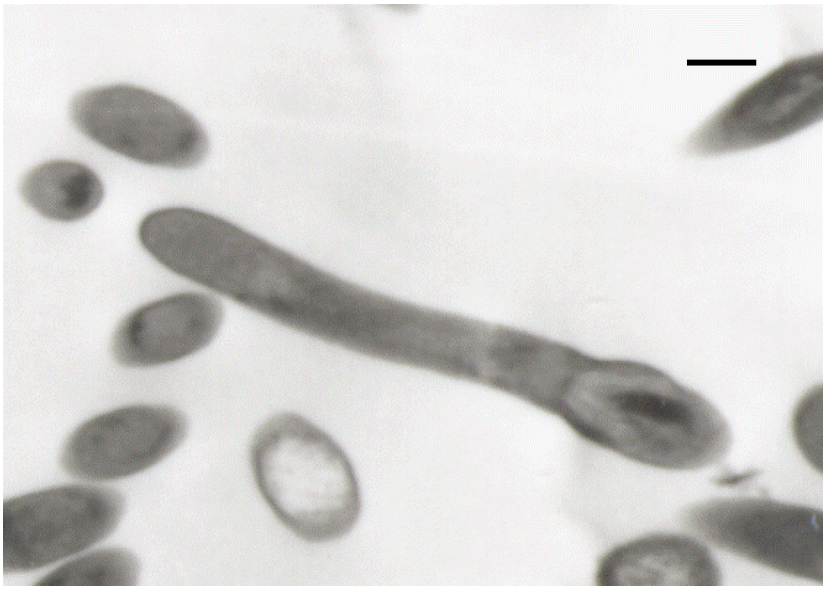


Figure 5.2: TEM showing terminal ellipsoid spore

Bar represents 0.5μm

5.3.3 GROWTH CHARACTERISTICS AND SUBSTRATE UTILISATION

Isolate C21^T was a strict aerobe and grew better with agitation than as stationary cultures. It grew in Medium D containing either yeast extract or

tryptone and the presence of both these substrates at concentrations less than 0.2% increased biomass and improved generation time (Figure 5.3).

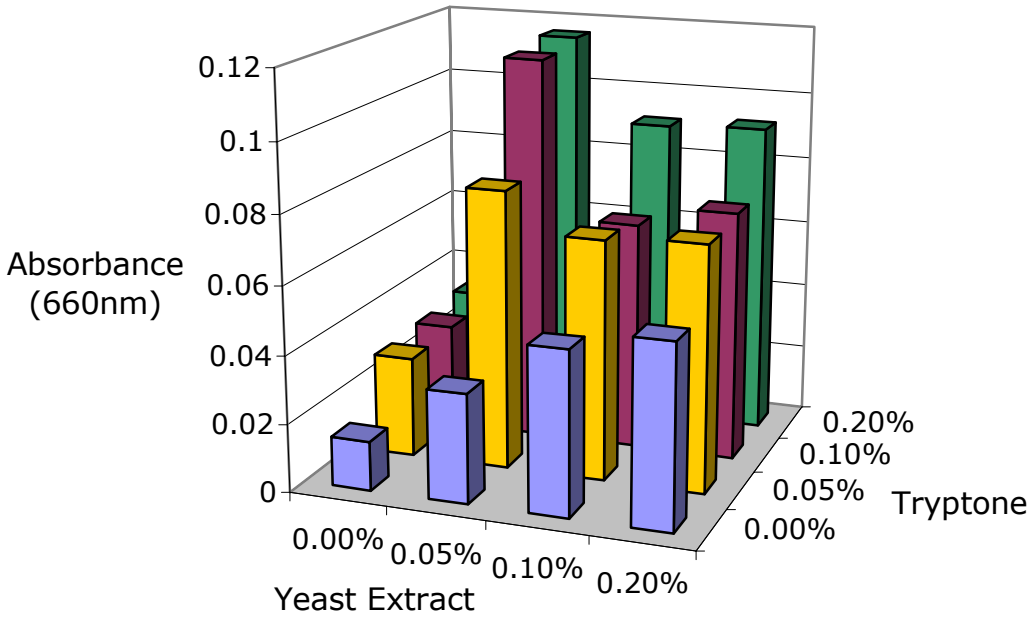


Figure 5.3: Effect of tryptone and yeast extract on isolate C21^T

Sucrose, cellobiose, glucose, dextrin, amylopectin, chitin, carboxymethyl-cellulose, xylan, inositol, arabinose, mannose, fructose, gelatin, starch, amylose, galactose, dextrose, xylose, maltose, L-sorbose, raffinose, organic acids (lactic acid, pyruvic acid, and benzoic acid), or casamino acids could not be used as sole carbon sources or when supplemented with yeast extract and tryptone (total final concentration of 0.2%).

The pH and temperature ranges (Figures 5.4 and 5.5 respectively) for growth in Media D containing 0.1% yeast extract and 0.1% tryptone was 70ºC (temperature growth range of 55ºC and 80ºC) and pH 8.5 (pH growth range of 6 and 10.5) respectively. A generation time of 90 minutes was obtained (Figure 5.6). Isolate C21^T did not require NaCl for growth and no growth was evident in media with NaCl concentrations greater than 1%. The growth of isolate C21^T was slower on Medium D agar plates than in Media D broth.

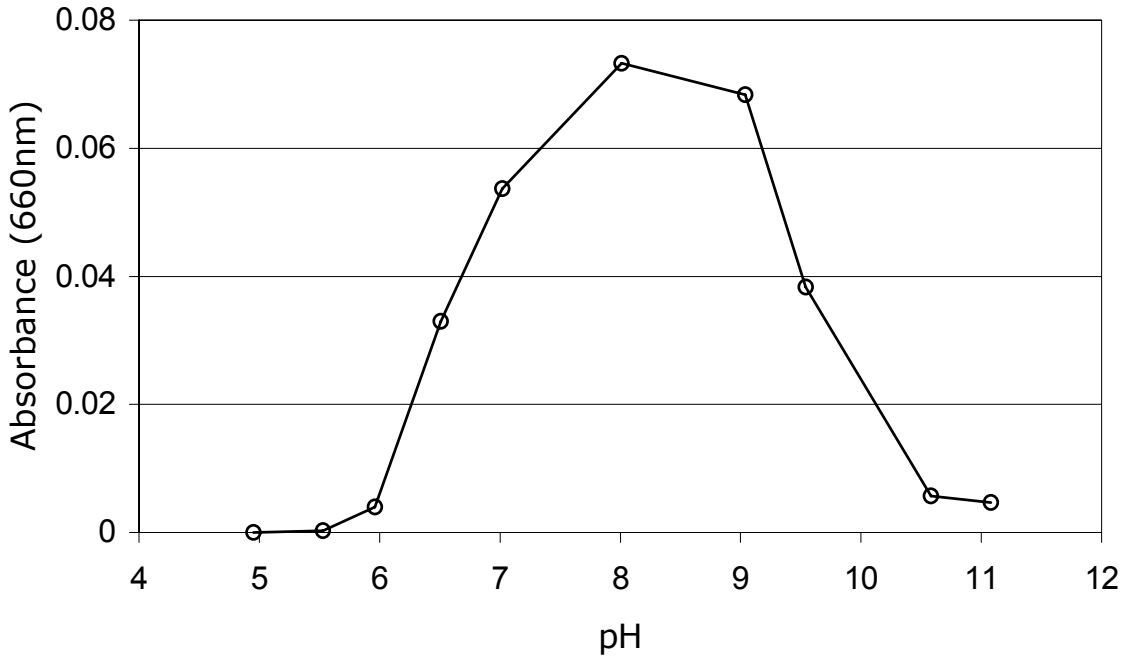


Figure 5.4: Effect of pH on growth of isolate C21^T

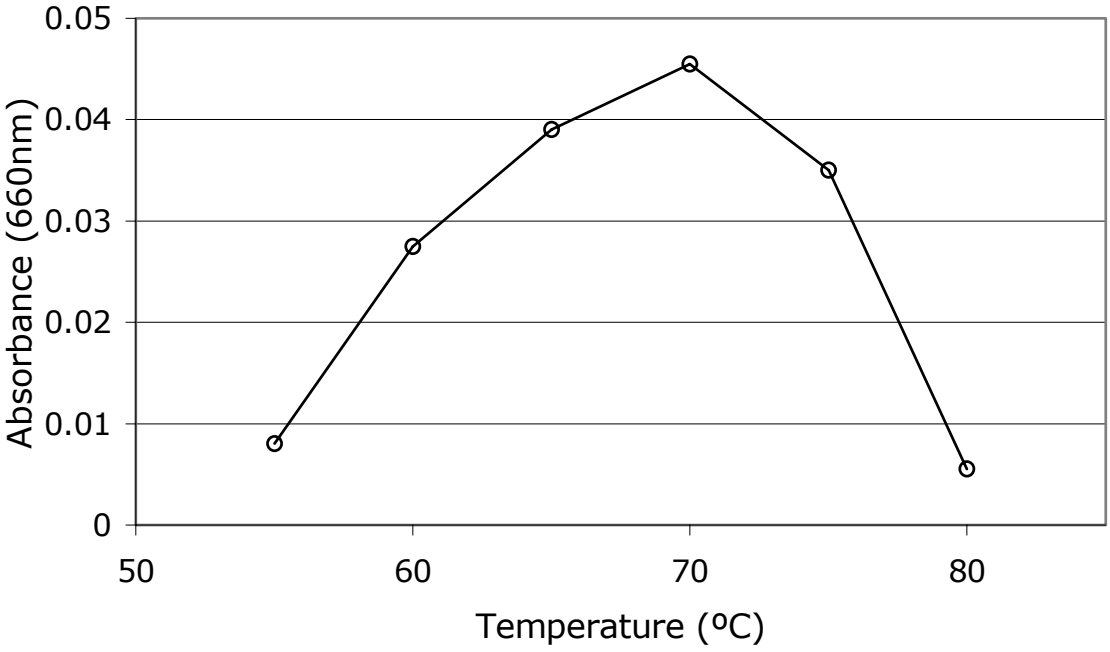


Figure 5.5: Effect of Temperature on growth of isolate C21^T

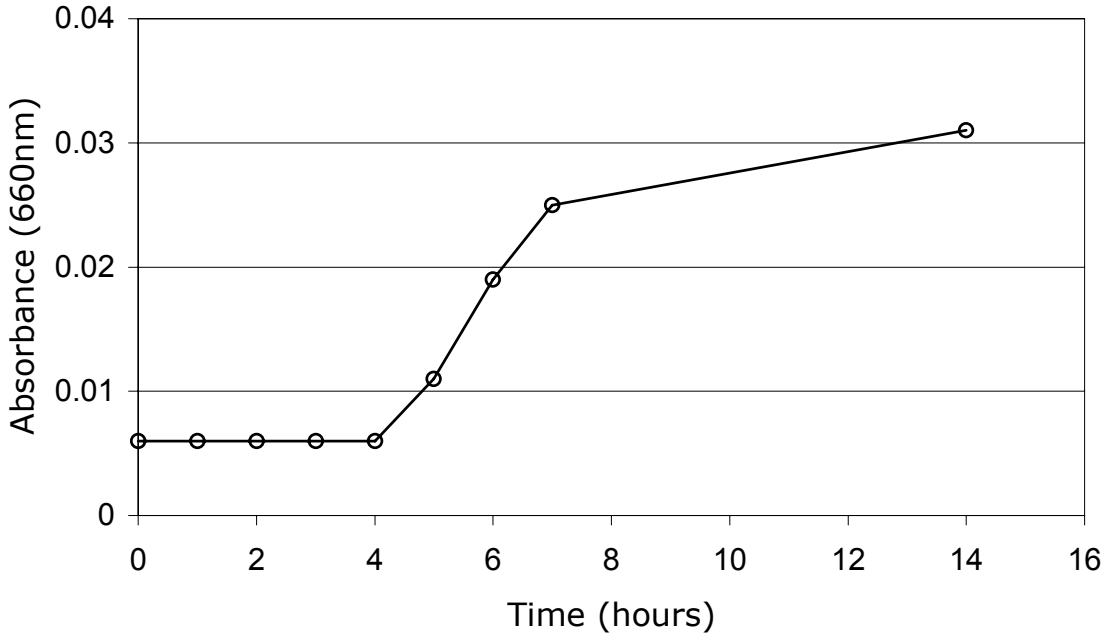


Figure 5.6: Growth curve of isolate C21^T

5.3.4 ANTIBIOTIC SUSCEPTIBILITY

The growth of isolate C21^T was sensitive to ampicillin, neomycin, penicillin, phosphomycin, polymixin B, streptomycin and tetracycline at concentrations of 10μg/mL, however growth was not inhibited by sodium azide at a concentration of 500μg/mL.

5.3.5 DNA BASE COMPOSITION AND DNA-DNA HYBRIDISATION

The DNA base composition of isolate C21^T was determined to be 71% mol%G+C (thermal denaturation). Quantitative DNA-DNA hybridisation experiments showed less than 5% genomic relatedness between isolate C21^T and *Thermaerobacter marianensis* str. 7p75a.

5.3.6 16S RRNA GENE SEQUENCE ANALYSIS

1552 nucleotides of the 16S rRNA gene of isolate C21^T, corresponding to position 7 to position 1541 of the 16S rRNA gene of *E. coli* (Winker & Woese, 1991) was generated using seven primers and has been deposited in Genbank with the accession number AF343566. The G+C content of the 16S rRNA gene sequence was 64%. The 16S rRNA gene sequence of isolate

C21^T has been deposited to Genbank and is listed in Appendix II. A phylogenetic analysis of this sequence with representative members of the Domain *Bacteria* revealed a relationship with members of the family *Syntrophomonodaceae*, order *Clostridiales,* class *Clostridia*, phylum *Firmicutes*. A more detailed analysis within this family showed that isolate C21^T exhibited high similarity to *Thermaerobacter marianensis* str. 7p75a (value of 98%) and bootstrap analysis gave a 100% confidence level for this relationship (Figure 5.7).

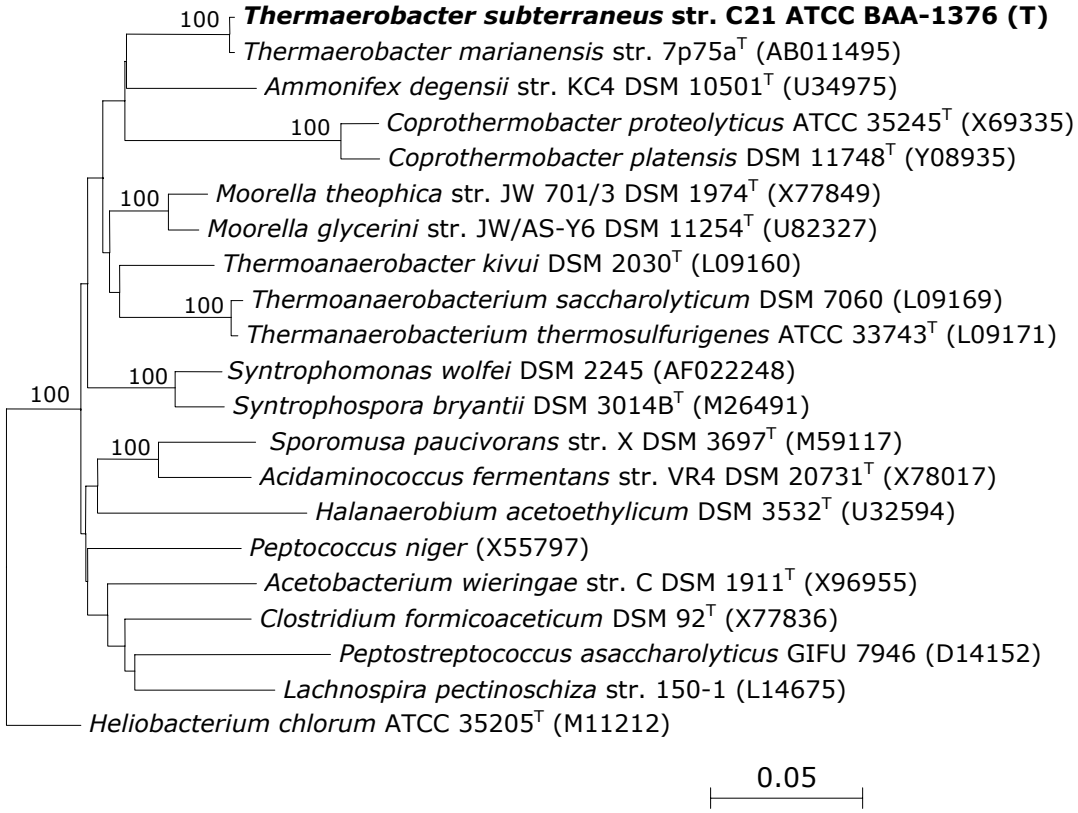


Figure 5.7: Phylogenetic placement of Isolate C21 within the phylum *Firmicutes*

Phylogenetic tree of representative prokaryotes from the phylum *Firmicutes* inferred from 16S rRNA gene sequences by using the neighbour-joining method of 1118 homologous positions of sequence from each organism. Numbers on the tree represent the bootstrap values over 70 out of 100 replicates. Scale bar indicates 5 substitutions per 100 bases. Numbers in parenthesis are GenBank accession numbers.

5.4 DISCUSSION

5.4.1 GENERAL DISCUSSION

A variety of different physiological groups of bacteria including sulfate reducers, carbohydrate fermenters, strict aerobes and strict anaerobes have been isolated from the Great Artesian Basin environment. These include *Desulfovibrio longreachensis* (Redburn & Patel, 1994), *Desulfotomaculum australicum* (Love *et al*., 1993), *Fervidobacterium gondwanense*, a phylogenetic deep member of the order *Thermotogales* (Andrews & Patel, 1996), members of the genera *Thermus* (Denman *et al*., 1991), *Bacillus* (Love *et al*., 1992), *Caloramator* and *Thermoanaerobacter* (Wynter *et al*., 1996), and as yet unnamed taxons. The isolation of strain C21^T from this unique non-volcanically heated subsurface aquifer extends the known microbial diversity of this environment. Isolate C21^T is a strictly aerobic, spore-forming thermophilic bacterium which has a typical Gram-positive type cell wall. Based on these properties, isolate C21^T resembles members of the genus *Bacillus* (Blanc *et al*., 1997) and *Saccharococcus* (Ahmad *et al*., 2000) at the exclusion of non-spore formers such as *Thermus* (Brock & Freeze, 1969; Denman *et al*., 1991). The mol%G+C content of the DNA of isolate C21^T is 71% which is much higher than that reported for members of the family *Bacillaceae* (de Bartolomeo *et al*., 1991).

Phylogenetic analysis of the 16S rRNA gene of isolate C21^T placed it as a member of the phylum *Firmicutes*, within the family *Thermoanaerobacteriaceae* of the order *Thermoanaerobacteriales* with *Thermaerobacter marianensi*s being the closest relative (similarity value of 98%). Interestingly, all members of the order *Thermoanaerobacteriales* possess an anaerobic metabolism (Collins *et al*., 1994) with the exception of *T. marianensis* (Takai *et al*., 1999) and isolate C21^T described in this study. This lack of congruence of physiology with phylogeny is notable but not unusual. For example, all members of the family *Bacillaceae* are strict aerobes or facultative anaerobes with the exception of *Bacillus infernus* which is an obligate anaerobe (Boone *et al*., 1995) necessitating an amendment to the genus description. These results therefore confirm that

physiologies do not have phylogenetic boundaries and that different metabolic variants can co-exist within a cohesive phylogenetic cluster.

Thermaerobacter marianensis was isolated from the world's deepest sea-floor (10897m), the Mariana Trench Challenger Deep (Takai *et al*., 1999). In the same paper, the authors have cited a personal communiqué from their colleague on a phylogenetically similar thermophilic bacterium (98% similarity based on the 16S rRNA gene sequence) isolated from a shallow hydrothermal vent. The increased hydrostatic pressure sensitivity of *T. marianensis* suggests the possibility that it may not be a normal inhabitant of the Trench environment and that it could have been deposited as a result of subduction activities which greatly perturb the upper mantle. However, the isolation of C21^T from the Great Artesian Basin does not support this hypothesis as it is a relatively recent rainwater recharged, closed environment (Habermahl, 1980).

T. marianensis and isolate C21^T share a number of characteristics including the high mol % G+C content of the DNA, an aerobic metabolism, and a Gram-positive cell wall ultrastructure. However, a number of differences also exist. Isolate C21^T is an alkalophile which does not require NaCl, does not use substrates other than yeast extract and tryptone for growth and produces spores whereas *T. marianensis* is an obligate halophile, is more nutritionally versatile and spores have not been detected (Table 5.1). In addition, isolate C21^T and *T. marianensis* have a DNA homology of less than 5%.

Isolate C21^T was isolated from a mix of bore run-off water and sediment taken from the bottom of the drain near the growth of the red mat (66ºC). However, the niche of isolate C21^T is different from the filamentous mats present. As such, the probability that phylotypes of isolate C21^T would be detected in the PCR-generated 16S rRNA gene clone libraries discussed in Chapter 3 is very low.

Based on the results presented above, the designation of isolate C21^T is proposed as a new member of the genus *Thermaerobacter*, *Thermaerobacter subterraneus* sp. nov. It is widely accepted that spore-

forming and non-spore-forming species can be included in the same genus as exemplified by members of the genus *Thermoanaerobacter* (Cayol *et al*., 1995).

Table 5.1: Characteristics of isolate C21^T and *Thermaerobacter marianensis* str. 7p75a

Characteristics	**Isolate C21^T** (a)	***T. marianensis* str. 7p75a** (b)
Habitat	Bore outflow, Great Artesian Basin of Australia	Challenger Deep sediment, Mariana Trench
Morphology (μm)	2-10 x 0.3	2-7 x 0.3-0.6
Presence of spores	Ellipsoidal, terminal, 2μm x 0.5μm	No
Growth conditions:		
Temperature growth range (°C)	55 to 80 (optimum 70)	50 to 80 (optimum 74-76)
pH growth range	6 to 10.5 (optimum 8.5)	5.4 to 9.5 (optimum 7 to 7.5)
Sodium chloride requirement	No	0.5 to 5% (optimum 2%)
Growth on:		
Yeast extract	Yes	Yes
Peptone	Yes	Yes
Amino acids	No	Yes
Carbohydrates	No	Yes
Carboxylic acids	No	Yes
Requirement of yeast extract or peptone for growth on carbohydrates	Not applicable	No
G+C content (mol %)	71 (thermal denaturation)	72.5 (HPLC)

(a) Data from these studies; (b) Data from Takai *et al*. (1999).

5.4.2 EMENDED DESCRIPTION OF *THERMAEROBACTER* GEN.

(Takai *et al*., 1999)

Thermaerobacter (Therm.ae.ro.bac'ter. Gr. adj. *Thermos* hot; Gr. n. *aer* air; M.L. *bacter* masc. Equivalent of Gr. neut. N. *bakterion* rod or staff; M.L. masc. N. *Thermaerobacter* rod which grows at high temperatures in the presence of air).

Rod-shaped, may or may not form spores. Gram-variable cells are non-motile and flagella are absent. Aerobic and thermophilic. Heterotrophic. Grow at neutral to alkaline pH. NaCl may or may not be required for growth. May utilise organic substrates such as yeast extract, peptone, cellulose, starch, chitin, casein, casamino acids, a variety of sugars, carboxylic acids and amino acids. The G+C content of genomic DNA is 71 to 73 mol%. Major cellular fatty acids are iso-$C_{17:0}$, $C_{14:1}$, anteiso-$C_{17:0}$ iso-$C_{15:0}$, anteiso-$C_{15:0}$, $C_{16:0}$, and $C_{18:0}$. On the basis of 16S rRNA gene analysis, the genus *Thermaerobacter* is most closely related to the genus *Moorella*. *Thermaerobacter* species habitats known so far include deep sea-floor environments, hydrothermal vents and subterranean thermal environments such as the Great Artesian Basin of Australia.

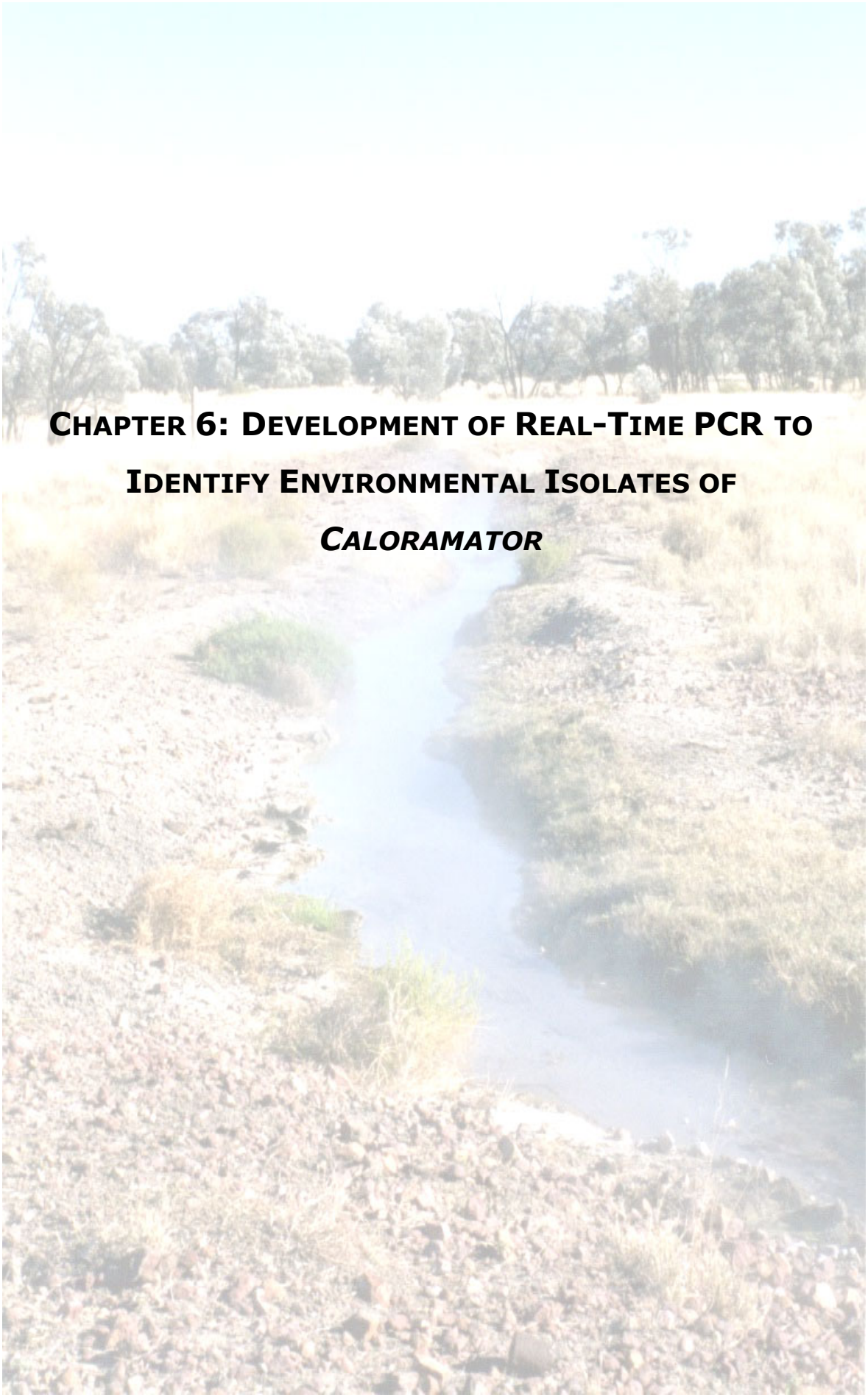
5.4.3 DESCRIPTION OF *THERMAEROBACTER SUBTERRRANEUS* SP. NOV.

Thermaerobacter subterraneus (sub. terr. aneus L. adj. sub under, beneath; L. n. *terra* earth, ground, L. masc. adj.*subterraneus* under the earth)

Cells are rod-shaped (2-10μm x 0.3μm) with rounded ends and occur singly or in pairs. Cells are non-motile and do not posses flagella. They stain Gram-negative but possess a Gram-positive cell wall ultrastructure. The cells form terminal ellipsoidal spores that distend the cells. Strictly aerobic. Temperature range for growth is 55-80ºC with the optimum being 70ºC. pH range for growth is 6-10.5 with an optimum of 8.5. The organism does not require NaCl, but is inhibited by NaCl concentrations higher than 1%. The organism grows on yeast extract and / or tryptone but not on any other

carbon sources as sole carbon/energy sources. Ampicillin, neomycin, penicillin, phosphomycin, polymixin B, streptomycin and tetracycline, but not sodium azide, inhibit growth. The G+C composition of genomic DNA is 71 mol%G+C. Phylogenetically related to *T. marianensis* (16S rRNA gene similarity value of 98%). The isolate was obtained from a sediment sample from the outflow of a Great Artesian Basin bore (the New Lorne Bore) in Queensland, Australia.

The type strain is *Thermaerobacter subterraneus* str. C21^T = ATCC BAA-137^T.



CHAPTER 6: DEVELOPMENT OF REAL-TIME PCR TO IDENTIFY ENVIRONMENTAL ISOLATES OF *CALORAMATOR*

6.1 INTRODUCTION

The economic importance of the Great Artesian Basin is great, especially in the more arid regions of Australia. Prokaryotic communities influence the standard and quantity of bore water. Populations of detrimental prokaryotes produce precipitates that cause problems with filtration and pumping. In addition the prokaryotes involved with these processes increase corrosion of the bore casings and cause a decline in the standard of water quality with adverse effects on taste, colour and odour. It is vital that detrimental populations can be monitored and minimised before the need for expensive and complex rehabilitation arises. Developing a rapid, standardised technique using new technologies (e.g. real-time PCR) will enable the relatively easy and cheap monitoring of at-risk bores that will provide early indications of infections of damaging prokaryotes.

6.1.1 RESEARCH UTILISING REAL-TIME PCR

The majority of previous research that has taken advantage of real-time detection during PCR concerns the detection and identification of pathogenic species of prokaryotes and viruses (Fortin *et al*., 2001; Nogva *et al*., 2000; Pirnay *et al*., 2000). Creating a standardised technique (e.g. DNA extraction and PCR conditions) has the further advantages as it enables the quantitation of the target DNA used in the PCR (Knerr *et al*., 1999; Lyons *et al*., 2000; Miley *et al*., 2000). There is little application of real-time PCR to monitoring environmental populations (Hermansson & Lindgren, 2001), however, the specificity and sensitivity of the assay, combined with high speed, robustness, reliability, and the possibility of automating the technique, lends itself to the detection of economically important (both clinical and environmental) species.

6.1.2 THE GENUS *CALORAMATOR*

Members of this genus were first described by Patel *et al.* (1987) as *Clostridium fervidus*. Further phylogenetic comparison of 16S rRNA gene sequences of members of the genus *Clostridium* required that *Clostridium*

fervidus be placed into a new genus and reclassified as *Caloramator fervidus* (Collins *et al*. 1994).

Members of the genus *Caloramator* are obligately anaerobic, Gram-negative, thermophilic rod-shaped sporulating microorganisms (Collins *et al*. 1994). Currently *Caloramator* comprises four species. Two species, *C. coolhaasii* and *C. proteoclasticus*, were isolated from enrichment cultures taken from anaerobic, thermophilic, granular sludge (Plugge *et al*., 2000; Tarlera *et al*., 1997). The remaining two species, *C. fervidus* and *C. indicus*, were isolated from non-volcanically heated waters of the Great Artesian Basin and from India respectively (Patel *et al*.; 1987, Chrisostomos *et al*., 1996).

As the presence of members of the genus *Caloramator* have been previously isolated from the Great Artesian Basin of Australia, initial isolations were carried out from sediment samples from the New Lorne bore. Using these isolates, a preliminary investigation involving adjacent hybridisation probes and the LightCycler™ was carried out to determine the applicability of this technique in the identification of environmental isolates belonging to the genus *Caloramator*.

6.2 MATERIALS AND METHODS

6.2.1 *CALORAMATOR* STRAINS 75-1 AND 75-2

Caloramator str 75-1 and *Caloramator* str 75-2 were obtained from a sediment slurry sample from the New Lorne bore at 66ºC (Section 2.4). The sediment slurry was used as the inoculum for a dilution series in TYEG (Section 2.3.4) and incubated at 50ºC. Strains 75-1 and 75-2 were isolated using the roll-tube technique in TYEG amended with 1.5% agar (Hungate, 1969). Subsequent 16S rRNA sequencing and phylogenetic analysis (Section 2.15) identified isolates 75-1 and 75-2 as members of the genus of *Caloramator*, closely related to *Caloramator coolhaasii* .

Bacillus str B4-1 was isolated from a sediment slurry sample from the New Lorne bore at 66ºC (Section 2.4). The sediment slurry was used as the

inoculum for a dilution series in Media D (Section 2.3.3) and incubated at 50ºC. *Bacillus* str B4-1 was isolated by streaking the enrichment on Media D amended with 2% agar and incubating again at 50ºC. Subsequent 16S rRNA gene sequencing and phylogenetic analysis (Section 2.15) identified isolate B4-1 as a member of the genus *Bacillus*, closely related to *Bacillus flavothermus*.

6.2.2 DNA EXTRACTION FOR PCR AND REAL-TIME PCR

Overnight cultures of *Caloramator* str 75-1 and *Caloramator* str 75-2 grown in TYEG at 50ºC had the chromosomal DNA extracted as detailed in Section 2.6.1. Chromosomal DNA extracted (Section 2.6.1) from overnight cultures of an environmental isolate, *Bacillus* str B4-1, was employed as a negative control for real-time PCR. The concentration of chromosomal DNA was determined as described in Section 2.12.

6.2.3 DEVELOPMENT OF ADJACENT HYBRIDISATION PROBES

The two adjacent hybridisation probes were designed according to the following specifications advised by Idaho Technology:

1. Probe T_M's should be near equal and 5 to 10ºC greater than primer T_M's.
2. The 3' end of the upstream probe should be labelled by fluorescein, which serves as the donor in FRET and blocks the extension from the probe.
3. The 5' end of the downstream probe should be labelled with Cy5, which serves as the acceptor in FRET, and the 3' end of the probe should be phosphorylated to block extension.
4. The probes should be separated by one base.
5. The probes should be placed on one strand near an amplification primer of the opposite strand.

The 16S rRNA gene was selected as the target due to the vast sequence data available and a selection of primers is already available for use (Table 2.2).

6.2.4 OPTIMISATION OF PCR USING THE RAPIDCYCLER

Reactions consisted of 1μL of 10x PCR buffer, 1μL of 2mM dNTPs (0.5mM dATP, 0.5mM dGTP, 0.5mM dCTP, and 0.5mM dTTP), 1μL of 5μM F3 primer

(Table 2.2), 1μL of 5μM Rd1 primer (Table 2.2), 0.08μL of 5U/μL of *Taq* DNA Polymerase (Promega Corp.), 1μL of chromosomal DNA, and 4.92μL of sterile ddH_2O.

To optimise the PCR, the annealing temperature was varied to determine the best possible amplification of the target sequence.

The PCR was carried out in a RapidCycler (Idaho Technology Inc., USA) with the following parameters: 1 cycle of 94ºC for 30 seconds; and 30 cycles of 94ºC for 0 seconds, 50ºC (55ºC, 58ºC or 60ºC) for 0 seconds, 74ºC for 30 seconds with a slope of 9.9.

The reaction mix was allowed to enter a 5-30μL borosilicate glass tube (1.0mm outer diameter, 0.8mm inner diameter) by capillary action. The ends were heat-sealed and the PCR started. Each PCR contained a negative control (1μL of sterile ddH_20) and a positive control (1μL of known amplifiable DNA instead of template DNA). The efficacy of the PCR was determined by agarose gel electrophoresis of the complete reaction volume (Section 2.7).

6.2.4 OPTIMISATION OF REAL-TIME PCR USING THE LIGHTCYCLER

Reaction samples were modified by the addition of the two hybridisation probes (Cal-FITC and 1046plus-Cy5). Reactions consisted of 1μL of 10x PCR buffer, 1μL of 2mM dNTPs (0.5mM dATP, 0.5mM dGTP, 0.5mM dCTP, and 0.5mM dTTP), 1μL of 5μM F3 primer (Table 2.2), 1μL of 5μM Rd1 primer (Table 2.2), 1μl of 2μM Cal-FITC, 1μL of 4μM 1046plus-Cy5, 0.08μL of 5U/μL of *Taq* DNA Polymerase (Promega Corp.), 1μL of chromosomal DNA, and 2.92μL of sterile ddH_2O.

The reaction mix was pipetted into a glass capillary tube and snap-sealed by a plastic cap. The PCR was carried out in a LightCycler™ (Idaho Technology Inc., USA). The PCR conditions optimised in Section 6.2.3 were used as the initial PCR conditions for real-time PCR, however, after optimisation the following parameters were selected: 1 cycle of 94ºC for 30 seconds; and 30 (or 45) cycles of 94ºC for 0 seconds, 55ºC for 20 seconds, 74ºC for 30 seconds with a ramp speed of 20ºC/sec. Fluorescence emissions were

monitored and recorded for 100mseconds during the annealing step (55ºC for 30 seconds) by the LightCycler™. At the end of each run the experimental data could be analysed with the software provided of imported into a spreadsheet for further manipulation. The effect of sample-to-sample variation was minimised by normalisation of the fluorescence data. Normalisation required the subtraction of the minimum value from each data point, dividing the result by the maximum value, and multiplying by 100.

Each run for the optimisation of the LightCycler™ and adjacent hybridisation probes contained a PCR negative control (1μL of sterile ddH_20), a PCR positive but hybridisation negative control (1μl of *Bacillus* str. B4-1 chromosomal DNA) and a PCR positive, hybridisation positive control (1μL of *Caloramator* str 75-1 or *Caloramator* str 75-2). The efficacy of the PCR was determined by agarose gel electrophoresis of the whole sample (see Section 2.7).

The melting characteristics of the hybridisation probes were determined by continuously monitoring the emission of fluorescence from 45ºC to 94ºC with a ramp speeds of 0.2ºC/sec. The speed at which the probes bind at 55ºC was determined by completely denaturing the hybridisation probes from the target DNA by heating at 94ºC for 1 minute, rapidly decreasing the temperature at 20ºC/sec to 55ºC and following the emission of fluorescence for 2 minutes.

6.3 RESULTS

6.3.1 DNA CONCENTRATION OF CHROMOSOMAL DNA

Chromosomal DNA was extracted from *Caloramator* str. 75-1, *Caloramator* str 75-2 and *Bacillus* str B4-1 and the concentration was determined spectrophotometrically. The concentration of chromosomal DNA *Caloramator* str. 75-1 was 8μg/mL, *Caloramator* str 75-2 was 6μg/mL, and *Bacillus* str B4-1 was 21μg/mL.

6.3.2 DESIGN OF ADJACENT HYBRIDISATION PROBES

Following the guidelines suggested by Idaho Technology, amplification primers selected for the 16S rRNA gene were F3 and Rd1. This pair amplified a region of approximately 640bp near the 3' end of the 16S rRNA gene and had very similar T_M's. To counter the cost of producing a different pair of hybridisation probes for each species, a region in the 16S rRNA gene was identified that was highly conserved adjacent to a highly variable region. The more expensive probe to manufacturer i.e. the Cy5-labelled probe would be designed against the conserved region, while the cheaper FITC-labelled probe would be designed against the variable region and would confer the specificity required for this technique. The two hybridisation probes were designated 1046plus-Cy5 and Cal-FITC. Sequences of the probes are shown in Table 6.1 and Figure 6.1 shows a partial 16S rRNA gene sequence alignment with the sites of hybridisation for the primers and probes.

Table 6.1: Adjacent Hybridisation Probes used in Real-Time PCR

Probe	Sequence (5'→3')
Cal-FITC	GCC-CTT-CGG-GGA-ACG-GTG-AGA-**FITC**
1046plus-Cy5	**Cy5**-AGG-TGI*-TGC-ATG-GIT-GTC-GTC-AGC-TCG-TGT-**PO_4**

* denotes deoxy-inosine.

Figure 6.1: Partial 16S rRNA gene sequence alignment showing sites of hybridisation for PCR primers and adjacent hybridisation probes

```
                    *       920         *       940         *       960
Ecoli     : AGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT
Bflav     : AGAGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT
Bac B4-1  : AGAGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT
Cindicus  : AGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGTTTAAT
Ccool     : AGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGTTTAAT
Cprot     : AGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGTTTAAT
Cferv     : AGACTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGTTTAAT
Cal 75-1  : AGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGTTTAAT
Cal_75-2  : AGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGTTTAAT

            (F3) AAACTCAAAGGAATTGACGG ->

                    *       980         *      1000         *      1020
Ecoli     : TCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACGGAAGTTTTCAGAGATGAG
Bflav     : TCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCCTGACAACCCGAGAGATCGG
Bac B4-1  : TCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCCTGACAACCCGAGAGATCGG
Cindicus  : TCGAAGCAACGCGAAGAACCTTACCAGGGCTTGACATCCACCGAACCCTGTGGAAACACG
Ccool     : TCGAAGCAACGCGAAGAACCTTACCAGGGCTTGACATCCACCGAACCCTGTGGAAACACG
Cprot     : TCGAAGCAACGCGAAGAACCTTACCAGGGCTTGACATCCACCGAACCCTGTGGAAACACG
Cferv     : TCGAAGCAACGCGAAGAACCTTACCAGGGCTTGACATCCACCGAACCCTGTGGAAACACG
Cal 75-1  : TCGAAGCAACGCGAAGAACCTTACCAGGGCTTGACATCCACCGAACCCTGTGGAAACACG
Cal_75-2  : TCGAAGCAACGCGAAGAACCTTACCAGGGCTTGACATCCACCGAACCCTGTGGAAACACG
```

```
                      *        1040         *      1060         *
Ecoli     : AATGTG--CCTTCGGG--AACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTT
Bflav     : GCGTTCCCCCTTCGGGGGGACAGGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC
Bac B4-1  : GCGTTCCCCCTTCGGGGGGACAGGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC
Cindicus  : GGGGTG-CCCTTCGGGG-AACGGTGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC
Ccool     : GGGGTG-CCCTTCGGGG-AACGGTGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC
Cprot     : GGGGTG-CCCTTCGGGG-AACGGTGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC
Cferv     : GGGGTGCCCCTTATGGGGAGCGGTGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC
Cal 75-1  : GGGGTG-CCCTTCGGGG-AACGGTGAGACAGGTGGTGCATGGTTGTTGTCAGTTCGTGTC
Cal_75-2  : GGGGTG-CCCTTCGGGG-AACGGTGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC

                G-CCCTTCGGGG-AACGGTGAGA AGGTGITGCATGGITGTCGTCAGCTCGTGT-PO4
               (Cal-FITC)          FITC Cy5              (1046plus-Cy5)

             1080         *      1100         *      1120         *
Ecoli     : GTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGG-T
Bflav     : GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGACCTTAGTTGCCAGCA-TT
Bac B4-1  : GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCTTCGACCTTAGTTGCCAGCA-TT
Cindicus  : GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGCCTTTAGTTGCCAGCAC-T
Ccool     : GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGCCTTTAGTTGCCAGCAA-T
Cprot     : GTGAGATGTTGGGTTAAGTCCCGCAACGAACGCAACCCTTACCTTTAGTTGCCACCAA-A
Cferv     : GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATCGTTAGTTGCCAGCAC-T
Cal 75-1  : GTGAGATGTTGGGTTAAGTCCCGCAAGGAGCGCAACCCCTGCCTTTAGTTGCCAGCAA-T
Cal_75-2  : GTGAGATGTGGGGTTAAGTCCCGNAAGGGGCGCAACCCCTGCCTTTAGTTGCCAGCAA-T

               1140         *      1160         *      1180         *
Ecoli     : CCGG-CCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGT
Bflav     : CAGT--TGGGCACTCTAAGGTGACTGCCGGCTAAAAGTCGGAGGAAGGTGGGGATGACGT
Bac B4-1  : CAGT--TGGGCACTCTAAGGTGACTGCCGGCTAAAAGTCGGAGGAAGGTGGGGATGACGT
Cindicus  : TCGGGGTGGGCACTCTAAAGGGACTGCCTGGGTTAACCAGGAGGAAGGTGGGGATGACGT
Ccool     : TCGG-TTGGGCACTCTAGAGGGACTGCCTGGGTTAACCAGGAGGAAGGTGGGGATGACGT
Cprot     : TCGG-TTGGGCACTCTAGAGGGACTACCTGGGTTACCCAGGAGGAAGGTGGGGATGACGT
Cferv     : TCGGGGTGGGCACTCTAACGAGACTGCCAGGGTTAACCTGGAGGAAGGTGGGGATGACGT
Cal 75-1  : TCGG-TTGGGCATTTTAGAGGGACTGCCTGGGTTAACCAGGAGGAAGGTGGGGATGACGT
Cal_75-2  : TNGG-TTGGGCACTCTAGAGGGACTGCCTGGGTTAACCAGGGGGAAGGTGGGGANGACGT

               1200         *      1220         *      1240         *
Ecoli     : CAAGTCATCATGGCCCTTACGACCAGGCTACACACGTGCTACAATGGCGCATACAAAGAG
Bflav     : CAAATCATCATGCCCCTTATGACCTGGCTACACACGTGCTACAATGGGCGGTACAAAGGG
Bac B4-1  : CAAATCATCATGCCCCTTATGACCTGGCTACACACGTGCTACAATGGGCGGTACAAAGGG
Cindicus  : CAAATCATCATGCCCCTTATGCTCTGGCTACACACGTGCTACAATGGCCGGTACAATGAG
Ccool     : CAAATCATCATGCCCCTTATGCTCTGGCTACACACGTGCTACAATGGCCGGTACAAAGAG
Cprot     : CAAATCATCATGCCCCTTATGCTCTGGCTACACACGTGCCACAATGGCCGGTACAATGAG
Cferv     : CAAATCATCATGCCCCTTATGCCCTGGCTACACACGTGCTACAATGGCCACTACAGAGAG
Cal 75-1  : CAAATCATCATGCCCCTTATGCTTTGGGTACACACGTGCTACAATGGCCGGTACAAAGAG
Cal_75-2  : CAAATCATCATGCCCATTATGCTGTGGATACACACGTGATACAATGGCCGGTACAAAGAG

              1260         *      1280         *      1300         *
Ecoli     : AAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCT
Bflav     : TTGCGAACCCGCGAGGGGGAGCCAATCCCAAAAAGCCGCTCTCAGTTCGGATTGCAGGCT
Bac B4-1  : TCGCGAACCCGCGAGGGGGAGCCAATCCCAAAAAGCCGCTCTCAGTTCGGATTGCAGGCT
Cindicus  : TTGCAAACCCGTGAGGGGGAGCTAATCTCA-AAAACCGGTCCCAGTTCGGATTGTAGGCT
Ccool     : AAGCAAGTCCGCGAGGAGGAGCCAATCTCA-AAAACCGGTCCCAGTTCGGATTGTAGGCT
Cprot     : TTGCAAACCCGCGAGGGGGAGCTAATCTCA-AAAACCGGTCCCAGTTCGGATTGTAGGCT
Cferv     : AAGCGAACCCGCGAGGGGGAGCGAAATCTTGAAAGGTGGTCCCAGTTCGGATTGCAGGCT
Cal 75-1  : AAGCAATACCGTGAGGAGGAGCCAATCTCA-AAAACCGGTCCCAGTTCGGATTGTAGGCT
Cal_75-2  : AAGCAATACCGTGAGGAGGAGCCAATCTCA-AAAACCGGTCCCAGTTCGGATTGTAGGGT

              1320         *      1340         *      1360         *
Ecoli     : GCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAA
Bflav     : GCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAA
Bac B4-1  : GCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAA
Cindicus  : GCAACTCGCCTACATGAAGCCGGAGTTGCTAGTAATCGCGGGTCAGCATGCCGCGGTGAA
Ccool     : GCAACTCGCCTACATGAAGCTGGAGTTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAA
Cprot     : GCAANTCGCCCACATGAAGCTGGAGTTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAA
Cferv     : GCAACTCGCCTGCATGAAGCCGGAGTTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAA
Cal 75-1  : GCAATTCGCCTACATGAAGCTGGAGTTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAA
Cal_75-2  : GCAATTCGCATACATGAAGCTGGAGTTGNTAGTAATCGCGGATCAGCATGCCGCGGTGAA
```

```
            1380         *      1400         *       1420         *
Ecoli     : TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA-TGGGAGTGGGTTGCAAAAGAA
Bflav     : TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA-CGAGAGTTTGCAACACCCGAN
Bac B4-1  : TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA-CGAGAGTTTGCAACACCCGAA
Cindicus  : TACGTTCCCGGGGCTTGTACACACCGCCCGTCACACCA-TGAGAGCCGGCAACACCCGAA
Ccool     : TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA-TGAGAGCCGGCAACACCCGAA
Cprot     : TACGTTCCCGGACCTTGTACACACCGCCCGTCACACCATTGAGAGCCGGCAACACCCGAA
Cferv     : TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA-TGAGAGCCGGCAACACCCGAA
Cal 75-1  : TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA-TGAGAGCCGGCAACACCCGAA
Cal_75-2  : TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA-TGAGAGCCGGCAACACCCGAA

              1440         *        1460         *      1480         *
Ecoli     : GTAGGTAGCTTAACC-TTCG-GGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGA
Bflav     : GTCGGTGAGGTAACCCTTACGGGAGCCAGCCGCCGAAGGTGGGGCAAATGATTGGGGTGA
Bac B4-1  : GTCGGTGAGGTAACCCTTACGGGAGCCAGCCGCCGAAGGTGGGGCAAATGATTGGGGTGA
Cindicus  : GCCAGTGGGCTAACCCGCAAGGGAGGCAGCTGTTGAAGGTGGGGCTGGTGATTGGGGTGA
Ccool     : GCCAGTGGGCTAACCCGCAAGGGAGGCAGCTGTCGAAGGTGGGGCTGGTGATTGGGGTGA
Cprot     : GCCAGTGGGCTAACCCTCAAGGGAGGCAGCTGTCGAA----------------------
Cferv     : GCCAGTGGGCTAACCCGAAA-GGAGGCAGCTGTCGAAGGTGGGGCTGGTGATTGGGGTGA
Cal 75-1  : GCCAGTGGGCTAACCCGCAAGGGAGGCAGCTGTCGAAGGTGGGGCTGGTGATTGGGGTGA
Cal_75-2  : GCCAGTGGGCTAACCCGCAAGGGAGGCAGCTGTTGAAGGTGGGGCTGGTGATTGGGGTGA

                1500         *      1520         *      1540
Ecoli     : AGTCGTAACAAGGTAACCGTAGGGGAACCTGCGGTTGGATCACCTCCTTA
Bflav     : AGTCGTAACAAGGTAGCCGTATCGGAAGGTGCGGCTGGAT----------
Bac B4-1  : AGTCGTAACAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCT--
Cindicus  : AGTCGTAACAAGGTAGCCGTAGGAGAACCTGCGGCTGGATCACCTCCTT-
Ccool     : AGTNGTAACAA-------------------------------------
Cprot     : ---------------------------------------------------
Cferv     : AGTCGTAACAAGGTAGCCGTAGGAGAACCTGCGGCTGGATCACCTCC---
Cal 75-1  : AGTTGTAACAAGGTAGCCGTAGGAGAACCTGCGGCTGGATCACCTCCT--
Cal_75-2  : ANTCGTAACAAGGTAGCCGTAGGAGAACCTGCGGCTGGATCACCTCCT--

                                          <- CCGACCTAGTGGAGAA (Rd1)
```

Abreviations: Ecoli *Escherichia coli* (J01695); Bflav *Bacillus flavothermus* DSM 2641 (Z26932); Bac_B4-1 *Bacillus* str B4-1; Cindicus *Caloramator indicus* ACM 3982^T (X75788); Ccool *Caloramator coolhaasii* str. Z^T (AF104215); Cprot *Caloramator proteolyticus*^{T} (X90488); Cferv *Caloramator fervidus* ATCC 43204^T (L09187); Cal_75-1 *Caloramator* str 75-1; Cal_75-2 – *Caloramator* str 75-2. GenBank accession numbers are in parenthesis.

The T_M's of the PCR amplification primers F3 and Rd1 were 52.8ºC and 51.7ºC respectively. The T_M's of the adjacent-adjacent hybridisation probes 1046plus-Cy5 and Cal-FITC were calculated to be 64.2ºC and 60.1ºC respectively, at least 8ºC higher than the amplification primers.

6.3.3 Optimal PCR Conditions

The optimal PCR conditions were determined to have an annealing temperature of 55ºC as is shown in Figure 6.2. At an annealing temperature of 50ºC, there is considerable smearing of the PCR product. At the higher temperatures of 58ºC and 60ºC, the amount of PCR product is much lower. At 55ºC the amount of amplified product was high and there was relatively little smearing, making it the best choice for the PCR. The minimal times for extension, annealing and denaturing did not require

adjusting as amplification did occur in all samples, and it was not necessary to extend times at this stage of optimisation.

6.3.4 OPTIMAL LIGHTCYCLER™ CONDITIONS

The thermal cycling was optimised for the PCR amplification of the target site using the RapidCycler. The transferral of the thermal cycling parameters to the LightCycler™ allowed the amplification of the target region of the 16S rRNA genes from the chromosomal DNA. However, the real-time monitoring of the hybridisation probes showed that the fluorescence detected by the LightCycler™ did not increase smoothly. In an attempt to increase the accuracy and consistency of the fluorescence signal detected, the hybridisation of the probes to the target DNA was followed for 2 minutes (Figure 6.3). This showed that after 10 seconds the probes had hybridised to approximately only 50% of the available template. To increase the sensitivity and accuracy of the readings, the annealing conditions were changed from 0 seconds at 55ºC to 20 seconds at 55ºC, after which time there is considerable more probe (approximately 70%) hybridised to the available template. This would reduce the introduced variability of the fluorescent measurements taken after only 0 seconds at 55ºC.

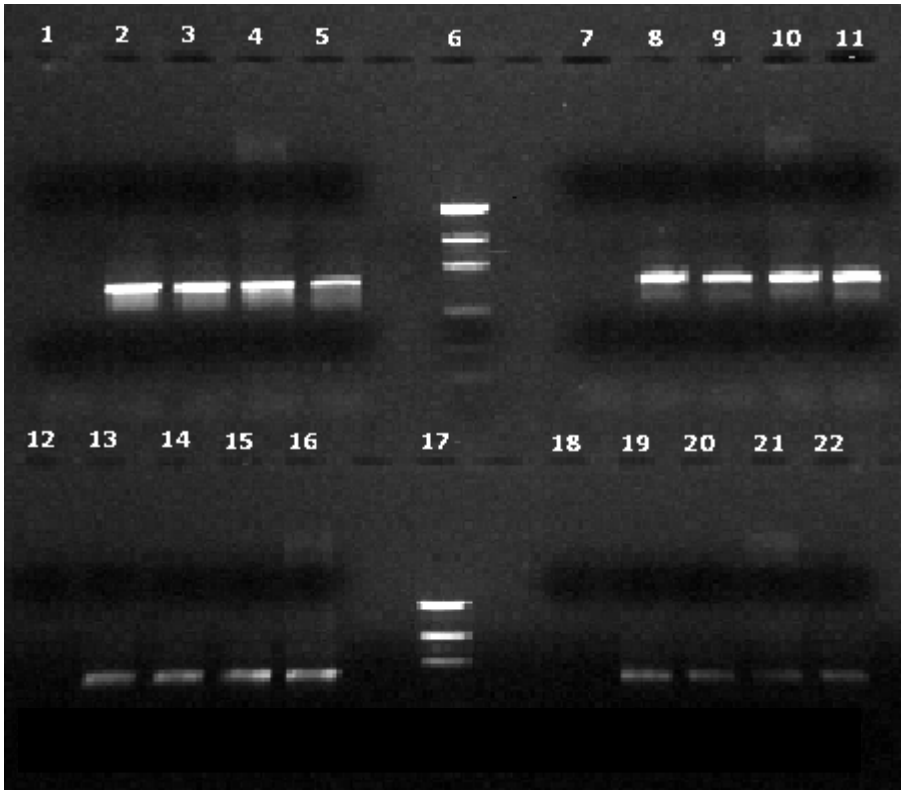


Figure 6.2: Effect of Annealing Temperature on PCR yield

Lanes 1-5: 50ºC anneal; Lanes 6-11: 55ºC anneal; Lanes 12-16: 58ºC anneal; Lanes 18-22: 60ºC anneal. Lanes 6 and 17: 500ng Low Mass Ladder. Order of samples: ddH_2O negative control; *Bacillus* str B4-1; *Caloramator* str 75-1; *Caloramator* str 75-1(duplicate) ; *Caloramator* str 75-2.

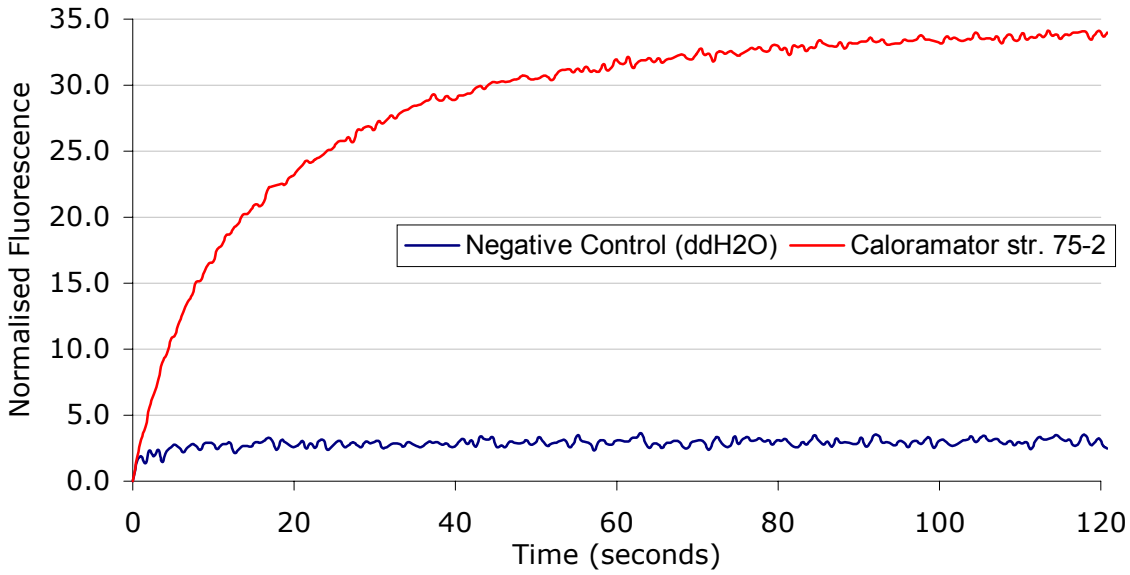


Figure 6.3: Binding of the hybridisation probes followed at 55ºC

Figure 6.4 shows the sensitivity of hybridisation probes with real-time PCR. Noticeable increases in fluorescence were detected in all *Caloramator* str 75-2 samples down to 6fg of template DNA. No fluorescence was detected in the ddH_2O amplification negative control, the *Bacillus* str B4-1 hybridisation negative control, or *Caloramator* str 75-2 samples with less than 6fg of template DNA added.

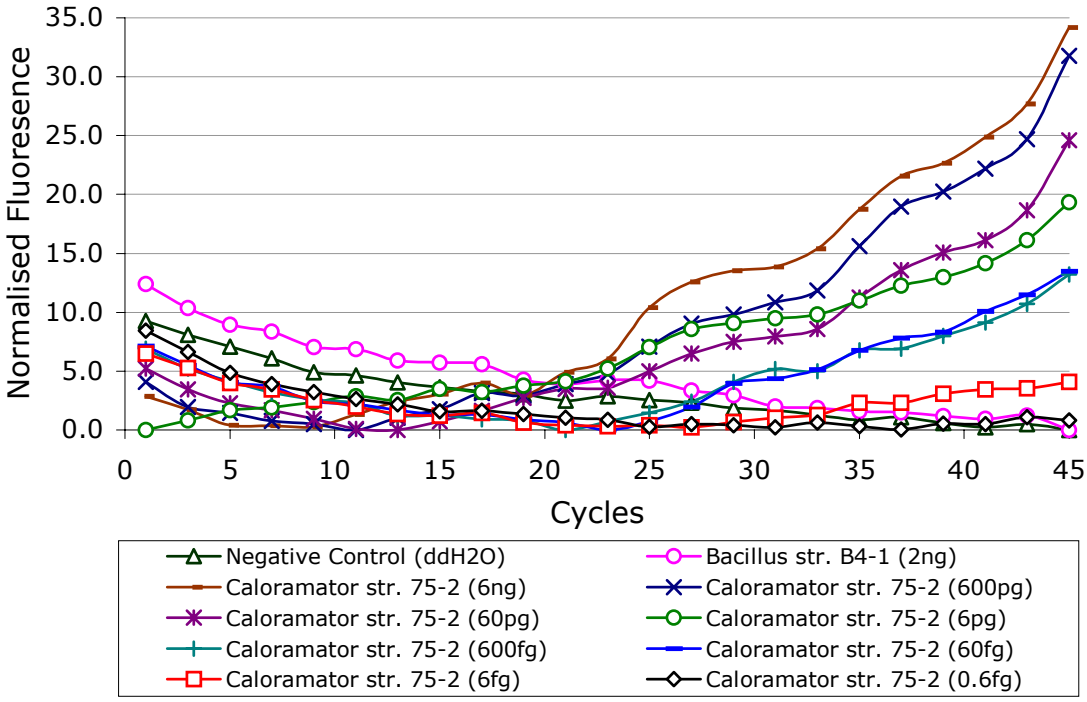


Figure 6.4: Real-time PCR specific for *Caloramator*

Increases in fluorescence were detected as early as cycle 10 for *Caloramator* str 75-2 (6ng) and as late as cycle 29 for the *Caloramator* str 75-2 (6fg).

Figure 6.5 is the agarose gel image of the samples after the real-time PCR and shows that amplification is noticeable in all samples apart from the ddH_2O amplification negative control and the *Caloramator* str 75-2 samples with less than 60fg of template DNA added.

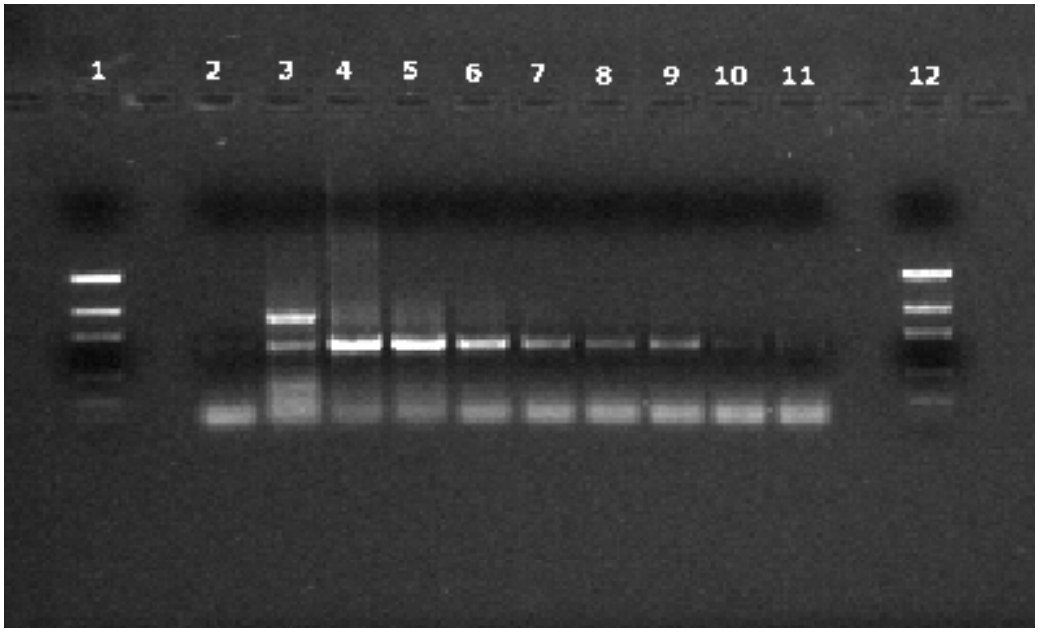


Figure 6.5: Agarose gel electrophoresis image of the Real-Time PCR dilution series

Lanes 1 and 12: 500ng Low Mass Ladder. Lane 2: ddH_2O negative control. Lane 3: *Bacillus* str B4-1 (20ng DNA). Lane 4-11: *Caloramator* str 75-2. Amounts of DNA are: 6ng; 600pg, 60pg, 6pg, 600fg, 60fg, 6fg, and 0.6fg from Lane 4 to Lane 11.

The increase in the fluorescence ratio for the environmental *Caloramator* isolates, but not for the *Bacillus* str B4-1 demonstrates that these probes can be used in the rapid identification of *Caloramator* isolates from natural ecosystems. The annealing temperature of 55ºC conferred acceptable specificity to the assay, and further modification of the PCR's conditions was considered unnecessary. Under these conditions, the fluorogenic probes started dissociating from the PCR product at approximately 65ºC and was completely dissociated at 75ºC as shown in Figure 6.6. The T_M calculated from first derivative analysis of this curve was 69ºC and will be specific for the dissociation of the hybridisation probes to the target region.

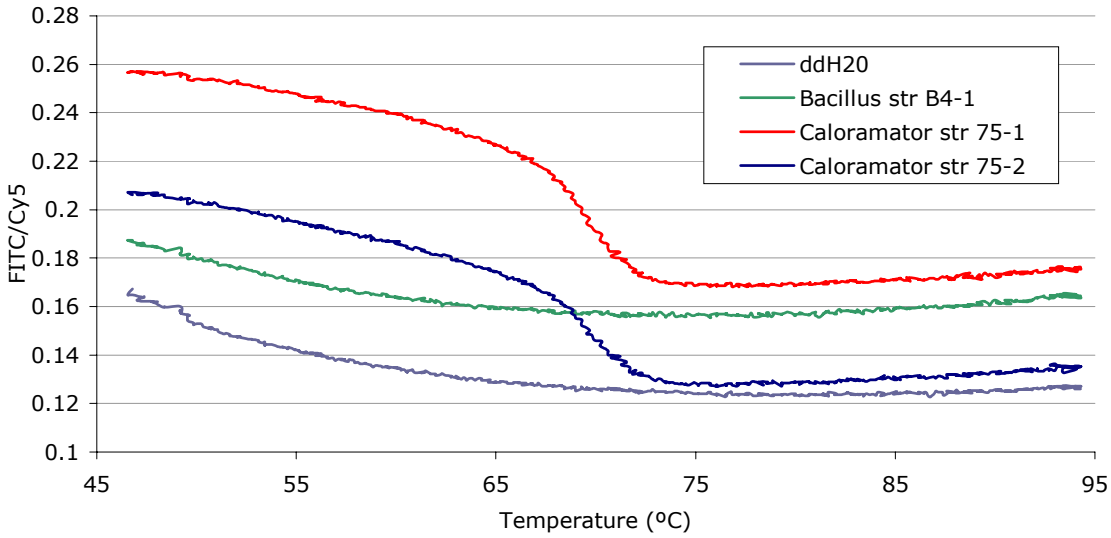


Figure 6.6: Melting curve showing gradual dissociation of the adjacent hybridisation probes

Dissociation of the adjacent hybridisation probes began at 65ºC and was complete at 75ºC. The Tm calculated by the first derivative analysis is 69ºC and will be specific for this set of probes and *Caloramator* species. No dissociation was detected in both negative controls.

6.4 DISCUSSION

6.4.1 OPTIMAL PCR CONDITIONS

At an annealing temperature of 50ºC, there is considerable smearing of the PCR product, an undesirable artefact of the low annealing temperature. At the higher temperatures of 58ºC and 60ºC, the amount of PCR product is much lower, a result of lower binding of the amplification primers. The optimal annealing temperature for PCR was regarded as 55ºC as the amount of amplified product was high and there was relatively little smearing at that annealing temperature.

6.4.2 HYBRIDISATION PROBE DESIGN

The 16S rRNA gene was selected as the target site for both amplification and identification, as a series of primers specific for this gene are well known. To allow for a wide range of applications and the cost of the Cy5-labelling procedure, this hybridisation probe was designed to bind to as many species as possible by designing it against a conserved region of the

16S rRNA gene. The fine precision of this technique was introduced from the cheaper, fluorescein-labelled probe that was designed against an adjacent, but variable region. Sequence analysis has shown that the 1046plus-Cy5 probe is complementary to the 16S rRNA gene from a wide number of species. The inclusion of dI in the oligonucleotide increases its' degeneracy. Sequence analysis of the Cal-FITC probe shows its specificity to the genus of *Caloramator*. To transfer this protocol to identify a different species of prokaryote, a new fluorescein-labelled probe constructed for the same region of the 16S rRNA gene is the only modification required.

6.4.3 OPTIMAL LIGHTCYCLER CONDITIONS

The initial thermal cycling conditions optimised on the RapidCycler transferred to the LightCycler™ amplified the target DNA well. However, the measurement of emitted fluorescence data was not consistent, showing inconsistencies between readings. To reduce this effect, the association of the probes were monitored at 55ºC (the temperature of annealing and recording) for 2 minutes. This showed that a reasonable majority of the probes did not hybridise with the target for 20 seconds after reaching the annealing temperature. To ensure that the stringency of the thermal cycling was reserved, the annealing temperature was not reduced, but the time was extended from 0 seconds to 20 seconds. This proved to stabilise the readings for the real-time monitoring of the PCR.

The slight decrease in normalised fluorescent readings during the initial cycles shown in Figure 6.4 is thought to be mainly due to a photobleaching effect on the fluorescein label used in the experiment. Fluorescein is known to be very susceptible to photobleaching effects (Song *et al*., 1995; Sjoback *et al*, 1995). In addition, quenching of the fluorescein-signal is also known to occur when labelled probes are hybridised to complementary sequences (Talavera *et al*, 2000).

6.4.4 THE MELTING PROFILE OF THE HYBRIDISATION PROBES

As both probes are required in close proximity to excite the fluorescein label of the Cal-FITC probe, if one probe dissociates from the target DNA, the

emission from fluorescein will reduce. The probes' melting profile showed a high uniformity with the T_M's calculated. Analysis of the melting profile of the probes showed an initial decrease of approximately 65ºC which corresponds well with the calculated T_M of the 1046plus-Cy5 probe which was 64.2ºC. The first derivative analysis of the melting profile gave a T_M of 69ºC that also corresponds well. The specific nature of the probe set (Ririe *et al*., 1997) will unambiguously provide a T_M of 69ºC when binding to the 16S rRNA gene of *Caloramator* species. The introduction of mismatches into the target region (i.e. from a different species) will reduce the strength of the hybridisation between the probes and the target DNA, reducing the observed T_M to a lower value.

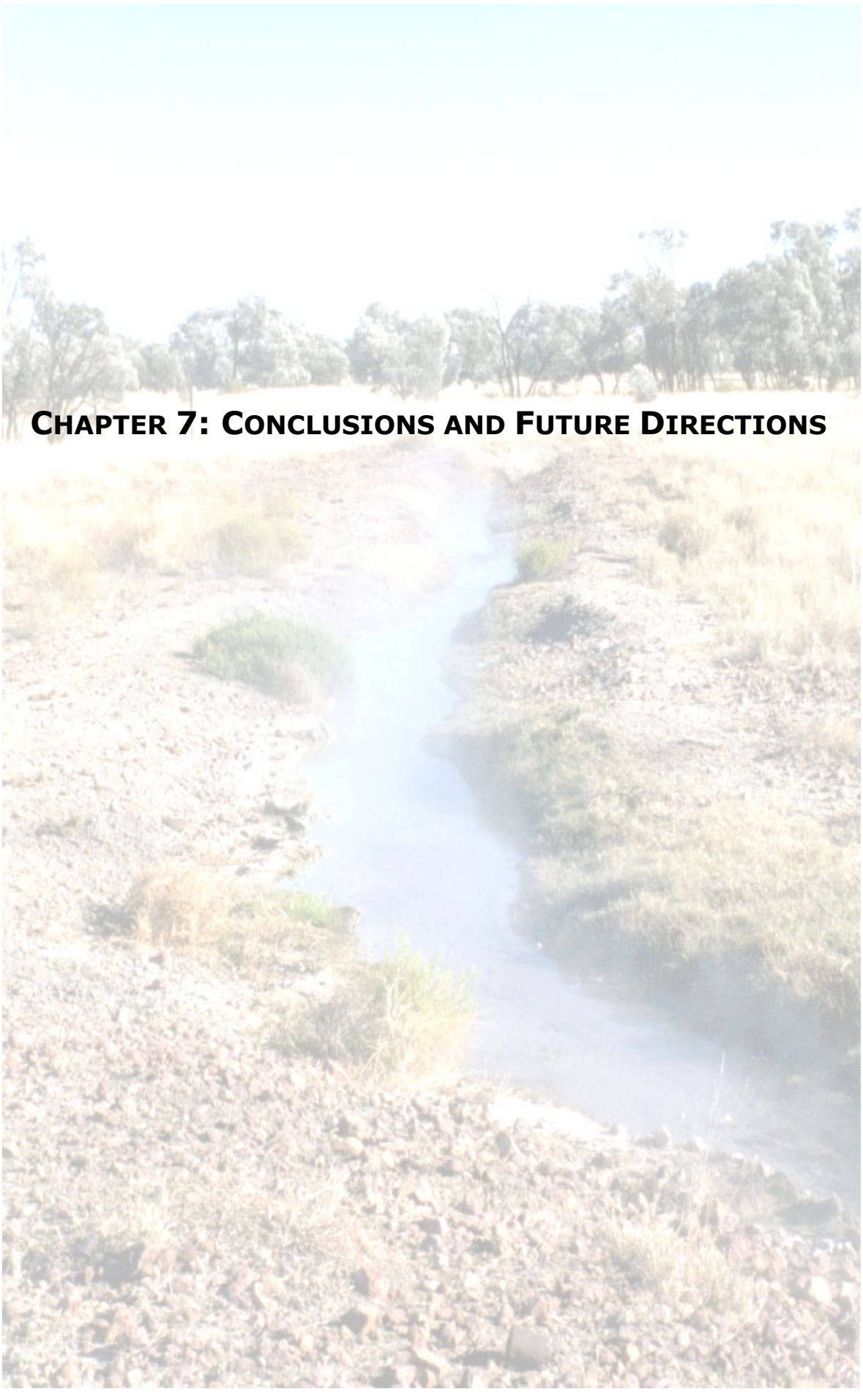
6.4.5 REAL-TIME PCR AS A TOOL FOR PROKARYOTIC ECOLOGY

The use of real-time PCR to identify and quantify pathogenic organisms has widely been published (Ballard *et al*., 2000; Bellin *et al*., 2001; Gut *et al*., 1999; Loeffler *et al*., 2000; Pirnay *et al*., 2000; Takeuchi *et al*., 1999). The economic importance of these pathogenic organisms cannot be discounted. However, the interest in thee transfer of this technology to monitor prokaryotic populations in the environment is relatively low (Becker *et al*., 2000; Hermansson & Lindgren, 2001).

This chapter reports the design of adjacent hybridisation probes for the identification of isolates of the genus *Caloramator*. It was limited to environmental isolates that, from prior 16S rRNA gene sequencing and phylogenetic analysis, were known members of this genus to optimise the procedure for their identification. This procedure is ready to be applied to unknown thermophilic strains isolated in TYEG media to aid in their rapid identification. The development of real-time PCR and this pair of probes to further detect *Caloramator* species in environmental samples is also a possible application.

The preliminary development of the Cal-FITC probe here allows the rapid identification of environmental isolates that belong to the genus *Caloramator*. The identification of different community members can be accomplished with the development of new probes designed to the variable

region upstream of the 1046plus-Cy5 probe, and used in conjunction with the non-specific 1046plus-Cy5 fluorogenic probe. As show in Section 6.3.4, the results of real-time PCR are dependent on initial concentrations of the target for PCR. This enables a standardised method to rapidly quantify and identify prokaryotes. Quantification and identification of different members of prokaryotic communities will provide a more complete understanding of the interactions between them. In addition to this, detrimental populations can be monitored and pre-emptive action taken before damage is caused.



CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS

7.1 CONCLUSIONS

This study describes the prokaryotic populations present in a single runoff drain from the New Lorne bore in central Queensland. The outlet temperature of 89ºC was a crucial factor in choosing the bore, as thermophilic communities were to be the subject of this thesis. A combination of culture independent and culture dependent techniques were used to characterise the prokaryotic communities present. The first part of this thesis (Chapter 3) characterised five microbial communities present in this high-temperature bore and drain using the culture independent technique of 16S rRNA gene amplification and cloning. Geographical limitations on species and genera have been examined for a variety of prokaryotes and Chapter 4 is an examination of *Thermus* and *Meiothermus* isolates and clones from this environment and expands the current knowledge of the ecological niches for these genera. Chapter 5 concentrated on the isolation and characterisation of a novel species, *Thermaerobacter subterraneus* str C21, isolated from the runoff drain. The development of real-time PCR to identify environmental isolates of *Caloramator* is discussed in Chapter 6.

The molecular study of the prokaryotic communities of the Great Artesian Basin of Australia has revealed that there are still many uncultivated species present within this environment. The characterisation of these new and novel prokaryotes will increase our knowledge of prokaryotic diversity. Although only beginning, directed cultivation techniques are being introduced in the field of microbial ecology (Hugenholtz, 2000). This will expand our understanding of phenotypic diversity.

Isolates of the thermophilic genera *Thermus* and *Meiothermus* have been shown to exhibit a limited biogeography due to the sporadic nature of thermal ecosystems. 16S rRNA gene sequence data obtained from isolates from the Great Artesian Basin and 16S rRNA gene clone libraries has shown that populations of *Thermus* and *Meiothermus* in the Great Artesian Basin are limited to several clusters and do not include representatives of all species within these genera. A comprehensive study of the available data

provides further evidence for the biogeographical limitations on several of these species, including *T. filiformus* (New Zealand), *T. brockianus* (USA) and *T. aquaticus* (USA).

The description and isolation of *Thermaerobacter subterraneus* str C21^T from the Great Artesian Basin is the first report of this genus from this unique thermal environment. It extends the ecological niche of the genus *Thermaerobacter*. *T. subterraneus* str C21^T and other unique prokaryotes from the Great Artesian Basin represent a potentially rich source of thermophilic enzymes for biotechnological use. The genome and proteins of *T. subterraneus* str C21^T should be a basis for further study as it will elucidate the phenotypic basis for the obligately aerobic nature of this genus, considering its phylogenetic placement within the obligately anaerobic class of *Firmicutes*.

The use of real-time PCR for the identification and quantification of pathogenic organisms is widespread, however, its use in environmental microbiology is very limited. The development of probes for specific prokaryotic populations is an initial step for the rapid monitoring of ecologically important members in communities. Its ability to quantify while identifying populations is essential in characterising prokaryotic communities using this method.

7.2 Future Directions

This relatively unknown resource of new prokaryotes potentially provides a source of innovative thermophilic enzymes and metabolites that may be exploited biotechnologically.

To understand fully the effect of prokaryotic populations have on our biosphere, much work needs to be directed towards characterising prokaryotic communities. An understanding of the genetic and phenotypic diversity of complex communities is required. Studies identifying active and dormant populations and the physical organisation of such communities will clarify the role of prokaryotic communities in the cycling of nutrients and in biosphere.

The scope of the phylogenetic analysis of the thermophilic communities was limited to the domain *Bacteria* due to time restrictions. The evidence provided here, however, does not exclude the presence of *Archaea* in the outflow of this bore. There is a good possibility that some groups of *Archaea* (i.e. the methanogens) inhabit this environment. The New Lorne bore, however, is relatively low in dissolved sulfur compounds like sulfate and sulfite, and the probability that the sulfur-dependent *Archaea* are present is low. Sulphur-dependent *Archaea* may be present in other areas of the Great Artesian Basin of Australia that have higher levels of sulphate (i.e. near the western margins). It is necessary to continue the search for *Archaea* in the Great Artesian Basin and further studies to determine their diversity and the extent of their habitat should be carried out.

Further examination of the Great Artesian Basin as an environment is required to recognise many of the factors influencing the prokaryotic diversity. A thorough characterisation of environmental conditions including the hydrochemical and subsurface data will provide a framework for the detection of the effects on prokaryotic diversity. This study concentrated on a sole bore, the New Lorne bore in Central Queensland. The hydrochemistry of the Great Artesian Basin varies, and comprehensive examination of the biodiversity of the Great Artesian Basin is required to fully understand the environmental effects on community structure.

Culture independent techniques based on the 16S rRNA gene for identification of phylogenetic groups will provide a resource of genetic information that can be used to detect groups in a variety of ways. The database can be exploited to develop DNA probes based on the 16S rRNA gene.

Population studies will be enhanced as these probes are applied in a number of ways. Real-time PCR and hybridisation probes have been used to rapidly enumerate and identify disease-causing prokaryotes, and are only recently being introduced in the field of microbial ecology. FISH can be utilised to enumerate groups, identify the spatial relationships between prokaryotes and the organisations of communities in nature. DGGE can be applied to monitor populations in prokaryotic communities.

Examinations of continuous cultures of mixed populations is required as it will elucidate the relationships between different species e.g. commensalism or syntrophism.

The economic importance of the Great Artesian Basin is great, especially in the more arid regions of Australia. Prokaryotic communities influence the standard and quantity of bore water. Populations of iron-oxidising prokaryotes produce insoluble ferric iron that then precipitates out of solution. This precipitate causes problems with filtration and pumping. In addition the prokaryotes increase corrosion of the bore casings. These prokaryotes cause a decline in the standard of water quality with adverse effects on taste, colour and odour. It is vital that detrimental populations can be minimised and monitored before the need for expensive and complex rehabilitation arises. Developing a rapid, standardised technique using new technologies (e.g. real-time PCR) will enable the relatively easy and cheap monitoring of at-risk bores that will provide early indications of infections of detrimental prokaryotes.

A considerable amount of research has been carried out on the genus *Thermus*, however, the monophyletic nature of its species is still under question. Numerical and chemotaxonomic studies do not provide mirror groups compared to the phylogenetic groups obtained from 16S rRNA gene sequences. To ensure that groups within this species are correctly placed phylogenetic, chemotaxonomic and numerical studies must be broadened to include new characteristics. The genomic variation between isolates shown by (Moreira *et al*., 1997) may provide insights into the phyletic nature of this genus. DNA-DNA hybridisation experiments may show a higher homology present within species than shown by RFLP-PFGE.

Further studies of isolates of *Thermus* from the Great Artesian Basin and other environments are required to elucidate its ecological nature. In addition, this may present new information regarding the phylogeny of this genus. From previous studies, it is known that there are some geographical limitations on members of the genus *Thermus*. A widespread approach to identify *Thermus* from worldwide environments will demonstrate that biodiversity can be linked to biogeography. This approach can be used to

study the geographical limitations of other genera and species that may be important for understanding biodiversity and the ecology of prokaryotes.

The universal root of the tree of life is the centre of much controversy. Studying the evolution of prokaryotic communities may clarify the true root of life through knowledge of how communities adapt and evolve to new environments. Genome sequencing, still in its infancy, may provide new theories of how life evolved on this planet.

The study of prokaryotic communities, especially those from extreme environments, may provide further insights into the stability of life at high temperatures. Research on prokaryotic communities from the Great Artesian Basin of Australia has much potential to clarify the ecological role of thermophiles and thermophilic communities.

APPENDICES

APPENDIX I: WORLD WIDE WEB RESOURECES

BioEdit: Biological Sequence Alignment Editor for Windows 95/98/NT
http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html

Ribosomal Database Project II
http://rdp.cme.msu.edu/html/index.html

TreeCon for Windows
http://www.evolutionsbiologie.uni-konstanz.de/peer-lab/treeconw.html

National Centre for Biotechnology Information
http://www.ncbi.nlm.nih.gov/

Comparative RNA Web Site
http://www.rna.icmb.utexas.edu/

Bergy's Manual Trust
http://server.mph.msu.edu/bergeys/

The Institue of Genomic Research
http://www.tigr.org/

APPENDIX II: GENBANK ACCESSION NUMBERS

Clone	Accession Number	Clone	Accession Number
Sed01	AF407673	G21	AF407703
Y03	AF407674	G24	AF407704
Y04	AF407675	G32	AF407705
Y10	AF407676	G34	AF407706
Y27	AF407677	G55	AF407707
Y30	AF407678	G58	AF407708
Y36	AF407679	G62	AF407709
Y63	AF407680	G73	AF407710
Y71	AF407681	G94	AF407711
Y88	AF407682	B01	AF407712
Y90	AF407683	B10	AF407713
R03	AF407684	B11	AF407714
R08	AF407685	B13	AF407715
R10	AF407686	B15	AF407716
R15	AF407687	B16	AF407717
R16	AF407688	B25	AF407718
R27	AF407689	B27	AF407719
R35	AF407690	B35	AF407720
R38	AF407691	B37	AF407721
R57	AF407692	B44	AF407722
R58	AF407693	B53	AF407723
R75	AF407694	B55	AF407724
R82	AF407695	B63	AF407725
G01	AF407696	B66	AF407726
G06	AF407697	B79	AF407727
G07	AF407698	B83	AF407728
G10	AF407699	B86	AF407729
G13	AF407700	B90	AF407730
G18	AF407701	B95	AF407731
G19	AF407702		

Isolate	Accession Number
C21	AF343566
Y70-05	AF407732
Y70-06	AF407733
Y70-07	AF407734
R70-06	AF407735
R70-07	AF407736
G70-05	AF407737
G70-06	AF407738
G70-07	AF407739
G70-08	AF407740
B70-04	AF407741
B70-05	AF407742
Y55-07	AF407743
Y55-08	AF407744
Y55-09	AF407745
R55-10	AF407749
R55-11	AF407750

REFERENCES

Ahmad, S., Scopes, R. K., Rees, G. N. & Patel, B. K. C. (2000). *Saccharococcus caldoxylosilyticus* sp. nov., an obligately thermophilic, xylose-utilising, endospore-forming bacterium. *International Journal of Systematic and Evolutionary Microbiology* **50**, 517-532.

Alfredsson, G. A., Kristjansson, J. K., Hjörleifdottir, S. & Stetter, K. O. (1988). *Rhodothermus marinus*, gen. nov., sp. nov., a thermophilic, halophilic bacterium from submarine hot springs in Iceland. *Journal of General Microbiology* **134**, 49-68.

Altshul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (2001). Basic local alignment search tool. *Journal of Molecular Biology* **215**(3), 403-410.

Amann, G., Stetter, K. O., Llobet-Brossa, E., Amann, R. & Anton, J. (2000). Direct proof for the presence and expression of two 5% different 16S rRNA genes in individual cells of *Haloarcula marismortui*. *Extremophiles* **4**(6), 373-376.

Amann, R. I. (1995). Fluorescently labelled, rRNA-targeted oligonucleotide probes in the study of microbial ecology. *Molecular Ecology* **4**(5), 543-553.

Amann, R. I., Ludwig, W. & Schleifer, K. H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiological Reviews* **59**(1), 143-169.

Andrews, K. T. & Patel, B. K. C. (1996). *Fervidobacterium godwanense* sp nov, a new thermophilic anaerobic bacterium isolated from nonvolcanically heated geothermal waters of the Great Artesian Basin of Australia. *International Journal of Systematic Bacteriology* **46**(1), 265-269.

Aravind, L., Tatusov, R. L., Wolf, Y. I., Walker, D. R. & Koonin, E. V. (1998). Evidence for massive gene exchange between archael and bacterial hyperthermophiles. *Trends in Genetics* **14**(11), 442-444.

Ash, C., Priest, F.G. & Collins, M.D. (1993). Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. *Antonie van Leeuwenhoek* **64**, 253-260.

Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R. & Wolfe, R. S. (1979). Methanogens: reevaulation of a unqie biological group. *Microbiology Reviews* **1**(2), 260-296.

Baldauf, S. L., Palmer, J. D. & Doolittle, W. F. (1996). The root of the universal tree and the origin of eukaryotes based on elongation factor phylogeny. *Proceedings of the National Academy of Sciences of the United States of America* **93**(15), 7749-7754.

Ballard, A. L., Fry, N. K., Chan, L., Surman, S. B., Lee, J. V., Harrison, T. G. & Towner, K. J. (2000). Detection of *Legionella pneumophila* using a real-time PCR hybridization assay. *Journal of Clinical Microbiology* **38**(11), 4215-4218.

Barns, S. M., Delwiche, C. F., Palmer, J. D. & Pace, N. R. (1996). Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. *Proceedings of the National Academy of Sciences of the United States of America* **93**(17), 9188-9193.

Barns, S. M., Fundyga, R. E., Jeffries, M. W. & Pace, N. R. (1994). Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. *Proceedings of the National Academy of Sciences of the United States of America* **91**(5), 1609-1613.

Bateson, M. M., Thibault, K. J. & Ward, D. M. (1990). Comparative analysis of 16S ribosomal RNA sequences of *Thermus* species. *Systematic and Applied Microbiology* **13**, 8-13.

Becker, S., Boger, P., Oehlmann, R. & Ernst, A. (2000). PCR bias in ecological analysis: a case study for quantitative *Taq* nuclease assays in analyses of microbial communities. *Applied and Environmental Microbiology* **66**(11), 4945-4953.

Beeder, J., Kåre, R., Rosnes, J. T., Torsvik, T. & Lien, T. (1994). *Archeoglobus fulgidus* isolated from hot North Sea oil field waters. *Applied and Environmental Microbiology* **60**(4), 1227-1231.

Beffa, T., Blanc, M., Lyon, P. F., Vogt, G., Marchiani, M., Fischer, J. L. & Aragno, M. (1996). Isolation of *Thermus* strains from hot composts (60 to 80ºC). *Applied and Environmental Microbiology* **62**(5), 1723-1727.

Bellin, T., Pulz, M., Matussek, A., Hempen, H. G. & Gunzer, F. (2001). Rapid detection of enterohemorrhagic Escherichia coli by real-time PCR with fluorescent hybridization probes. *Journal of Clinical Microbiology* **39**(1), 370-374.

Benlloch, S., Martínez-Murcia, A. J. & Rodríguez-Valera, F. (1995). Sequencing of *Bacterial* and *Archaeal* 16S rRNA genes directly amplified from a hypersaline environment. *Systematic and Applied Microbiology* **18**, 574-581.

Benson, D. A., Boguski, M. S., Lipman, D. J., Ostell, J., Ouellette, B. F., Rapp, B. A. & Wheeler, D. L. (1999). GenBank. *Nucleic Acids Research* **27**(1), 12-17.

Bintrim, S. B., Donohue, T. J., Handelsman, J., Roberts, G. P. & Goodman, R. M. (1997). Molecular phylogeny of archaea from soil. *Proceedings of the National Academy of Sciences of the United States of America* **94**(1), 277-282.

Blanc, M., Marilley, L., Beffa, T. & Aragno, M. (1997). Rapid identification of heterotrophic, thermophilic, spore-forming bacteria isolated from hot composts. *International Journal of Systematic Bacteriology* **47**(4), 1246-1248.

Blochl, 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 degrees C. *Extremophiles* **1**(1), 14-21.

Bocchetta, M., Gribaldo, S., Sanangelantoni, A. & Cammarano, P. (2000). Phylogenetic depth of the bacterial genera Aquifex and Thermotoga inferred from analysis of ribosomal protein, elongation factor, and RNA polymerase subunit sequences. *Journal of Molecular Evolution* **50**(4), 366-380.

Bond, P. L., Hugenholtz, P., Keller, J. & Blackall, L. L. (1995). Bacterial community structures of phosphate- and non-phosphate-removing activated sludges from sequencing batch reactors. *Applied and Environmental Microbiology* **61**(5), 1910-1916.

Boone, D. R. & Castenholz, R. W. (2001). *The Archaea and the deeply branching and phototrophic Bacteria*. 2nd edit. Bergey's Manual of Systematic Bacteriology (Garrity, G. M., Ed.), 1, Springer, New York.

Boone, D. R., Liu, Y. T., Zhao, Z. J., Balkwill, D. L., Drake, G. R., Stevens, T. O. & Aldrich, H. C. (1995). *Bacillus infernus* sp nov, an Fe(III)- and Mn(IV)-reducing anaerobe from the deep terrestrial subsurface. *International Journal of Systematic Bacteriology* **45**(3), 441-448.

Boucher, Y. & Doolittle, W. F. (2000). The role of lateral gene transfer in the evolution of isoprenoid biosynthesis pathway. *Molecular Microbiology* **37**(4), 703-716.

Brandt, M. E., Padhy, A. A., Mayer, L. W. & Holloway, B. P. (1998). Utility of random amplified polymorphic DNA PCR and TaqMan automated detection in molecular identification of *Aspergillus fumigatus*. *Journal of Clinical Microbiology* **36**(7), 2057-2062.

Britschgi, T. B. & Giovannoni, S. J. (1991). Phylogenetic analysis of a natural marine bacterioplankton population by rRNA gene cloning and sequencing. *Applied and Environmental Microbiology* **57**(6), 1707-1713.

Brock, T. D. (1967). Micro-organisms adapted to high temperatures. *Nature* **214**, 882-885.

Brock, T. D. & Freeze, H. (1969). *Thermus aquaticus* gen. nov., a nonsporulating extreme thermophile. *Journal of Bacteriology* **98**, 289-297.

Brown, J. K. (1994). Bootstrap hypothesis tests for evolutionary trees and other dendrograms. *Proceedings of the National Academy of Sciences of the United States of America* **91**(25), 12293-12297.

Bulygina, E. S., Galchenko, V. F., Govorukhina, N. I., Netrusov, A. I., Nikitin, D. I., Trotsenko, Y. A. & Chumakov, K. M. (1990). Taxonomic studies on methylotrophic bacteria by 5S ribosomal RNA sequencing. *Journal of General Microbiology* **136**(Pt 3), 441-446.

Burggraff, S., Olsen, G. j., Stetter, K. O. & Woese, C. R. (1992). A phylogenetic analysis of *Aquifex pyrophilus*. *Systematic and Applied Microbiology* **15**, 352-356.

Burrell, P. C., Keller, J. & Blackall, L. L. (1998). Microbiology of a nitrite-oxidizing bioreactor. *Applied and Environmental Microbiology* **64**(5), 1878-1883.

Byers, H. K., Patel, B. & Stackebrandt, E. (1997). Design and application of four oligonucleotide probes specific for *Thermus* species. *Systematic and Applied Microbiology* **20**, 248-254.

Byers, H. K., Stackebrandt, E., Hayward, C. & Blackall, L. L. (1998). Molecular investigation of a microbial mat associated with the Great Artesian Basin. *FEMS Microbiology Ecology* **25**, 391-403.

Canganella, F. & Trovatelli, L. D. (1995). Ecological and physiological studies on thermophilic bacilli from sulfataric hot springs of central Italy. *Journal of Basic Microbiology* **35**(1), 9-19.

Castenholz, R. W. (1969). Thermophilic blue-green algae and the thermal environment. *Bacteriology Reviews* **33**(4), 476-504.

Cayol, J. L., Ollivier, B., Patel, B. K. C., Magot, M., Ageron, E., Grimont, P. A. D. & Garcia, J. L. (1995). Description of *Thermoanaerobacter brockii* subsp. nov., isolated from a deep subsurface French oil well, a proposal to reclassify *Thermoanaerobacter finii* as *Thermoanaerobacter brockii* subsp. *finii*comb. nov., and an amended description of *Thermoanaerobacter brockii*. *International Journal of Systematic Bacteriology* **45**, 783-789.

Chandler, D. P., Brockman, F. J., Bailey, T. J. & Fredrickson, J. K. (1998). Phylogenetic diversity of *Archaea* and *Bacteria* in a deep subsurface paleosol. *Microbial Ecology* **36**, 37-50.

Chang, Y.-J., Peacock, A. D., Long, P. E., Stephen, J. R., McKinley, J. P., Macnaughton, S. J., Anwar Hussain, A. K. M., Saxton, A. M. & White, D. C. (2001). Diversity and characterization of sulfate-reducing Bacteria in groundwater at a uranium mill tailings site. *Applied and Environmental Microbiology* **67**(7), 3149-3160.

Chen, C., Lin, L., Peng, Q., Ben, K. & Zhao, S. *Meiothermus rosaceus*, a rose-pigmented thermophile bacteria from hot springs at Tengchong Rehai in Yunnan, P.R. China. *Unpublished*.

Chen, M. Y. & Tsay, S. S. Microbial resources of hot springs of Taiwan. *Unpublished*.

Chrisostomos, S., Patel, B. K. C., Dwivedi, P. P. & Denman, S. E. (1996). *Caloramator indicus* sp. nov., a new thermophilic anaerobic bacterium isolated from the deep-seated nonvolcanically heated waters of an Indian artesian aquifer. *International Journal of Systematic Bacteriology* **46**(2), 497-501.

Chung, A. P., Rainey, F., Nobre, M. F., Burghardt, J. & da Costa, M. S. (1997). *Meiothermus cerbereus* sp. nov., a new slightly thermophilic species with high levels of 3-hydroxy fatty acids. *International Journal of Systematic Bacteriology* **47**(4), 1225-1230.

Chung, A. P., Rainey, F. A., Valente, M., Nobre, M. F. & da Costa, M. S. (2000). *Thermus igniterrae* sp. nov. and *Thermus antranikianii* sp. nov., two new species from Iceland. *International Journal of Systematic and Evolutionary Microbiology* **50**(1), 209-217.

Cline, J., Braman, J. C. & Hogrefe, H. H. (1996). PCR fidelity of *Pfu* DNA polymerase and other thermostable DNA polymerase. *Nucleic Acids Research* **24**(18), 3546-3551.

Collee, J. G., Fraser, A. G., Marmion, B. P. & Simmons, A., Eds. (1996). Mackie and McCartney Practical Medical Microbiology. 14 edit. New York: Churchill Livingstone.

Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J., Fernendez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. & Farraw, J. A. E. (1994). The phylogeny of the Genus *Clostridium*: proposal of five new genera and eleven new species combinations. *International Journal of Systematic Bacteriology* **44**(4), 812-826.

Cometta, S., Sonnleitner, B., Sidler, W. & Fiechter, A. (1982). Population distribution of aerobic extremely thermophilic microorganisms in an Icelandic natural hot spring. *European Journal of Applied and Microbiological Biotechnology* **16**, 151-156.

Cowan, D. A. (1997). Thermophilic proteins: stability and function in aqueous and organic solvents. *Comparative Biochemistry and Physiology Part A: Physiology* **118**(3), 429-438.

de Bartolomeo, A., Trorra, F., La Rosa, F., Saltalamachio, G. & Mastrandrea, V. (1991). Numerical analysis and DNA base compositions of some thermophilic *Bacillus* species. *International Journal of Systematic Bacteriology* **41**(4), 502-509.

de la Tour, C. B., Portemer, C., Nadal, M., Stetter, K. O., Forterre, P. & Duguet, M. (1990). Reverse gyrase, a hallmark of the hyperthermophilic *archaebacteria*. *Journal of Bacteriology* **172**, 6803-6808.

Denman, S., Hampson, K. & Patel, B. K. C. (1991). Isolation of strains of *Thermus aquaticus* from the Australian Artesian Basin and a simple and rapid procedure for the preparation of their plasmids. *FEMS Microbiology Letters* **82**, 73-78.

Doolittle, W. F. (1999). Phylogenetic classification and the universal tree. *Science* **284**(5423), 2124-2129.

Dower, W. J., Miller, J. F. & Rangsdale, C. W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Research* **16**, 6127.

Dunbar, J., Takala, S., Barns, S. M., Davis, J. A. & Kuske, C. R. (1999). Levels of bacterial community diversity in four arid soils compared by

cultivation and 16S rRNA gene cloning. *Applied and Environmental Microbiology* **65**(4), 1662-1669.

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

Edmonds, C. G., Crain, P. F., Gupta, R., Hashizume, T., Hocart, C. H., Kowalak, J. A. & al, e. (1991). Posttranscriptional modification of tRNA in thermophilic archaea (archaebacteria). *Journal of Bacteriology* **173**, 3138-3148.

Ehrich, S., Behrens, D., Lebedeva, E., Ludwig, W. & Bock, E. (1995). A new obligately chemolithoautotrophic, nitrite-oxidizing bacterium, *Nitrospira moscoviensis* sp. nov. and its phylogenetic relationship. *Archives of Microbiology* **164**(1), 16-23.

Eisen, J. A. (2000). Horizontal gene transfer among microbial genomes: new insights from complete genome analysis. *Current Opinion in Genetics & Development* **10**, 606-611.

Ekendahl, S., Arlinger, J., Ståhl, F. & Pedersen, K. (1994). Characterization of attached bacterial populations in deep granitic groundwater from the Stripa research mine by 16S rRNA gene sequenccing and scanning electron microscopy. *Microbiology* **140**, 1575-1583.

Embley, T. M., Thomas, R. & Williams, R. (1993). Reduced thermophilic bias in the 16S rDNA sequence from *Thermus ruber* provides further support for a relationship between *Thermus* and *Deinococcus*. *Systematic and Applied Microbiology* **16**, 25-29.

Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric DNA-DNA hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *International Journal of Systematic Bacteriology* **39**, 224-229.

Fardeau, M. L., Ollivier, B., Patel, B. K., Magot, M., Thomas, P., Rimbault, A., Rocchiccioli, F. & Garcia, J. L. (1997). *Thermotoga hypogea* sp. nov., a xylanolytic, thermophilic bacterium from an oil-producing well. *International Journal of Systematic Bacteriology* **47**(4), 1013-1019.

Farrelly, V., Rainey, F. A. & Stackebrandt, E. (1995). Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Applied and Environmental Microbiology* **61**(7), 2798-801.

Felsenstein, J. (1978). Cases in which parsimony or compatibility methods will be positively misleading. *Systematics in Zoology* **27**, 401-410.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution* **17**, 368-376.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783-791.

Felske, A., Engelen, B., Nübel, V. & Backhaus, H. (1996). Direct ribosome isolation from soil to extract bacterial rRNA for community analysis. *Applied and Environmental Microbiology* **62**(11), 4162-4167.

Fergusan, R. L., Buckley, E. N. & Palumbo, A. V. (1984). Response of marine bacterioplankton to differential filtration and confinement. *Applied and Environmental Microbiology* **47**, 49-55.

Ferreira, A. C., Nobre, M. F., Rainey, F. A., Silva, M. T., Wait, R., Burghardt, J., Chung, A. P. & da Costa, M. S. (1997). *Deinococcus geothermalis* sp. nov. *and Deinococcus murrayi* sp. nov., two extremely radiation-resistant and slightly thermophilic species from hot springs. *International Journal of Systematic Bacteriology* **47**(4), 939-947.

Ferris, M. J., Muyzer, G. & Ward, D. M. (1996a). Denaturing gradient gel electrophoresis profiles of 16S rRNA defined populations inhabiting a hot spring microbial mat community. *Applied and Environmental Microbiology* **62**(2), 340-346.

Ferris, M. J., Ruffroberts, A. L., Kopczynski, E. D., Bateson, M. M. & Ward, D. M. (1996b). Enrichment culture and microscopy conceal diverse thermophilic *Synechococcus* populations in a single hot spring microbial mat habitat. *Applied and Environmental Microbiology* **62**(3), 1045-1050.

Ferris, M. J. & Ward, D. M. (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**(4), 1375-1381.

Forterre, P. & Philippe, H. (1999). Where is the root of the universal tree of life? *BioEssays* **21**(10), 871-879.

Fortin, N. Y., Mulchandani, A. & Chen, W. (2001). Use of real-time polymerase chain reaction and molecular beacons for the detection of *Escherichia coli* O157:H7. *Analytical Biochemistry* **289**(2), 281-288.

Frostegård, Å., Courtois, S., Ramisse, V., Clerc, S., Bernillon, D., Le Gall, F., Jeannin, P., Nesme, X. & Simonet, P. (1999). Quantification of bias related to the extraction of DNA directly from soils. *Applied and Environmental Microbiology* **65**(12), 5409-5420.

Fuhrman, J. A., Lee, S. H., Masuchi, Y., Davis, A. A. & Wilcox, R. M. (1994). Characterization of marine prokaryotic communities via DNA and RNA. *Microbial Ecology* **28**, 133-145.

Giovannoni, S. J., Rappé, M. S., Vergin, K. L. & Adair, N. L. (1996). 16S rRNA genes reveal stratified open ocean bacterioplankton populations related to the green non-sulfur bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **93**(15), 7979-7984.

Giovannoni, S. J., Schabtach, E. & Castenholz, R. W. (1987). *Isosphaera pallida*, gen and comb. nov., a gliding, budding eubacterium from hot springs. *Archives of Microbiology* **147**, 276-284.

Godfroy, A., Lesongeur, F., Raguénès, G., Quérellou, J., Antoine, E., Meunier, J.-R., Guezennez, J. & Barbier, G. (1997). *Thermococcus hydrothermalis* sp. nov., a new hyperthermophilic archaeon isolated from a

deep-sea hydrothermal vent. *International Journal of Systematic Bacteriology* **47**(3), 622-626.

Grassia, G. S., McLean, K. M., Glénat, P., Bauld, J. & Sheehy, A. J. (1996). A systematic survey for thermophilic fermentative *Bacteria* and *Archaea* in high temperature petroleum reservoirs. *FEMS Microbial Ecology* **21**, 47-58.

Gray, J. P. & Herwig, R. P. (1996). Phylogenetic analysis of the bacterial communities in marine sediments. *Applied and Environmental Microbiology* **62**(11), 4049-4059.

Greene, A. C., Patel, B. K. C. & Sheehy, A. J. (1997). *Deferribacter thermophilus* gen. nov., sp. nov., a novel thermophilic manganese- and iron-reducing bacterium isolated from a petroleum reservoir. *International Journal of Systematic Bacteriology* **47**(2), 505-509.

Grishin, N. V. (1999). A novel approach to phylogeny reconstruction from protein sequences. *Journal of Molecular Evolution* **48**(3), 264-273.

Guipaud, O., Marguet, E., Knoll, K. M., Bouthier de la Tour, C. & Forterre, P. (1997). Both DNA gyrase and reverse gyrase are present in the hyperthermophilic bacterium *Thermotoga maritima*. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 10606-10611.

Gupta, R. S. (2000). The phylogeny of proteobacteria: relationships to other eubacterial phyla and eukaryotes. *FEMS Microbiology Reviews* **24**(4), 367-402.

Gupta, R. S., Mukhtar, T. & Singh, B. (1999). Evolutionary relationships among photosynthetic prokaryotes (*Heliobacterium chlorum*, *Chloroflexus auranticus*, cyanobacteria, *Chlorobium tepidum*, and proteobacteria): implications regarding the origin of photosynthesis. *Molecular Microbiology* **32**(5), 893-906.

Gut, M., Leutenegger, C. M., Huder, J. B., Pedersen, N. C. & Lutz, H. (1999). One-tube fluorogenic reverse transcription-polymerase chain

reaction for the quantitation of feline coronaviruses. *Journal of Virological Methods* **77**(1), 37-46.

Gutell, R. R., Subashchandran, S., Schnare, M., Du, Y., Lin, N., Madabusi, L., Muller, K., Pande, N., Yu, N., Shang, Z., Date, S., Konings, D., Schweiker, V., Weiser, B. & Cannone, J. J. (2001). Comparative Sequence Analysis and the Prediction of RNA Structure, and the Web. *Unpublished*.

Habermahl, M. A. (1980). The Great Artesian Basin. *BMR Journal of Australian Geology and Geophysics* **5**, 9-38.

Habermahl, M. A. (1996). *Mesozic Geology of the Eastern Australia Plate*.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **48**, 95-98.

Harmsen, H. J., Prieur, D. & Jeanthon, C. (1997a). Group-specific 16S rRNA targeted oligonucleotide probes to identify thermophilic bacteria in marine hydrothermal vents. *Applied and Environmental Microbiology* **63**(10), 4061-4068.

Harmsen, H. J. M., Prieur, D. & Jeanthon, C. (1997b). Distribution of microorganisms in deep-sea hydrothermal vent chimneys investigated by whole-cell hybridization and enrichment culture of thermophilic subpopulations. *Applied and Environmental Microbiology* **63**(7), 2876-2883.

Hartford, T. & Sneath, P. H. A. (1988). Distortion of taxonomic structure from DNA relationships due to different choice of refernce strains. *Systematic and Applied Microbiology* **10**, 241-250.

Hensel, R., Demharter, W., Kandler, O., Kroppenstedt, R. M. & Stackebrandt, E. (1986). Chemotaxonomic and molecular-genetic studies of the genus *Thermus*: evidence for a phylogenetic relationship of *Thermus aquaticus* and *Thermus ruber* to the genus *Deinococcus*. *International Journal of Systematic Bacteriology* **36**(3), 444-453.

Hermansson, A. & Lindgren, P. (2001). Quantification of ammonia-oxidizing bacteria in arable soil by real-time PCR. *Applied and Environmental Microbiology* **67**(2), 972-976.

Heuer, H., Krsek, M., Baker, P., Smalla, K. & Wellington, E. M. H. (1997). Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Applied and Environmental Microbiology* **63**(8), 3233-3241.

Hillier, J. (1996). *Mesozoic Geology of Eastern Australia Plate Conference*.

Hiraishi, A., Kishimoto, N., Kosako, Y., Wakao, N. & Tano, T. (1995). Phylogenetic position of menaquinone-containing acidophilic chemo-organotroph *Acidobacterium capsulatum*. *FEMS Microbiology Letters* **132**, 91-94.

Hori, H. & Osawa, S. (1979). Evolutionary change in 5S RNA secondary structure and a phylogenic tree of 54 5S RNA species. *Proceedings of the National Academy of Sciences of the United States of America* **76**(1), 381-385.

Hornsby, L. A. & Horan, N. J. (1994). Isolation of filamentous bacteria from activated sludge using micromanipulation. *Water Resources* **28**(9), 2033-2034.

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. *International Journal of Systematic Bacteriology* **48**(1), 31-38.

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

Hudson, J. A., Morgan, H. W. & Daniel, R. M. (1987). Numerical classification of some *Thermus* isolates from Icelandic hot springs. *Systematic and Applied Microbiology*, **9**, 218-223.

Hudson, J. A., Morgan, H. W. & Daniel, R. M. (1989). Numerical classification of *Thermus* isolates from globally distributed hot springs. *Systematic and Applied Microbiology* **11**, 250-256.

Hugenholtz, P. (2000). *Ninth International Congress for Culture Collections, Brisbane, Australia*.

Hugenholtz, P., Goebel, B. M. & Pace, N. R. (1998a). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *Journal of Bacteriology* **180**(18), 4765-4774.

Hugenholtz, P. & Pace, N. R. (1996). Identifying microbial diversity in the natural environment: a molecular phylogenetic approach. *Trends in Biotechnology* **14**(6), 190-197.

Hugenholtz, P., Pitulle, C., Hershberger, K. L. & Pace, N. R. (1998b). Novel division level bacterial diversity in a Yellowstone hot spring. *Journal of Bacteriology* **180**(2), 366-376.

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

Ishiguro, M., Kaneko, S., Kuno, A., Koyama, Y., Yoshida, S., Park, G., Sakakibara, Y., Kusakabe, I. & Kobayashi, H. Purification and characterization of the recombinant *Thermus* sp. strain T2 alpha-galactosidase expressed in *Escherichia coli*. *Unpublished*.

Jackson, C. R., Harper, J. P., Willoughby, D., Roden, E. E. & Churchill, P. F. (1997). A simple, efficient method for the separation of humic substances and DNA from environmental samples. *Applied and Environmental Microbiology* **63**(12), 4993-4995.

Jaenicke, R. (1996). Gylceraldehyde-3-phosphate dehydrogenase from *Thermotoga maritima*: strategies of protein stabilization. *FEMS Microbiology Reviews* **18**(2-3), 215-224.

Jones, J. G. (1977). The effect on environmental factors on estimated viable and total populations of planktonic bacteria in lakes and experimental enclosures. *Freshwater Biology* **7**, 67-91.

Jones, R. A., Jermiin, L. S., Easteal, S., Patel, B. K. & Beacham, I. R. (1999). Amylase and 16S rRNA genes from a hyperthermophilic archaebacterium. *Journal of Applied Microbiology* **86**(1), 93-107.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian protein metabolism* (Munro, H. H., ed.), pp. 21-132. Academic Press, New York.

Kämpfer, P., Erhart, R., Beimfor, C., Böhringer, M., Wagner, M. & Amann, R. (1996). Characterization of bacterial communities from activated sludge: Culture-dependent numerical identification versus *in situ* identification using group- and genus-specific rRNA-targeted oligonucleotide probes. *Microbial Ecology* **32**(2), 101-102.

Kawasumi, T., Igarashi, Y., Kodama, T. & Minoda, Y. (1984). *Hydrogenobacter thermophilus* gen. nov., sp. nov., an extremely thermophilic, aerobic, hydrogen-oxidizing bacterium. *International Journal of Systematic Bacteriology* **34**(1), 5-10.

Keller, M., Braun, F. J., Dirmeier, R., Hafenbradl, D. B., S., Rachel, R. & Stetter, K. O. (1995). *Thermococcus alcaliphilus* sp nov, a new hyperthermophilic Archaeum growing on polysulfide at alkaline pH. *Archives of Microbiology* **164**(6), 390-395.

Kieft, T. L., Fredrickson, J. K., Onstott, T. C., Gorby, Y. A., Kostandarithes, H. M., Bailey, T. J., Kennedy, D. W., Li, S. W., Plymale, A. E., Spadoni, C. M. & Gray, M. S. (1999). Dissimilatory reduction of Fe(III) and other electron acceptors by a *Thermus* isolate. *Applied and Environmental Microbiology* **65**(3), 1214-1221.

Knerr, I., Repp, R., Dotsch, J., Gratzki, N., Hanze, J., Kapellen, T. & Rascher, W. (1999). Quantitation of gene expression by real-time PCR disproves a "retroviral hypothesis" for childhood-onset diabetes mellitus. *Pediatric Research* **46**(1), 57-60.

Kogure, K., Simidu, U. & Taga, N. (1979). A tentative direct micrscopic method for counting living marine bacteria. *Canadian Journal of Microbiology* **25**, 415-420.

Kogure, K., Simidu, U. & Taga, N. (1980). Distribution of viable marine bacteria in neritic seawater around Japan. *Canadian Journal of Microbiology* **26**, 318-323.

Kollman, J. M. & Doolittle, R. F. (2000). Determining the relative rates of change for prokaryotic and eukaryotic proteins with anciently duplicated paralogs. *Journal of Molecular Evolution* **51**(2), 173-181.

Kopczynski, E. D., Bateson, M. M. & Ward, D. M. (1994). Recognition of chimeric small-subunit ribosomal DNAs composed of genes from uncultured microorganisms. *Applied and Environmental Microbiology* **60**(2), 746-748.

Kowalchuk, G. A., Stephen, J. R., De Boer, W., Prosser, J. I., Embley, T. M. & Woldendorp, J. W. (1997). Analysis of ammonia-oxidizing bacteria of the b subdivision of the class *Proteobacteria* in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR amplified 16S ribosomal DNA fragments. *Applied and Environmental Microbiology* **63**(4), 1489-1497.

Kristjánssen, J. K. & Alfredsson, G. A. (1983). Distribution of *Thermus* spp. in Icelandic hot springs and a thermal gradient. *Applied and Environmental Microbiology* **45**(6), 1785-1789.

Kristjánssen, J. K. & Hregguidssen, G. O. (1995). Ecology and habitats of extremophiles. *World Journal of Microbiology and Biotechnology* **11**, 17-25.

Kristjánsson, J. K., Hjörleifsdóttir, S., Marteinsson, V. T. & Alfredsson, G. A. (1994). *Thermus scotoductus*, sp. nov., a pigment-producing thermophilic

bacterium from hot tap water in Iceland and including *Thermus* sp. X-1. *Systematic and Applied Microbiology* **17**, 44-50.

Kristjánsson, J. K., Hreggvidsson, G. O. & Alfredsson, G. A. (1986). Isolation of halotolerant *Thermus* spp. from submarine hot springs in Iceland. *Applied and Environmental Microbiology* **52**, 1313-1316.

Kudo, T., Ohkuma, M., Moriya, S., Noda, S. & Ohtoko, K. (1998). Molecular phylogenetic identification of the intestinal anaerobic microbial community in the hindgut of the termite, *Reticulitermes speratus*, without cultivation. *Extremophiles* **2**(3), 155-161.

Kusunoki, S., Ezaki, T., Tamesada, M., Hatanaka, Y., Asano, K., Hishimoto, Y. & Yabuchi, E. (1991). Application of colorimetric microdilution plate hybridization for rapid genetic identification of 22 *Mycobacterium* species. *Journal of Clinical Microbiology* **29**, 1596-1603.

Kyrpides, N. C. & Olsen, G. J. (1999). Archaeal and bacterial hyperthermophiles: horizontal gene exchange or common ancestry? *Trends in Genetics* **15**(8), 298-299.

Ladenstein, R. & Antranikian, G. (1998). Proteins from hyperthermophiles: stability and enzymatic catalysis close to the boiling point of water. *Advances in Biochemical Engineering and Biotechnology* **61**, 37-85.

Lane, D., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L. & Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 6955-6959.

Langworthy, T. A. & Pond, J. L. (1986). Membranes and lipids of thermophiles. In *Thermophiles: general, molecular and applied microbiology* (Brock, T. D., ed.), pp. 107-135. John Wiley and Sons, New York, USA.

Larson, L., Nielsen, P. & Ahring, B. K. (1997). *Thermoanaerobacter mathranii* sp. nov., an ethanol-producing, extremely thermophilic anaerobic bacterium from a hot spring in Iceland. *Archives in Microbiology* **168**(2), 114-119.

Leff, L. G., Dana, J. R., McArthur, J. V. & Shimkets, L. J. (1995). Comparison of methods of DNA extraction from stream sediments. *Applied and Environmental Microbiology* **61**(3), 1141-1143.

Liesack, W., Weyland, H. & Stackebrandt, E. (1991). Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria. *Microbial Ecology* **21**, 191-198.

Loeffler, J., Henke, N., Hebart, H., Schmidt, D., Hagmeyer, L., Schumacher, U. & Einsele, H. (2000). Quantification of fungal DNA by using fluorescence resonance energy transfer and the light cycler system. *Journal of Clinical Microbiology* **38**(2), 586-590.

Loginova, L. G., Egorova, L. A., Golovacheva, R. S. & Seregina, L. M. (1975). A new species of obligate-thermophilic non-sporeforming bacteria belonging to the genus *Thermus*. *Izvestiia Akademii Nauk SSSR Seriya Biologicheskaya* **2**, 305-307.

Loginova, L. G., Egorova, L. A., Golovacheva, R. S. & Seregina, L. M. (1984). *Thermus ruber* sp. nov., nom. rev. *International Journal of Systematic Bacteriology* **34**(4), 498-499.

Love, C. A., Patel, B. K. C., Nichols, P. D. & Stackebrandt, E. (1993). *Desulfotomaculum australicum*, sp nov, a thermophilic sulfate-reducing bacterium isolated from the Great Artesian Basin of Australia. *Systematic and Applied Microbiology* **16**(2), 244-251.

Love, C. A., Scott, J. C. & Patel, B. K. C. (1992). 16S rRNA based phylogenetic studies of thermophilic bacteria from the Great Artesian Basin of Australia. *Australian Microbiologist* **13**(3), A18.

Ludwig, W., Neumaier, J., Klugbauer, N., Brockman, E., Roller, C., Jilg, S., Reetz, K., Schachtner, I., Ludvigsen, A., Bachleitner, M., Fischer, U. & Schleifer, K. H. (1993). Phylogenetic relationships of *Bacteria* based on comparative sequence analysis of elongation factor Tu and ATP-synthase beta-subunit genes. *Antonie Van Leeuwenhoek* **64**(3-4), 285-305.

Ludwig, W. & Schleifer, K.-H. (1999). Phylogeny of *Bacteria* beyond the 16S rRNA standard. *ASM News* **65**(11), 752-757.

Ludwig, W. & Schleifer, K. H. (1994). Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. *FEMS Microbiology Reviews* **15**(2-3), 155-173.

Lyons, S. R., Griffen, A. L. & Leys, E. J. (2000). Quantitative real-time PCR for *Porphyromonas gingivalis* and total bacteria. *Journal of Clinical Microbiology* **38**(6), 2362-2365.

Macy, J. M., Snellen, J. E. & Hungate, R. E. (1972). Use of syringe methods for anaerobiosis. *American Journal of Clinical Nutrition* **25**, 1318-1323.

Madigan, M. T., Martinko, J. M. & Parker, J. (2000). *Brock Biology of Microorganisms*. 9 edit, Prentice-Hall Inc.

Magot, M., Ollivier, B. & Patel, B. K. (2000). Microbiology of petroleum reservoirs. *Antonie Van Leeuwenhoek* **77**(2), 103-116.

Magot, M., Ravot, G., Campaignolle, X., Ollivier, B., Patel, B. K. C., Fardeau, M. L., Thomas, P., Crolet, J. L. & Garcia, J. L. (1997). *Dethiosulfovibrio peptidovorans* gen. nov. sp. nov., a new anaerobic slightly halophilic, thiosulfate-reducing bacterium from corroding offshore oil wells. *International Journal of Systematic Bacteriology* **47**(3), 818-824.

Maidak, B. L., Cole, J. R., Lilburn, T. G., Parker, C. T. J., Saxman, P. R., Stredwick, J. M., Garrity, G. M., Li, B., Olsen, G. J., Pramanik, S., Schmidt, T. M. & Tiedje, J. M. (2000). The RDP (Ribosomal Database Project) continues. *Nucleic Acids Research* **28**(1), 173-174.

Maidak, B. L., Cole, J. R., Lilburn, T. G., Parker Jr, C. T., Saxman, P. R., Farris, R. J., Garrity, G. M., Olsen, G. J., Schmidt, T. M. & Tiedje, J. M. (2001). The RDP-II (Ribosomal database project). *Nucleic Acids Research* **29**(1), 173-174.

Maidak, B. L., Cole, J. R., Parker, C. T. J., Garrity, G. M., Larsen, N., Li, B., Lilburn, T. G., McCaughey, M. J., Olsen, G. J., Overbeek, R., Pramanik, S.,

Schmidt, T. M., Tiedje, J. M. & Woese, C. R. (1999). A new version of the RDP (Ribosomal Database Project). *Nucleic Acids Research* **27**(1), 171-173.

Manaia, C. M., B., H., Gutierrez, M. C., Gillis, M., Ventosa, A., Kersters, K. & da Costa, M. S. (1994). Halotolerant *Thermus* strains from marine and terrestrial hot springs belong to *Thermus thermophilus* (ex *Oshima* and *Imahori*, 1974) nom. rev. emend. *Systematic and Applied Microbiology* **17**, 526-532.

Manaia, C. M. & da Costa, M. S. (1991). Characterization of halotolerant *Thermus* isolates from shallow marine hot springs on S. Miguel, Azores. *Journal of General Microbiology* **137**, 2643-2648.

Margoliash, E. & Smith, E. L. (1965). Structural and functional aspects of *cytochrome c* in relation to evolution. In *Evolving Genes and Proteins* (Bryson, V. & Vogel, H. J., eds.), pp. 221-242. Academic Press, New York, USA.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from bacteria. *Journal of Molecular Biology* **3**, 208-218.

Marteinsson, V. T., Birrien, J. L., Kristjánsson, J. K. & Prieur, D. (1995). First isolation of thermophilic aerobic non-sporolating heterotrophic bacteria from deep-sea hydrothermal vent. *FEMS Microbiology Ecology* **18**, 163-174.

Marteinsson, V. T., Birrien, J. L. & Prieur, D. (1997). *In situ* enrichment and isolation of thermophilic microorganisms from deep-sea vent environments. *Canadian Journal of Microbiology* **43**(7), 694-697.

Marteinsson, V. T., Birrien, J. L., Raguénès, G., da Costa, M. S. & Prieur, D. (1999). Isolation and characterization of *Thermus thermophilus* Gy1211 from a deep-sea hydrothermal vent. *Extremophiles* **3**(4), 247-51.

Martin, W. (1999). Mosiac bacterial chromosomes: a challenge *en route* to a tree of genomes. *BioEssays* **21**, 99-104.

Maruyama, A., Honda, D., Yamamoto, H., Kitamura, K. & Higashihara, T. (2000). Phylogenetic analysis of psychrophilic bacteria isolated from the

Japan Trench, including a description of the deep-sea species *Psychrobacter pacificensis* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* **50**, 835-846.

Mazor, E. (1995). Stagnant, aquifer concept. 1. Large scale artesian systems - Great Artesian Basin, Australia. *Journal of Hydrology* **173**(1-4), 219-240.

McCaig, A. E., Embley, T. M. & Prosser, J. I. (1994). Molecular analysis of enrichment cultures of marine ammonia oxidisers. *FEMS Microbiology Letters* **120**, 363-368.

Miley, W. J., Suryanarayana, K., Manns, A., Kubota, R., Jacobson, S., Lifson, J. D. & Waters, D. (2000). Real-time polymerase chain reaction assay for cell-associated HTLV type I DNA viral load. *AIDS and Research in Human Retroviruses* **16**(7), 665-675.

Miller, D. N., Bryant, J. E., Madsen, E. L. & Ghiorse, W. C. (1999). Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. *Applied and Environmental Microbiology* **65**(11), 4715-4724.

Miller, S. R., Wingard, C. E. & Castenholz, R. W. (1998). Effects of visible light and UV radiation on photosynthesis in a population of a hot spring cyanobacterium, a *Synechococcus* sp., subjected to high-temperature stress. *Applied and Environmental Microbiology* **64**(10), 3893-3899.

Mobarry, B. K., Mobarry, B. K., Wagner, M., Urbain, V., Rittmann, B. E. & Stahl, D. A. (1996). Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Applied and Environmental Microbiology* **62**(6), 2156-2162.

Moffett, B. F., Walsh, K. A., Harris, J. A. & Hill, T. C. J. (2000). Analysis of bacterial community structure using 16S rDNA analysis. *Anaerobe* **6**, 129-131.

Moreira, L. M., da Costa, M. S. & Sá-Correia, I. (1995). Plasmid RFLP profiling and DNA homology in *Thermus* isolated from hot springs of different geographical areas. *Archives of Microbiology* **164**, 7-15.

Moreira, L. M., da Costa, M. S. & Sá-Correia, I. (1997). Comparative genomic analysis of isolates belonging to the six species of the genus *Thermus* using pulsed-field gel electrophoresis and ribotyping. *Archives of Microbiology* **168**(2), 92-101.

Moyer, C. L., Dobbs, F. C. & Karl, D. M. (1995). Phylogentic diversity of the bacterial community from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. *Applied and Environmental Microbiology* **61**(4), 1555-1562.

Mudd, G. M. (2000). Mound springs of the Great Artesian Basin in South Australia: a case study from Olympic Dam. *Environmental Geology* **39**(5), 463-475.

Munster, M. J., Munster, A. P., Woodrow, J. R. & Sharp, R. J. (1986). Isolation and preliminary taxonomic studies of *Thermus* strains isolated from Yellowstone National Park, USA. *Journal of General Microbiology* **132**, 1677-1683.

Murzina, N. V., Vorozheykina, D. P. & Marvienko, N. I. (1988). Nucleotide sequence of *Thermus thermophilus* HB8 gene coding 16S rRNA. *Nucleic Acids Research* **16**, 8172.

Muyzer, G. (1999). DGGE/TGGE a method for identifying genes from natural ecosystems. *Current Opinions in Microbiology* **2**(3), 317-322.

Muyzer, G., de Waal, E. C. & Uitterlinden, A. G. (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**(3), 695-700.

Muyzer, G. & Ramsing, N. B. (1995). Molecular methods to study the organization of microbial communities. *Water Science and Technology* **32**(8), 1-9.

Nilsen, R. K., Beeder, J., Thorstenson, T. & Torsvik, T. (1996). Distribution of thermophilic marine sulfate reducers in North Sea oil field waters and oil reservoirs. *Applied and Environmental Microbiology* **62**(5), 1793-1798.

Nogva, H. K., Rudi, K., Naterstad, K., Holck, A. & Lillehaug, D. (2000). Application of 5'-nuclease PCR for quantitative detection of *Listeria monocytogenes* in pure cultures, water, skim milk, and unpasteurized whole milk. *Applied and Environmental Microbiology* **66**(10), 4266-4271.

Nold, S. C. & Ward, D. M. (1995). Diverse *Thermus* species inhabit a single hot spring microbial mat. *Systematic and Applied Microbiology* **18**, 274-278.

Nübel, U., Garcia-Pichel, F., Kühl, M. & Muyzer, G. (1999). Quantifying microbial diversity: morphotypes, 16S rRNA genes, and carotenoids of oxygenic phototrophs in microbial mats. *Applied and Environmental Microbiology* **65**(2), 422-430.

O'Donnell, A. G. & Gorres, H. E. (1999). 16S rDNA methods in soil microbiology. *Current Opinions in Biotechnology* **10**(3), 225-229.

Oren, A., Ventosa, A., Carmen Gutiérrez, M. & Kamekura, M. (1999). *Haloarcula quadrata* sp nov., a square, motile arcgaeon isolated from a brine pool in Sinai (Egypt). *International Journal of Systematic Bacteriology* **49**, 1149-1155.

Oshima, T. & Imahori, K. (1971). Isolation of an extreme thermophile and thermostability of of its transfer ribonucleic acid and ribosomes. *Journal of General and Applied Microbiology* **17**, 513-517.

Pace, N. R. (1997). A molecular view of microbial diversity and the biosphere. *Science* **276**, 734-740.

Patel, B. K. C., Monk, C., Littleworth, H., Morgan, H. W. & Daniel, R. M. (1987). *Clostridium fervidus* sp. nov., a new chemoorganotrophic acetogenic thermophile. *International Journal of Systematic Bacteriology* **37**(2), 123-126.

Patel, B. K. C., Morgan, H. W. & Daniel, R. M. (1985). *Fervidobacterium nodosum* gen. nov., a new chemoorganotrophic caldoactive anaerobic bacterium. *Archives of Microbiology* **14**, 63-69.

Pirnay, J. P., De Vos, D., Duinslaeger, L., Reper, P., Vandenvelde, C., Cornelis, P. & Vanderkelen, A. (2000). Quantitation of *Pseudomonas aeruginosa* in wound biopsy samples: from bacterial culture to rapid 'real-time' polymerase chain reaction. *Critical Care* **4**(4), 255-261.

Plugge, C. M., Zoetendal, E. G. & Stams, A. J. (2000). *Caloramator coolhaasii* sp. nov., a glutamate-degrading, moderately thermophilic anaerobe. *International Journal of Systematic and Evolutionary Microbiology* **50**(3), 1155-62.

Polz, M. F. & Cavanaugh, C. (1998). Bias in template-to-product in multitemplate PCR. *Applied and Environmental Microbiology* **64**(10), 3724-3730.

Porteous, L. A., Armstrong, J. L., Seidler, R. J. & Watrud, L. S. (1994). An effective method to extract DNA from environmental samples for polymerase chain reaction amplification and DNA fingerprint analysis. *Current Microbiology* **29**(5), 301-307.

Prieur, D. (1997). Microbiology of deep-sea hydrothermal vents. *Techniques in Biotechnology* **15**, 242-244.

Redburn, A. C. & Patel, B. K. (1994). *Desulfovibrio longreachii* sp. nov., a sulfate-reducing bacterium isolated from the Great Artesian Basin of Australia. *FEMS Microbiology Letters* **115**(1), 33-38.

Redburn, A. C. & Patel, B. K. C. (1993). Phylogenetic analysis of *Desulfotomaculum thermobenzoicum* using polymerase chain reaction-amplified 16S rRNA-specific DNA. *FEMS Microbiology Letters* **113**, 81-86.

Reizer, J., Grossowicz, N. & Barenholz, Y. (1985). The effect of growth temperature on the thermotropic behaviour of the membranes of a thermophilic *Bacillus*. Composition-structure-function relationships. *Biochimica et Biophysica Acta* **815**(2), 268-280.

Reysenbach, A. L., Ehringer, M. & Herhberger, K. (2000). Microbial diversity at 83ºC in Calcite Springs, Yellowstone National Park: another environment where the *Aquificales* and "Korarchaeota" coexist. *Extremophiles* **4**, 61-67.

Reysenbach, A. L., Wickham, G. S. & Pace, N. R. (1994). Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone National Park. *Applied and Environmental Microbiology* **60**(6), 2113-2119.

Ririe, K. M., Rasmussen, R. P. & Wittwer, C. T. (1997). Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Analytical Biochemistry* **245**, 154-160.

Risatti, J. B., Capman, W. C. & Stahl, D. A. (1994). Community structure of a microbial mat: the phylogenetic dimension. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 10179-10177.

Robison-Cox, J. F., Bateson, M. M. & Ward, D. M. (1995). Evaluation of nearest-neighbour methods for detection of chimeric small-subunit rRNA sequences. *Applied and Environmental Microbiology* **61**(4), 1240-1245.

Rogers, M. J., Simmons, J., Walker, R. T., Weisburg, W. G., Woese, C. R., Tanner, R. S., Robinson, I. M., Stahl, D. A., Olsen, G., Leach, R. H. & Maniloff, J. (1985). Construction of the *Mycoplasma* evolutionary tree from 5S rRNA sequence data. *Proceedings of the National Academy of Sciences of the United States of America* **82**(4), 1160-1164.

Roller, C., Wagner, M., Amann, R., Ludwig, W. & Schleifer, K.-H. (1994). *In situ* probing of Gram-positive bacteria with high DNA G+C content using 23S rRNA-targeted oligonucleotides. *Microbiology* **140**, 2849-2858.

Ruff-Roberts, A. L., Kuenen, J. G. & Ward, D. M. (1994). Distribution of cultivated and uncultivated *Cyanobacteria* and *Chloroflexus*-like bacteria in hot spring microbial mats. *Applied and Environmental Microbiology* **60**(2), 697-704.

Saiki, T., Kimura, R. & Arimua, K. (1972). Isolation and characterization of extremely thermophilic bacteria from hot springs. *Agricultural and Biological Chemistry* **36**, 2357-2366.

Saitou, N. & Imanishi, T. (1989). Relative efficiencies of the Fitch-Margoliash, maximum parsimony, maximum likelihood, minimum evolution, and neighbor-joining methods of phylogenetic tree construction in obtain the correct tree. *Molecular and Biological Evolution* **6**, 514-525.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular and Biological Evolution* **4**, 406-425.

Sako, Y., Takai, K., Ishida, Y., Uchida, A. & Katayama, Y. (1996). *Rhodothermus obamensis* sp. nov., a modern lineage of extremely thermophilic marine bacteria. *International Journal of Systematic Bacteriology* **46**(4), 1099-1104.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular cloning: a laboratory manual*. 2nd edit, Cold Spring Harbour Laboratory Press, New York.

Santegoeds, C. M., Nold, S. C. & Ward, D. M. (1996). Denaturing gradient gel electrophoresis used to monitor the enrichment culture of aerobic chemoorganotrophic bacteria from a hot spring microbial mat. *Applied and Environmental Microbiology* **62**(11), 3922-3928.

Santos, M. A., Williams, R. A. D. & da Costa, M. S. (1989). Numerical taxonomy of *Thermus* isolates from hot springs in Portugal. *Systematic and Applied Microbiology* **12**, 310-315.

Saul, D. J., Reeves, R. A., Morgan, H. W. & Bergquist, P. L. (1999). *Thermus* diversity and strain loss during enrichment. *FEMS Microbiology Ecology* **30**(2), 157-162.

Saul, D. J., Rodrigo, A. G., Reeves, R. A., Williams, L. C., Borges, K. M., Morgan, H. W. & Bergquist, P. L. (1993). Phylogeny of twenty *Thermus*

isolates constructed from 16S rRNA gene sequence data. *International Journal of Systematic Bacteriology* **43**(4), 754-760.

Schade, M. & Lemmer, H. (1994). Counting bacteria of selected metabolic groups in activated sludge - an assessment of methods. *Water Science & Technology* **29**(7), 75-79.

Schadt, E. E., Sinsheimer, J. S. & Lange, K. (1998). Computational advances in maximum likelihood methods for molecular phylogeny. *Genome Research* **8**(3), 222-233.

Schäfer, G., Engelhard, M. & Müller, V. (1999). Bioenergetics of the *Archaea*. *Microbiology and Molecular Biology Reviews* **63**(3), 570-620.

Schleper, C., Holben, W. & Klenk, H. P. (1997). Recovery of crenarchaeotal ribosomal DNA sequences from freshwater-lake sediments. *Applied and Environmental Microbiology* **63**(1), 321-323.

Schmidt, T. M., DeLong, E. F. & Pace, N. R. (1991). Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. *Journal of Bacteriology* **173**(14), 4371-4378.

Scholz, S., Sonnenbichler, J., Schafer, W. & Hensel, R. (1992). Di-myo-inositol-1,1'-phosphate: a new inositol phosphate isolated from *Pyrococcus woesei*. *FEBS Letters* **306**, 239-242.

Sharp, R. J. & Williams, R. A. D. (1988). Properties of Thermus ruber strains isolated from Icelandic hot springs and DNA:DNA homology of Thermus ruber and Thermus aquaticus. Applied and Environmental Microbiology **54**(8), 2049-2053.

Shida, O., Takagi, H., Kadowaki, K., Nakamura, L.K. & Komagata, K. (1997). Transfer of *Bacillus alginolyticus*, *Bacillus chondroitinus*, *Bacillus curdlanolyticus*, *Bacillus glucanolyticus*, *Bacillus kobensis*, and *Bacillus thiaminolyticus* to the genus *Paenibacillus* and emended description of the genus *Paenibacillus*. *International Journal , of Systematic Bacteriology* **47**, 289-298.

Shoichet, B. K., Baase, W. A., Kuroki, R. & Matthewss, B. (1995). A relationship between protein stability and protein function. Proceedings of the National Academy of Sciences of the United States of America **92**, 452-456.

Sjoback, R., Nygren, J., and Kubista, M. (1995) Adsorption and fluorescence properties of fluorescein. *Spectrochimica Acta A* **51**, 7

Skirnisdottir, S., Hreggvidsson, G. O., Hjorleifsdottir, S., Marteinsson, V. T., Petursdottir, S. P., Holst, O. & Kristjansson, J. K. The influence of sulfide and temperature on the species composition and community structure of hot spring microbial mats. *Unpublished*.

Sneath, P. H. A. & Sokal, R. R. (1973). *Numerical taxonomy: the principles and practice of numerical classification*, W.H. Freeman, San Francisco, USA.

Snel, B., Bork, P. & Huynen, M. A. (1999). Genome phylogeny based on gene content. *Nature and Genetics* **21**(1), 108-110.

Song, L., Hennick, E.J., Young, I.T., and Tanke, H.J. (1995). Photobleaching kinetics of fluorescein in quantitative fluorescence microscopy. *Biophysics Journal* **68**, 2588-2600.

Stackebrandt, E. (1988). Phylogenetic relationships vs. phenotypic diversity: how to achieve a phylogenetic classification system of the eubacteria. *Canadian Journal of Microbiology* **34**(4), 552-556.

Stahl, D. A., Lane, D. J., Olsen, G. J. & Pace, N. R. (1985). Characterization of a Yellowstone hot spring microbial community by 5S rRNA sequences. *Applied and Environmental Microbiology* **49**(6), 1379-1384.

Staley, J. T. & Konopka, A. (1985). Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annual Reviews in Microbiology* **39**, 321-346.

Suzuki, M. T. & Giovannoni, S. J. (1996). Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Applied and Environmental Microbiology* **62**(2), 625-630.

Takai, K., Inoue, A. & Horikoshi, K. (1999). *Thermaerobacter marianensis* gen. nov., sp. nov., an aerobic extremely thermophilic marine bacterium from the 11000 m deep Mariana Trench. *International Journal of Systematic Bacteriology* **49**, 619-628.

Takami, H., Inoue, A., Fuji, F. & Horikoshi, K. (1997). Microbial flora in the deepest sea mud of the Mariana trench. *FEMS Microbiology Letters* **152**, 279-285.

Takeuchi, T., Katsume, A., Tanaka, T., Abe, A., Inoue, K., Tsukiyama-Kohara, K., Kawaguchi, R., Tanaka, S. & Kohara, M. (1999). Real-time detection system for quantification of hepatitis C virus genome. *Gastroenterology* **116**(3), 636-642.

Takai, K. & Horikoshi, K. (2000). Thermosipho japonicus sp. nov., an extremely thermophilic bacterium isolated from a deep-sea hydrothermal vent in Japan. *Extremophiles* **4**(1), 9-17.

Talavera, E.M., Afkir, M., Salto, R., Vargas, A.M., and Alvarez-Pez, J.M. (2000). Fluorescence-labelled DNA probes to detect complementary sequences in homogeneous media*. Journal of Photochemistry and Photobiology*. **59**, 9-14.

Tarlera, S., Muxí, L., Soubes, M. & Stams, A. J. M. (1997). *Caloramator proteoclasticus* sp. nov., a new moderately thermophilic anaerobic proteolytic bacterium. *International Journal of Systematic Bacteriology* **47**(3), 651-656.

Taylor, C. D., Wirsen, C. O. & Gaill, F. (1999). Rapid microbial production of filamentous sulfur mats at hydrothermal vents. *Applied and Environmental Microbiology* **65**(5), 2253-2255.

Tenreiro, S., Nobre, M. F. & da Costa, M. S. (1995). *Thermus silvanus* sp. nov. and *Thermus chliarophilus* sp. nov., two new species related to *Thermus ruber* but with lower growth temperatures. *International Journal of Systematic Bacteriology* **45**(4), 633-639.

Tenreiro, S., Nobre, M. F., Rainey, F. A., Miguel, C. & da Costa, M. S. (1997). *Thermonema rossianum* sp nov, a new thermophilic and slightly halophilic species from saline hot springs in Naples, Italy. *International Journal of Systematic Bacteriology* **47**(1), 122-126.

Thorne, J. L., Kishino, H. & Felsenstein, J. (1992). Inching toward reality: an improved likelihood model of sequence evolution. *Journal of Molecular Evolution* **34**, 3-16.

Torsvik, V., Goksoyr, J. & Daae, F. L. (1990). High diversity of soil bacteria. *Applied and Environmental Microbiology* **56**, 782-787.

Tsai, Y.-L. & Olson, B. H. (1992). Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. *Applied and Environmental Microbiology* **58**(7), 2292-2295.

Van de Peer, Y. & De Wachter, R. (1994). TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Computing Applications in Bioscience* **10**, 569-570.

Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K. & Swings, J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiology Reviews* **60**(2), 407-438.

Vieille, C., Burdette, D. S. & Zeikus, J. G. (1996). Thermozymes. *Biotechnology Annual Reviews* **2**, 1-83.

Viikari, L., Kantelinen, A., Sundquist, J., Linko, M. (1994). Xylanases in bleaching: From an idea to the industry. *FEMS Microbiology Reviews* **13**, 335-350.

Voordouw, G., Armstrong, S. M., Reimer, M. F., Fouts, B., Telang, A. J., Shen, Y. & Gevertz, D. (1996). Characterization of 16S rRNA genes from oil field microbial communities indicates the presence of a variety of sulfate-reducing, fermentative and sulfide-oxidizing bacteria. *Applied and Environmental Microbiology* **62**(5), 1623-1629.

Wagner, M., Amann, R., Lemmer, H. & Schleifer, K. H. (1993). Probing activated sludge with *Proteobacteria*-specific oligonucleotides: inadequecy of culture-dependent methods for describing microbial community structures. *Applied and Environmental Microbiology* **59**, 1520-1525.

Wagner, M., Aßmus, B., Hartmann, A., Hutzler, P. & Amman, R. (1994a). *In situ* analysis of microbial consortia in activated sludge using fluorescently labelled, rRNA-targeted oligonucleotide probes and confocal scanning laser microscopy. *Journal of Microscopy* **176**(3), 181-187.

Wagner, M., Erhart, R., Manz, W., Amann, R., Lemmer, H., Wedi, D. & Schleifer, K. H. (1994b). Development of an rRNA-targeted oligonucleotid probe specific for the genus *Acinetobacter* and its application for in situ monitoring in activated sludge. *Applied and Environmental Microbiology* **60**, 792-800.

Wagner, M., Roger, A. J., Flax, J. I., Brusseau, G. Y. & Stahl, D. A. (1998). Phylogeny of dissimilatory sulfite reducates supports an early origin of sulphate respiration. *Journal of Bacteriology* **180**(11), 2975-2982.

Wang, G., C.-Y. & Wang, Y. (1997). Frequency of formation of chimeric molecules as a consequence of PCR coamplification of 16S rRNA genes from mixed bacterial genomes. *Applied & Environmental Microbiology* **63**(12), 4645-4650.

Ward, D. M., Ferris, M. J., Nold, S. C. & Bateson, M. M. (1998). A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. *Microbiology and Molecular Biology Reviews* **62**(4), 1353-1370.

Ward, D. M., Santegoeds, C. M., Nold, S. C., Ramsing, N. B., Ferris, M. J. & Bateson, M. M. (1997). Biodiversity within hot spring microbial mat communities: molecular monitoring of enrichment cultures. *Antonie Van Leeuwenhoek* **71**(1-2), 143-150.

Ward, D. M., Weller, R. & Bateson, M. M. (1990). 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* **345**(3), 63-64.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**(2), 697-703.

Winker, S. & Woese, C. R. (1991). A definition of the Domains *Archaea*, *Bacteria*, and *Eucarya* in terms of small subunit ribosomal RNA characteristics. *Systematic and Applied Microbiology* **14**, 305-310.

Wintzingerode, F. V., Göbel, U. B. & Stackebrandt, E. (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiology Reviews* **21**, 213-229.

Wise, M. G., McArthur, J. V. & Shimkets, L. J. (1997). Bacterial diversity of a Carolina bay as determined by 16S rRNA gene analysis: confirmation of novel taxa. *Applied and Environmental Microbiology* **63**(4), 1505-1514.

Wittwer, C. T., Ririew, K. M., Andrew, R. V., David, D. A., Gundry, R. A. & Balis, U. J. (1997). The LightCycler(TM). A microvolume multisample fluorimeter with rapid temperature control. *BioTechniques* **22**(1), 176-181.

Woese, C. R. (1987). Bacterial evolution. *Microbiological Reviews* **51**, 221-271.

Woese, C. R., Kandler, O. & Wheelis, M. L. (1990). Towards a natural system of organisms: proposal for the domains *Archaea*, *Bacteria*, and *Eukarya*. *Proceedings of the National Academy of Sciences, U.S.A* **87**, 4576.

Wolin, E. A., Wolin, M. J. & Wolfe, R. S. (1963). Formation of methane from bacterial extracts. *The Journal of Biological Chemistry* **238**, 2882-2886.

Woo, T. H. S., Patel, B. K. C., Cinco, M., Smythe, L. D., Symonds, M. L., Norris, M. A. & Dohnt, M. F. (1998). Real-time homogeneous assay of rapid cycle polymerase chain reaction product for identification of *Leptonema illini*. *Analytical Biochemistry* **259**, 112-117.

Wynter, C., Patel, B. K., Bain, P., de Jersey, J., Hamilton, S. & Inkerman, P. A. (1996). A novel thermostable dextranase from a *Thermoanaerobacter*

species cultured from the geothermal waters of the Great Artesian Basin of Australia. *FEMS Microbiology Letters* **140**(2-3), 271-276.

Yamamoto, H., Hiraishi, A., Kato, K., Chiura, H. X., Maki, Y. & 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**(5), 1680-1687.

Zarda, B., Hahn, D., Chatzinotas, A., Schönhuber, W., Neef, A., Amann, R. I. & Zeyer, J. (1997). Analysis of bacterial community structure in bulk soil by *in situ* hybridization. *Archives of Microbiology* **168**, 185-192.

Zeikus, J. G., Hegge, P. W. & Anderson, M. A. (1979). *Thermoanaerobium brockii* gen. nov. and spec. nov., a new chemoorganotrophic, caldoactive, anaerobic bacterium. *Archives of Microbiology* **122**, 41-48.

Zhou, M. Y., Clark, S. E. & Gomez-Sanchez, C. E. (1995). Universal cloning method by TA strategy. *BioTechniques* **19**(1), 34-35.