Biodiscovery and Biodiversity of Antarctic Bacteria

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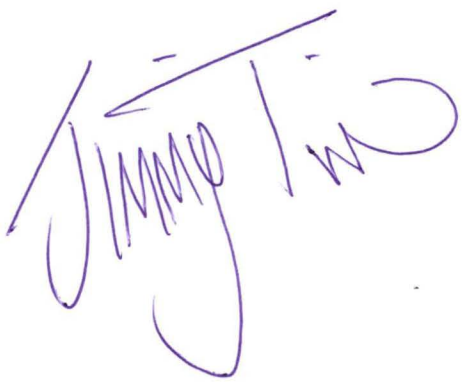
Declaration

This thesis contains no material that has been accepted for the award of any other degree or diploma in any tertiary institution. To the best of my knowledge, this thesis does not contain material written or published by another person, except where due reference is made



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Abbreviations

The following abbreviations and acronyms have been used in this thesis:

16S rRNA gene	16 subunit ribosomal ribonucleic acid
ACE CRC	Antarctic Climate and Ecosystem Cooperative Research Centre
alk	Alkane monooxygenase gene
BH	Bushnell Haas
Cat. #	Catalogue number
CFB	*Cytophaga-Flavobacterium-Bacteriodes* phylum
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DSMZ	Deutsche sammlung von mikroorganismen und zellkulturen (German collection of microorganisms and cell cultures)
G+C	Guanine plus Cytosine
GYM	Glucose yeast malt media
JCM	Japan collection of microorganisms
Kyr	Thousand year(s)
MA	Marine Agar
MDS	Multi-dimensional scaling
MK	Menaquinone
No.	Number
ndo	Naphthalene dioxygenase gene
OA	Oatmeal Agar
PCR	Polymerase Chain Reaction
PKS-II	Polyketide synthase type II
Pty ltd	Proprietary limited
RT	Room temperature
SAB	Special Antarctic blend diesel
SEM	Scanning electron microscope
Spp.	Species
t-RFLP	Terminal restriction fragment length polymorphism
TSA	Tryptic soy agar
TSA-ye	Tryptic soy agar supplemented with yeast extract
TSB	Tryptic soy broth
UTAA	University of Tasmania Antarctic Actinobacteria (collection)

Abstract

This PhD dissertation explores aspects of the microbial ecology of Antarctica, focussing on the taxonomy and physiological attributes of Antarctic bacteria, as well as investigating the bacterial diversity of previously uncharacterised Antarctic environments. The University of Tasmania has in its possession a collection of 1600 *Actinobacteria* strains previously isolated from Antarctic and sub-Antarctic soils. A polyphasic study involving both phylogenetic and phenotypic comparisons was carried out to identify any potentially novel bacterial strains. Five isolates of interest were identified, belonging to the genera *Arthrobacter*, *Promicromonospora*, and *Rhodococcus*. Further studies revealed one *Rhodococcus* strain exhibited the ability to utilise an unusually wide range of hydrocarbons including both straight chain alkanes and polyaromatic hydrocarbons. During this study it was noted that members of the genus *Streptomyces* dominated this archive of *Actinobacteria*, and subsequent antimicrobial screening showed a range of potentially novel anti-microbial compounds effective against the food pathogen *Listeria monocytogenes*. In addition to the characterisation of soil *Actinobacteria*, a preliminary look at the bacterial composition of several unique Antarctic environments was undertaken to explore ecological niches that may also play host to novel strains or interesting physiological abilities. Eight epiglacial lakes from the Framnes Mountains were compared using t-RFLP bacterial community profiling. These perennially frozen freshwater lakes possess alkaline waters up to pH 11 and have a similar chemical composition. The community structure differed from one lake to another, but each was dominated by Cyanobacterial with either Bacteriodetes group or Proteobacteria

species and eukaryotic algae. To continue the characterisation of unique Antarctic sites, ice was chosen for study as it covers almost the entire continent and its surrounding coastline, with ancient glacial ice providing a snapshot into the past. Initially, marine ice accreted beneath the Amery Ice Shelf was examined for its microbial constituents. The bacteria trapped along this core were found to be heterogeneous, with some of the segments of ice possessing cultureable bacteria, revealing either the cryoprotective nature of some Antarctic bacteria found in low levels throughout the marine ice or that the ice contains liquid brine pockets which act as microhabitats. Following on from this work, ice from the Law Dome glacier dating back 115, 755, and 2467 years was investigated. The bacterial DNA recovered from the ice identifies bacterial members that may be useful in studies in evolution and adaptation. The results from this dissertation highlights the fact there is much still to be learnt about the microbial ecology of Antarctica.

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This thesis is dedicated to my late grandmother Hilja Kunttu (1915-2005), who passed away 13 days prior to her 90th birthday. Stories of her endurance and triumph through hardship give me constant motivation through my own life.

"Do, or do not. There is no try."

Yoda (The Empire Strikes Back)

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Bittencourt, C., Twin, J., Ross, T., Bowman, J. (200x). *Anti-microbial activity of Antarctic Actinobacteria against various strains of Listeria monocytogenes.* Paper in preparation.

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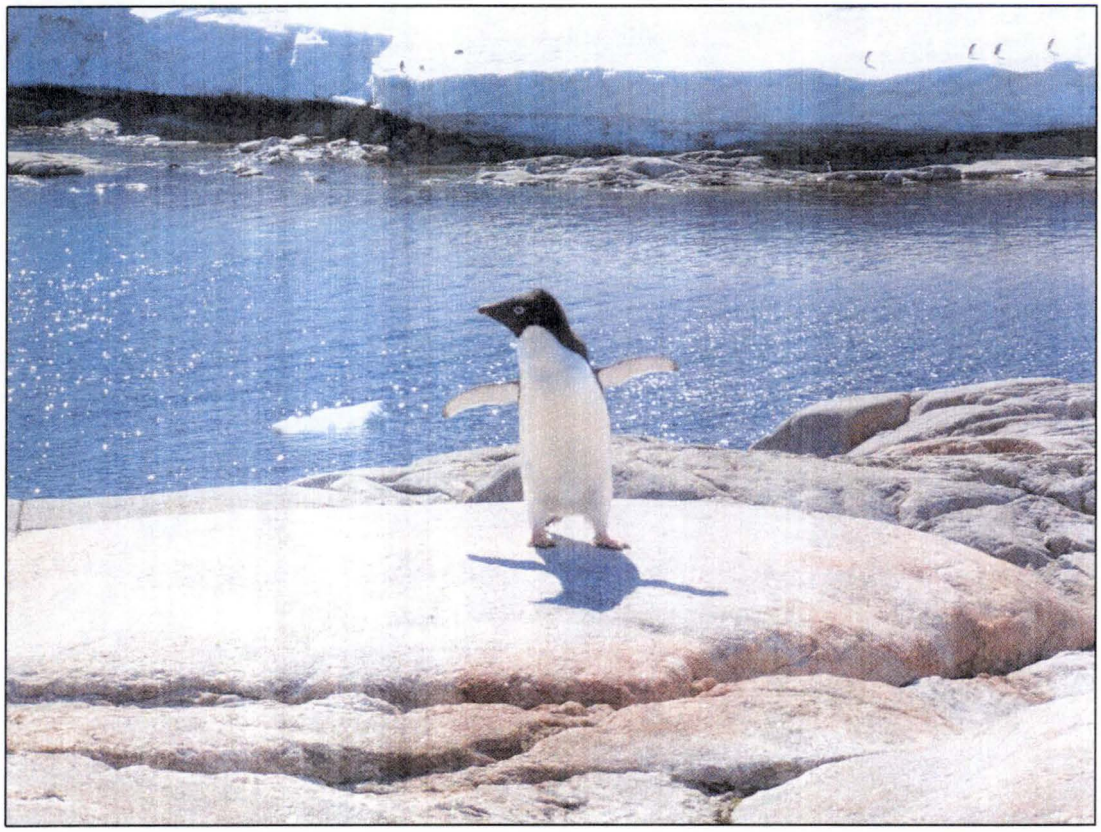
Chapter 1 Overview



Adelie Penguin

Shirley Island, Antarctica

Photo taken by Mr Jimmy Twin

1.1 Introduction

The first noted instance of a bacterium was in 1674 by Anton van Leeuwenhoek, regarded by many as the "father of microbiology". These single celled organisms were dubbed animalcules and were visualised on a single-lens microscope that he created (van Leeuwenhoek, 1753a, van LeeuwenHoek, 1753b). In recent years our understanding of the microbial diversity of the entire planet has improved tremendously. This has been in response to the implementation of new technologies that are becoming increasingly powerful, accurate, and affordable. An awareness of the important contribution bacteria make to the planet is becoming more apparent as further data becomes available. Still today however, much of the Earth's systems are not fully understood and many environments exist that have not been explored. Antarctica is no exception to this, and has emerged from being regarded as one of the most hostile environments on Earth that is devoid of life, to a continent that is host to a plethora of varied environments and ecosystems.

Antarctica is the southernmost landmass on the planet, encompassing the South Pole. It is the coldest, driest, windiest, and highest elevation (on average) of all the continents on Earth. Apart from penguins, seals, migrating birds and simple vegetation, no higher order life are present. As such microbial life is the dominating force in these harsh conditions and a great deal of research has been carried out in the field of Antarctic microbiology. The culmination of this research effort has resulted in a greater understanding about life on the planet Earth, and lead to new biotechnological discoveries. To demonstrate the impact Antarctic microbiology has played on the taxonomy of bacteria, during the past five years alone (February 2003-

February 2008), 19 novel bacterial genera and 58 novel bacterial species isolated from Antarctic and sub-Antarctic environments were described in the International Journal of Systematic and Evolutionary Microbiology (http://ijs.sgmjournals.org/).

1.2 Antarctic Microbiology

1.2.1 Antarctic soil and rocks

The soil in Antarctica is typically dry unless fed by glacial water, often highly alkaline, and is continually exposed to some of the highest UV radiation on the planet. Desert soils are low in carbon and nitrogen and those influenced by vegetation (mosses, lichens, liverworts, lichens, and algae) or higher order animals (penguins, migrating flight birds, and seals) are relatively organic rich in an otherwise nutrient deficient environment. For much of the year the soil is subject to cold or freezing conditions and in summer can be subjected to a daily freeze-thaw (Aislabie et al., 2004, Cowan & Tow, 2004). The microorganisms that inhabit this type of environment are often highly robust and take advantage of the summer period for growth (Delille & Perret, 1989, Straka & Stokes, 1960). Bacteria of the phyla *Actinobacteri*a are routinely detected, in addition to *Acidobacteria*, *Bacteriodetes*, *Cyanobacteria*, *Firmicutes*, *Proteobacteria*, and *Thermus-Deinococci* (Aislabie et al., 2006, Saul et al., 2005, Smith et al., 2006). *Actinobacteri*a from Antarctic soils in particular have received much interest for their ability to produce enzymes able to degrade compounds such as keratin and hydrocarbons (Bej et al., 2000, Gushterova et al., 2005), and the production of novel antimicrobial compounds, such as the

antibiotic and anti-tumour compound frigocyclinone from a *Streptomyces griseus* strain (Bruntner et al., 2005).

1.2.2 Antarctic lakes

One of the most diverse ecosystem types in Antarctica, the lakes can vary greatly, from being oligohaline to hypersaline, oligotrophic to organic rich, and many possess either a seasonal or perennial ice cover, and are often meromictic (Cowan & Tow, 2004). As expected, the biological communities vary as well according to unique ecological niches that may be present, but endemic organisms such as members of *Cyanobacteria* are highly prevalent, particularly in microbial mats (Taton et al., 2006). The Antarctic lakes studied have proved to be a rich source of cold active compounds such as chitinase genes (Gilbert et al., 2004), antifreeze proteins (Xiao et al., 2005), and polyunsaturated fatty acids (Nichols et al., 1993). Antarctic lakes have also been identified as being ideal candidates for studying evolutionary adaptation to the many stresses the biota of these lakes have to endure (Laybourn-Parry & Pearce, 2007).

Despite the progress in Antarctic lake limnology, there are 192 documented subglacial lakes under the Antarctic continental ice sheet (Popov & Masolov, 2007, Siegert et al., 2005) that remain unsampled to date. The largest of these lakes, Lake Vostok, has gathered attention recently as a potential analogue for life on other planetary bodies within our solar system such as Europa (Marion et al., 2003). Drilling has commenced to sample this lake (3623 metres)(Petit et al., 1999), and Vostok accretion ice was found to contain bacteria related to *Brachybacteria*, *Methylobacterium*, *Paenibacillus*, and *Sphingomonas* (Christner et al., 2001). Lakes

that form on rock/ice interfaces, known as epiglacial lakes, also are grossly understudied and to date no studies have been published on the microbiology of this lake type. This is surprising as this lake type has been documented since the 1950s and may be the most common lake type in Antarctica (Gibson, 2005, Pickard, 1982).

1.2.3 Continental Ice

Approximately 98% of Antarctica is covered in continental ice, the majority of lakes are either perennially or seasonally covered in ice, and the Antarctic continent is surrounded by seasonal sea ice (CIA, 2008). Although not typically regarded as a true habitat, ice is certainly a capture event, freezing bacterial cells and their nucleic acid in a snapshot of time, and in the case of Antarctica, this can date back approximately 8 million years as in the case of ice in the Dry Valleys of the Transantarctic Mountains. Evidence of *Alphaproteobacteria*, *Acidobacteria*, *Betaproteobacteria*, *Firmicutes*, and members of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) division were found in the most ancient ice from Antarctica (Bidle et al., 2007), and studies into ancient ice worldwide implicate *Proteobacteria*, spore-forming *Bacilli* and *Clostridia*, and the *Actinobacteria* genus *Arthrobacter* as the most robust for the detection of their nucleic acid in ice (Willerslev et al., 2004a).

1.2.4 Antarctic marine environments

The coastal waters off Antarctica and its associated sea-ice also have proven to be of great biotechnological interest. Novel cold-active enzymes are abundant in both the sea water and underlying sediment (Kawalec et al., 1997, Lonhienne et al., 2001b, Lonhienne et al., 2001c, Turkiewicz et al., 1999), as well as bioemulsifiers (Yakimov

et al., 1999) and a variety of secondary metabolites (Ivanova et al., 2001). Sea ice harbours brine channels and pockets that harbour unique microhabitats found to be a source of compounds of interest such as novel antifreeze proteins (Raymond et al., 2007).

1.3 Aims of this project

The aim of this project is quite straightforward, that is to expand the knowledge of Antarctic microbiology. Firstly, it is imperative for all work conducted in the field of microbiology that there is a solid grounding on the taxonomy of the bacteria of interest in a given environment. It was therefore chosen that a taxonomic screening of cultured Antarctic bacteria of the phyla *Actinobacteria* be carried out, with any isolates encountered being assessed as to whether they are of a previously undescribed novel species (Chapter Two). The potentially useful physiological attributes these isolates possess that are attributed to conditioning in the Antarctic environment will also be explored in this dissertation, concentrating on the potential for bioremediation (Chapter Three) and production of novel antimicrobial compounds (Chapter Four). This being said, there are many novel ecological niches unique to Antarctica that have not been investigated for their microbial diversity. This dissertation will also then cover select environments of interest with this aim in mind. There is a definite lack of knowledge regarding epiglacial lakes and they will form the focus of this section of the study (Chapter Five). Finally it must not be forgotten that as well as being a fascinating pristine environment on this planet, Antarctica also guards a snapshot into the past dating back to Gondwana land, and

work will be discussed that explores the emerging area of molecular archaeology keeping with the theme of Antarctic bacteria, looking at both marine-derived ice and glacial ice (Chapter Six). This PhD dissertation entitled "The biodiscovery and biodiversity of Antarctic bacteria" follows the Australian Government Antarctic Division's vision that all research on Antarctica be carried out with the aim of it being valued, protected, and understood, and supports the notion that the current knowledge of Antarctic microbiology is only the tip of the iceberg.

Chapter 2 Taxonomy of Antarctic *Actinobacteria* isolates

Resupply at Davis Station, Antarctica

The ice-free regions around Davis Station and the Vestfold Hills were the primary samples sites for the UTAA collection

Photo taken by Mr Jimmy Twin

2.1 Introduction

Gram-positive bacteria possessing a high guanine plus cytosine (G+C) content have been found to have a diverse cellular morphology and inhabit a wide range of ecological niches. 16S rRNA gene sequence analysis has shown the high G+C Gram-positive bacteria form a large, coherent phylogenetic cluster that has become known as the phylum *Actinobacteria* (Bergey et al., 1984, Stackebrandt et al., 1997). Actinobacterial cells can appear as cocci, have a rod-coccus growth cycle, possess fragmenting hyphal forms, and even be comprised of permanent and highly differential branched mycelia. The colony colour, shape, and texture differ greatly between members of *Actinobacteria*, and what similarities are seen often does not correlate with phylogeny. When a member of *Actinobacteria*, *Actinomyces bovis* was first characterised, it was actually mistaken as a fungus. It was found to be a causative agent for a disease called Actinomycosis ("lumpy jaw" with cattle), and only when colony morphologies inside tissue were analysed, researchers found that these *Actinomyces* formed radially arranged hyphae, that true fungi did not. Because of this, early work focussed on *Actinobacteria* as human pathogens, and this phylum is responsible for many diseases such as nocardiosis, tuberculosis, diphtheria, and leprosy (McNeil & Brown, 1994). However more importantly, *Actinobacteria* are ubiquitous in nature, having been isolated from both natural and man-made environments spanning the globe. Their role in the environment varies, with members being important recyclers of a broad range of carbon substrates, as well as being saprophytic, parasitic, and also maintaining mutualistic relationships with hosts. *Actinobacteria* are known to play an important part in soil ecosystems (Goodfellow, 1983).

2.1.1 The University of Tasmanian Antarctic Actinobacteria collection

The University of Tasmania has in its possession 1600 *Actinobacteria* isolates from soils of Antarctic and sub-Antarctic ice-free regions. For the purposes of this study this archive will be referred to as the University of Tasmania Antarctic *Actinobacteria* (UTAA) collection. These cultures arose from a collaborative project between the University of Tasmania and Cerylid Biosciences Ltd. The purpose of that project was to discover novel human pharmaceuticals. The contracts concerning these isolates have since expired and so the collection now is the property of the University of Tasmania.

The UTAA collection was obtained by sampling soils from various Antarctic and sub-Antarctic during summer periods of 1999-2001. In total, 700 individual samples were collected; primarily from within the Davis Station limits and the surrounding Vestfold Hill region (68°33′S, 78°15′E) with 293 samples taken from sub-Antarctic Macquarie Island (54°36′S, 158°54′E). Further information on each sample site and the bacteria isolated are included on the Appendix CD. As far as the identification of these isolates, all work done previously has been only through morphological evaluation. The information given states these isolates are of the phylum *Actinobacteria*, with the majority (74%) of the collection belonging to the genus *Streptomyces*.

Having access to the UTAA collection presented a unique opportunity to explore the taxonomy of *Actinobacteria* and of Antarctic microbiota. This chapter will discuss the results of this polyphasic taxonomic screen of the UTAA collection for novel actinobacterial species. This was achieved by initially selecting candidate bacterial

genera of interest via 16S rRNA gene sequencing, followed by an approach that included analysis of the G+C content, fatty acid composition, and carbon utilisation patterns of the selected strains to ascertain if any are novel or warranting further investigation. The genera covered in this study included in particular *Arthrobacter*, *Promicromonospora,* and *Rhodococcus*.

2.1.2 Arthrobacter

Arthrobacter is a genus belonging to the family *Micrococcaceae* (Conn & Dimmick, 1947, Euzeby, 2006). *Arthrobacter* spp. are aerobic, non-sporulating, catalase positive bacteria that have cells that exhibit a coryneform-like morphology that stain Gram positive. Member species usually exhibit a rod-coccus growth cycle (Conn & Dimmick, 1947, Keddie et al., 1986, Koch et al., 1995). Species within *Arthrobacter* can be divided into Group I and Group II, according to their cell wall peptidoglycan and menaquinone compositions (Keddie et al., 1986, Schleifer & Kandler, 1972, Stackebrandt et al., 1983, Stackebrandt & Schumann, 2000). Members of Group I are described as having an A3α peptidoglycan type, consisting of L-amino acids, and exhibiting the presence of a dihydrogenated menaquinone with nine isoprenoid units, denoted MK-9(H_2). Most species of *Arthrobacter* are Group I, including the type species *Arthrobacter globiformis*. Group II *Arthrobacter spp.* have an A4α peptidoglycan type, consisting of dicarboxylic amino acids and an unsaturated menaquinone with eight, nine, or ten isoprenoid units; MK-8(H_0), MK-9(H_0), or MK-10(H_0) respectively. Group II *Arthrobacter* spp. also cluster together in a separate branch when *Arthrobacter* 16S rRNA gene sequences are compared in phylogenetic trees.

The genus *Arthrobacter* is frequently found in Antarctic samples, with 5 out of the 60 validly described species of *Arthrobacter* being originally isolated from Antarctica: *A. ardleyensis* from lake and deep-sea sediment, *A. flavus* from a Dry Valley pond, *A. gangotriensis* and *A. kerguelensis* from an Indian Antarctic station, and *A. roseus* from a cyanobacterial mat sample (Chen et al., 2005, Gupta et al., 2004, Reddy, 2002, Reddy et al., 2000). Reddy *et al* also noted Antarctic *Arthrobacter* having several characteristics that they considered distinct, including cold adaptation, possessing glucose as a cell wall sugar, and not being able to hydrolyse starch (Reddy et al., 2000). Several undescribed *Arthrobacter* spp. isolated from Antarctica have beneficial cold temperature biotechnological applications, such as *Arthrobacter* strain TAD20 producing cold active chitobiase and chitinase that breaks down chitin, and *Arthrobacter* strain DS2-3R producing a citrate synthetase used in site-directed mutagenesis (Gerike et al., 2001, Lonhienne et al., 2001a, Lonhienne et al., 2001c). It has also been suggested that *Arthrobacter* spp. may be able to "out survive" spore forming bacteria in a frozen environment, as *Arthrobacter* DNA was detected in higher abundance in ancient Antarctic and Siberian permafrost samples (Willerslev et al., 2004a).

2.1.3 Taxonomy of Promicromonospora

Considered a relatively rare genus, *Promicromonospora* currently consists of four validly described species, *Promicromonospora citrea*, the type species, *P. aerolata*, *P. sukumoe*, and *P. vindobonensis* (Busse et al., 2003, Krasil'nikov et al., 1961, Takahashi et al., 1988). Because of the lack of comparable data, the defining characteristics of *Promicromonospora* are relatively simple. Members of this genus are characterised as having a nocardioform life cycle where mycelia are produced

that fragment into nonmotile coccoid/rod elements. Unlike *Arthrobacter*, *Promicromonospora* cannot be defined upon its peptidoglycan amino acid content with both A3α and A4α types being described. In all valid species to date, the menaquinone content detected is that of MK-9(H_4), and the major fatty acids being anteiso $C_{15:0}$ and iso $C_{15\,0}$. The G+C content of *Promicromonospora* spp. ranges from 70-75 mol% (Zhiheng et al., 1997). The 16S rRNA gene similarity within this genus is remarkably high, with suggestion that any percentage lower than 98.6% could be used as a cut-off for a presumptive novel species, provided the isolate does not cluster with *Promicromonospora*'s closest phylogenetic relatives *Rarobacter*, *Cellulosimicrobium*, *Oerskovia*, and *Cellulomonas* (Busse et al., 2003, Schumann et al., 2001). Because of this high percentage similarity, it is essential that DNA-DNA hybridisation data be used to clearly define separation speciation. Not surprisingly, there has been little industrial microbiology performed on *Promicromonospora* spp. although it has been reported that *P. sukumoe* produces the antibiotic 7-hydro-8-methyl-pteroyl glutamylglutamic acid and exhibits antagonistic activity against *Fusarium oxysporum* (Han et al., 1999, Murata et al., 1987). There is potential for this genus to be of biotechnological use as it is found in composts, has been associated with earthworms, and demonstrates the ability to lyse yeasts (Chernyakovskaya et al., 2004, Tiunov & Dobrovolskaya, 2002).

2.1.4 Taxonomy of Rhodococcus

Belonging to the family *Nocardiaceae*, members of the genus *Rhodococcus* are aerobic, non-motile, catalase-positive bacteria that stain Gram-positive to Gram-variable. Having a nocardioform cell cycle, cell morphologies change from being a short rod or cocci; continue being short rods; or having a more complex life cycle

producing filaments, branching patterns or branched hyphae. The cell wall of *Rhodococcus* spp. consists of peptidoglycan type A1γ with *meso*-$A2_{pm}$ as the diamino acid and muramic acid with N-glycolyl residues. Arabinose and galactose are the predominant sugars of capsular polysaccharides. The major menaquinone present is MK-8(H_2) and the major fatty acids include saturated and unsaturated chain components with compounds forming 10-methyl branched fatty acids. The G+C content of the *Rhodococcus* genome ranges from 63-73 mol% (Bell et al., 1998, Goodfellow et al., 1998). The biotechnological potential of this genus is enormous. *Rhodococcus* spp. are reported to be one of the major contributors to the natural bioremediation of oil contaminated sites in Antarctica (Luz et al., 2004, Whyte et al., 2002a) and are producers of industrially important compounds such as nitrile hydratase that is used for the low temperature synthesis of acrylamide (Watanabe et al., 1987).

2.2 Materials and methods

2.2.1 Origin, isolation, and maintenance of bacterial cultures

Strains UTAA053 and UTAA143 were isolated from samples collected from a lichen valley site (69°23'S 78°19'E) using Marine Agar (MA) and SC media (SC) respectively. Bacteria UTAA121 and UTAA124 were isolated from Gardner Island (68°35'S 77°52'E) where there were obvious signs of penguin influence. The isolation medium used for these two organisms was SC media. Bacterium UTAA127 was isolated from the old science lab entrance area at Davis Base (68°35'S 77°58'E) using AV agar (AV). All isolates in this study was purified using Oatmeal Agar (OA), glycerol stocks of each isolate were then stored at -20°C. These glycerol stocks were then utilised in this study. Any subsequent subculturing used MA plates. Bacterial colony characterisation was carried out on Nutrient Agar (NA). All culturing in this study was at 20°C unless otherwise mentioned. The recipes for the media listed above are available in the Appendix CD.

2.2.2 Reference strains

The reference strains used in this study were obtained from the Japan Collection of Microorganisms (RIKEN BioResource Center, Japan): *Arthrobacter flavus* JCM11496^T, *Arthrobacter globiformis* JCM1332^T, *Arthrobacter rhombi* JCM11678^T, *Promicromonospora citrea* JCM3051^T, *Promicromonospora sukumoe* JCM6845^T, *Rhodococcus coprophilus* JCM3200^T, *Rhodococcus fascians* JCM10002^T, and *Rhodococcus rhodochrous* JCM3202^T.

2.2.3 DNA extraction of bacterial colonies

A pure bacterial colony from an agar plate was placed in a 1.5mL screw capped tube containing 0.1mm, 0.5mm, and 1.0mm silicon-zirconium beads (Daintree Scientific) in 500µL saline EDTA. This cell suspension was frozen using liquid nitrogen, followed by shaking the tubes in a bead-beating device for 2 cycles of 10 seconds at 5000rpm to disrupt cells. Cellular debris and beads were pelleted via centrifugation at 10000xg for 5 minutes in a bench top centrifuge with the upper supernatant undergoing two chloroform:isoamyl alcohol (24:1) washes. The resulting DNA containing fraction then underwent purification using the UltraClean 15 DNA Purification Kit (MO BIO Laboratories, Inc., Cat #12100-300) following manufacturer's instructions.

2.2.4 PCR amplification of 16S rRNA gene

The 16S rRNA gene sequences of the DNA extracted were PCR amplified as follows. 5µL of template DNA was added to 45µL of a master mix which consisted of 25µL of HotStarTaq Master Mix (Qiagen Pty Ltd Cat. # 203445), 5pmol of each primer, making up to the required volume with Qiagen supplied dH_2O. The HotStarTaq Master Mix contains 2.5 units of HotStarTaq DNA Polymerase, buffer containing 1.5mM $MgCl_2$, and 200µM of each dNTP. The primers used were the universal bacterial primers 10f (5'-GAGTTTGATCCTGGCTCAG-3') and either 1492r (5'-TACGGYTACCTTGTTACGACTT-3') or 1520r (5'-AGAAAGGAGGTGATCCAGCC-3') (Lane, 1991). Amplicons were generated using a MJ Research PTC-200 peltier thermal cycler using the following program: one cycle of 15 minutes at 95°C; 30 cycles of 1 minute at 94°C, 1 minute

at 52°C, 1.5 minute at 72°C; and a final extension step of 10 minutes at 72°C. The resulting PCR product was then purified using a QIAquick Spin column (Qiagen Pty Ltd Cat. # 28106), and visualised on a 1.5% agarose gel stained with 500ng/ml ethidium bromide.

2.2.5 DNA sequencing of PCR products

DNA sequences were obtained from purified PCR products using a CEQ™ 8000 Genetic Analysis System (Beckman Coulter Inc). A slight modification of the standard protocol recommended with the GenomeLab DTCS Quick Start Kit (Beckman Coulter Inc., Cat #608120) was used to set up sequencing reactions. The sequencing mix consisted of 4μL DTCS Quickstart mix, 2μL of primer at a concentration of 2pmol/μL, 10μL sterile milliQ H_2O, plus 4μL of purified PCR product. The primers used for sequencing comprised of the original primers used in section 2.2.4 plus 519f (5'-GWATTACCGCGGGKGCTG-3'), and 907r (5'-CCGTCAATTCCTTTGAGTTT-3') (Giovannoni, 1991). The creation of labelled product was carried out on a MJ Research PTC-200 peltier thermal cycler using the following program: 30 cycles of 20 seconds at 96°C, 20 seconds at 50°C, and 4 minutes at 60°C. Prior to being sequenced the labelled PCR products were desalted using 100% and 70% ethanol washes as recommended by Beckman Coulter Inc.

2.2.6 Sequence analyses

Raw sequence files (*.scf) were exported into the Sequencher 4.5 program (Gene Codes Corporation) where chromatograms were analysed and sequence fragments were aligned. Consensus sequences were then compared against other sequences on

the Genbank database (Benson et al., 2003) using the BLASTN function (Altschul et al., 1990). The closest matches of each clone, as well as the closest named species were imported into the program Seaview (Galtier et al., 1996), where all sequences were aligned using the ClustalW function. Neighbour joining trees calculated using the Kimura 2 parameter model were then constructed using the program Phylowin (Galtier et al., 1996) with the resulting tree output made into a publishable quality using Adobe Photoshop CS 8.0 or Adobe Illustrator CS3 13.0.0 (Adobe Systems Inc.).

2.2.7 DNA base composition and DNA-DNA hybridisation

Pure bacterial cultures were subcultured onto 20 MA plates until confluent growth was observed. DNA was extracted from this biomass according to the protocol developed by Marmur & Doty (1962). The G+C content of the purified DNA was determined from thermal denaturation profiles (Sly et al., 1986). To perform DNA-DNA hybridisation studies on UTAA143 and *P. sukumoe*, 5g of wet biomass was submitted to Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany). The procedure followed there is described briefly as follows. DNA was initially isolated using a French pressure cells (Thermo Spectronic), purified by chromatography on hydroxyapatite (Cashion et al., 1977), and DNA-DNA hybridisation was carried out using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6x6 multicell changer and temperature controller (Varian) (Deley et al., 1970, Huss et al., 1983).

2.2.8 Phenotypic characterisation of bacterial stains

Gram-stain, catalase and oxidase tests were carried out using standard methods (Smibert & Kreig, 1994). Tests for motility were conducted by light microscopic observations of wet mounts. Growth was tested on MA streaked plates held at 2°C, 4°C, 8°C, 10°C, 20°C, 25°C, 30°C, 37°C, 40°C, and 55°C (dry heat incubators or temperature monitored cool rooms) for a period of up to a month with growth checked daily. To assess the growth of each bacterial isolate at different pH values single pure colonies were suspended in a series of 30ml Tryptone Soya Broths (TSB) with pH readings ranging from 4.1-13.9 (adjusted using HCl/NaOH). All broths were tested for their pH before autoclaving, after autoclaving, and control broths were tested again after the growth study. Growth was determined by the presence of visible turbidity after incubation shaking at 150-160rpm for one week at 20°C. Additional biochemical tests including carbon utilisation were carried out by using the API Coryne and API 50CH kits (bioMérieux Pty Ltd, Cat #20900, #50300), as well as the Biolog GP2 MicroPlate (Oxoid Australia Pty Ltd, Cat #B`1104) following the manufacturer's instructions.

2.2.9 Scanning Electron Microscopy

Cells were prepared for viewing under a Scanning Electron Microscope (SEM) briefly as follows: clean glass slides were treated with a 0.1% poly-l-lysine solution (Sigma Aldrich, Cat #P8920) for 30 minutes at room temperature (RT). Cell suspensions were then placed onto the slides and incubated for 30 minutes again at RT to allow adherence of cells. Slides were then rinsed with dH_2O followed by a primary fixation step with 2.5% glutaraldehyde (in dH_2O) for 30 minutes at RT

followed by three rinses with dH_2O. A secondary fixation of the cells was carried out by the addition of 1% osmium tetroxide (in dH_2O) for 20 minutes at RT. After 5 rinses in dH_2O the now fixed cells underwent a gradient of dehydrating ethanol washes from 25% to 100%. Each wash was for 5 minutes, and immediately after the final wash, the slides with cells were frozen using liquid nitrogen and dried using a vacuum freeze-dryer (Dynavac) for 24 hours. Slides were then sputter coated with gold before viewing on a SEM under supervision of staff at the University of Tasmania Central Science Laboratory (Hobart). Images were edited using Adobe Photoshop CS 8.0 (Adobe Systems Inc.).

2.2.10 Fatty acid methyl ester (FAME) and cell wall menaquinone analysis

Biomass was obtained by culturing the bacteria to be analysed in Glucose-Yeast-Malt broth (GYM) for 5 days at 20°C. The biomass was then resuspended into a small amount of 0.1% NaCl and the cells then lyophilised using a vacuum freeze-dryer (Dynavac). Cellular fatty acid profiles were obtained using GC and GC-MS procedures (Nichols et al., 1997). Prior to analysis, fatty acids were derivatised using dimethyldisulphide in order to determine the geometry and position of double bonds, numbering from the methyl end (ω) of the fatty acid (Nichols et al., 1986). Isoprenoid quinones from the aforementioned lyophilised cell extracts were analysed at the University of Tasmania Central Science Laboratory (Hobart) using reversed-phase liquid chromatography (Moss & Guerrant, 1983).

2.2.11 Cell wall analysis

Amino acids were extracted and derivatised using the EZ:faast kit (Phenomenex Inc., Cat #KG0-7168) following manufacturer's instructions, and were determined by GC-MS by comparison with a standard amino acid solution. Cell wall sugars were derivatised using sodium borohydride and analysed using a GC-MS procedure (Fox et al., 1990). Sugars were identified by the comparison of retention times to known standards. The sugars included in the standard were arabinose, galactose, glucose, mannose, inositol, rhamnose, and xylose.

2.3 Results and Discussion

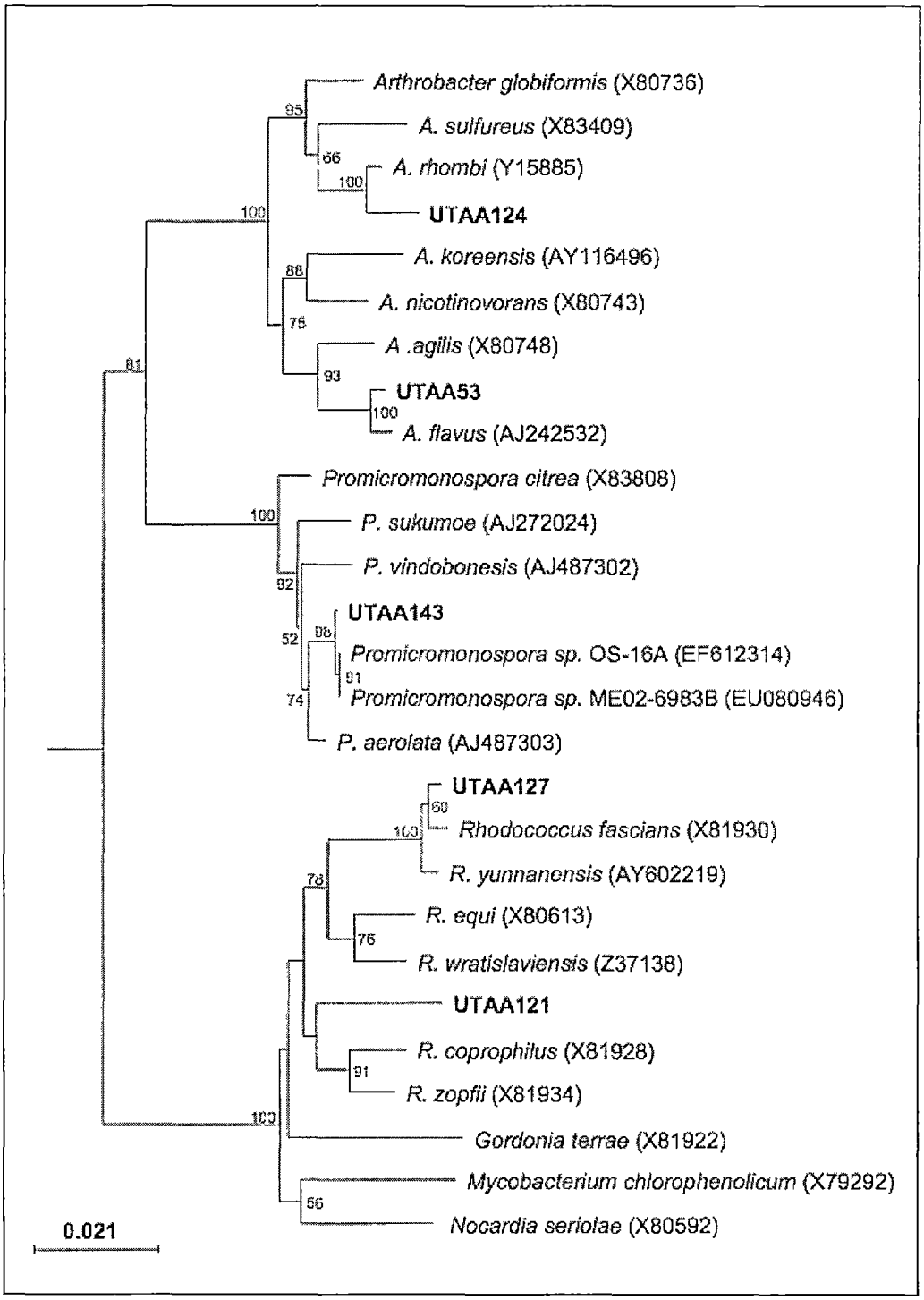
2.3.1 16S rRNA gene sequencing of UTAA isolates

The first 150 *Actinobacteria* isolates in the UTAA underwent partial 16S rRNA gene sequencing. Of these, 112 were identified as being of the genus *Streptomyces*, 9 of the genus *Nocardia*, 6 of *Rhodococcus*, 3 *Arthrobacter*, and 1 each of *Dietzia* and *Promicromonospora*. Of the remaining, 16 were unable to be either cultured or its DNA extracted, and 1 isolate appeared to be a *Flavobacterium* spp. (class Flavobacteria and phylum *Bacteroidetes*). Due to the intrinsic difficulties in characterising members of the genus *Streptomyces* as mentioned earlier, these isolates were ignored for this part of the study.

Five isolates were chosen for further study that belonged to rare genera or may have useful features. The 16S rRNA gene similarities of these isolates to their closest validly described neighbours are shown in the form of a phylogenetic tree in Figure 2i. Two of these isolates, UTAA053 and UTAA124, belong to the genus *Arthrobacter*. *Arthrobacter* UTAA053 and its closest phylogenetic neighbour *A. flavus* possess a 16S rRNA gene similarity of 98.0% over 1122bp. A longer sequence for *A. flavus* was not possible as the sequence submitted to Genbank was found to be chimeric. UTAA124 and *A. rhombi* have a similarity of 98.4% of a 1369bp region. One of the UTAA isolates in this study, *UTAA143*, groups with members of the genus *Promicromonospora*, sharing a 98.4% 16S rRNA gene identity with *P. sukumoe*, and a 97.8% similarity with *P. citrea* over a 1382bp region. The two remaining UTAA isolates, UTAA121 and UTAA127, cluster with the genus

Rhodococcus. UTAA121 forms a clear separate branch within this genus with its closest match in terms of 16S rRNA gene identity being 95.7% with *R. coprophilus* over a 1433bp region. UTAA127 clusters with *R. yunnanensis* and *R. fascians* with 16S rRNA gene similarities both of 98.9% over a 1395bp region. This corresponds to a 15bp difference with *R. yunnanensis* and *R. fascians* in different regions of the 16S rRNA gene fragment.

Figure 2i **16S rRNA gene Neighbour Joining tree of UTAA isolates with closest relatives. *Bifidobacterium bidifum* (S83624) and *Rubrobacter radiotolerans* (U65647) were used as outgroups, and comparisons were made using the Kimura two-parameter model with 1000 bootstrap replicates. The scale bar indicates 0.021 changes per nucleotide.**


Arthrobacter globiformis (X80736)
A. sulfureus (X83409)
A. rhombi (Y15885)
UTAA124
A. koreensis (AY116496)
A. nicotinovorans (X80743)
A .agilis (X80748)
UTAA53
A. flavus (AJ242532)
Promicromonospora citrea (X83808)
P. sukumoe (AJ272024)
P. vindobonesis (AJ487302)
UTAA143
Promicromonospora sp. OS-16A (EF612314)
Promicromonospora sp. ME02-6983B (EU080946)
P. aerolata (AJ487303)
UTAA127
Rhodococcus fascians (X81930)
R. yunnanensis (AY602219)
R. equi (X80613)
R. wratislaviensis (Z37138)
UTAA121
R. coprophilus (X81928)
R. zopfii (X81934)
Gordonia terrae (X81922)
Mycobacterium chlorophenolicum (X79292)
Nocardia seriolae (X80592)
0.021

2.3.2 Biochemical and carbon utilisation differentiation between strains

Table 2a summarises the differential characteristics between UTAA strains and their closest related valid species (section 2.3.1). All of the strains tested were Gram-positive, catalase positive, and were non-motile and gave negative results for the utilisation of gentibiose, D-tagatose, D-fucose, L-fucose, and L-arabitol; and β-glucoronidase activity.

2.3.3 Arthrobacter isolate UTAA053

UTAA053 was isolated from Lichen Valley in the Vestfold Hills region of Antarctica (69°23'S 78°19'E). Cells are aerobic, stain Gram-positive, and show signs of a rod-cocci growth cycle. No spores or spore-like elements were observed. On NA plates colonies are cream in colour whilst *A. flavus* colonies are typically yellow. UTAA053 was found to metabolise in the presence of the following substrates (Biolog GP2): acetic acid, n-acetyl L-glutamic acid, D-alanine, L-alanine, L-alaninamide, L-alanyl-glycine, L-asparagine, dextrin, esculin, fructose-6-Phosphate, D-gluconic acid, D-glucose, glucose-1-phosphate, glucose-6-phosphate, L-glutamic acid, glycogen, glycyl-l-glutamic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-phenylacetic acid, inosine, α-keto glutaric acid, lactamide, L-lactic acid, lactulose, D-mallic acid, L-mallic acid, D-maltose, maltotriose, mannan, α-methyl-D-galactoside, β-methyl-D-galactoside, α-methyl-D-glucoside, β-methyl-D-glucoside, α-methyl-D-mannoside, mono-methyl succinate, propionic acid, L-pyroglutamic acid, salicin, L-serine, stachyose, succinamic acid, succinic acid, D-trehalose, Tween 40, and Tween 80.

Table 2a

A comparison of biochemical and carbon utilisation characteristics of UTAA strains compared to their closest 16S rRNA neighbour

	UTAA053	*A. flavus*	UTAA124	*A. rhombi*	UTAA143	*P. sukumoe*	UTAA121	*R. coprophilus*	UTAA127	*R. fascians*	*R. yunnanensis**
Biochemical traits:											
Oxidase	+	-	-	+	+	ND	-	ND	-	-	-
Nitrate reduction	+	-	+	+	-	-	+	+	-	-	-
Pyrazinamidase	(+)	+	(+)	+	-	-	(+)	-	(+)	(+)	
Pyrolidonyl arylamidase	+	+	+	+	-	+	+	-	-	-	
Alkaline phosphatase	+	+	+	+	+	+	+	-	(+)	+	+
β-galactosidase	+	-	-	+	+	+	-	-	(+)	-	-
α-glucosidase	+	+	+	+	+	+	+	-	-	-	-
n-acetyl-beta-glucosaminidase	(+)	-	-	-	+	+	-	-	-	-	-
beta-glucosidase (esculin)	+	-	+	-	+	+	+	-	-	-	-
Urease	-	-	+	-	-	-	+	-	-	-	-
Hydrolysis of gelatin	+	-	+	+	-	-	+	-	-	-	-
Utilisation of:											
N-Acetylglucosamine	+	-	-	-	-	-	-	-	-	-	ND
D-Arabinose	-	-	-	-	+	-	-	-	-	-	ND
L-Arabinose	-	-	-	-	+	+	-	-	+	+	+
D-Arabitol	-	-	(+)	-	+	+	+	-	+	+	+
Erythritol	-	-	-	-	-	-	+	-	-	-	ND
Esculin	+	+	-	+	+	+	+	-	-	-	+
D-Glucose	+	-	-	-	+	+	-	-	+	-	ND
Glycerol	-	-	-	-	-	+	+	-	+	+	ND
Glycogen	+	-	-	-	+	+	-	-	-	-	ND
Inositol	-	-	-	-	-	-	+	-	-	-	-
D-Lactose	-	-	-	-	+	+	-	-	+	-	+
D-Maltose	+	-	-	-	+	+	-	-	-	-	+
D-Mannitol	-	-	-	-	+	+	+	-	+	+	+
D-Melbiose	-	-	-	-	+	-	-	-	+	-	ND
D-Melezitose	-	-	-	-	+	-	-	-	+	-	ND
Methyl-βd-xylopryanoside	-	-	-	-	+	+	-	-	-	+	ND
D-Ribose	-	-	-	-	+	+	+	-	-	(+)	+
Salicin	+	-	-	-	+	+	-	-	-	-	ND
D-Trehalose	+	-	-	-	+	+	+	-	+	(+)	+
D-Xylose	-	-	-	-	+	+	-	-	-	(+)	+

* - Data obtained from (Zhang et al , 2005) + = positive, (+) = weakly positive; - = negative; ND = No Data Items shaded in grey are differing characteristics between UTAA strain and JCM Type strain

Growth for UTAA053 occurred between 2°C and 37°C, within the pH ranges of 5.1 and 10.2, and tolerates up to 10 %, but not 13% (w/v) NaCl, whilst *A. flavus* grows between 5°C and 30°C, pH 6-9, and up to 11.5% (w/v) NaCl. The predominant acids in the cellular fatty acid profile are $C_{16.0}$, $C_{17\ 1}$, and cyl $C_{18\ 0}$, and *A. flavus* has been found to possess $C_{14\ 0}$, $C_{15\ 0}$, anteiso-$C_{15\ 0}$, $C_{16\ 0}$, iso-$C_{16\ 0}$, $C_{16\ 1}$, $C_{17.0}$, anteiso-$C_{17.0}$, iso-$C_{18\ 0}$, $C_{18\cdot0}$, $C_{20\ 0}$. Both UTAA053 and *A. flavus* possess an A3α peptidoglycan type with the major quinone being MK-9(H_2), consistent with Group I *Arthrobacter*. The DNA G+C content of UTAA053 and *A. flavus* are 62.2 and 64±2 mol% respectively (Reddy et al., 2000). The main cell wall sugar was identified as being galactose. In light of differences found in pigmentation and various biochemical and carbon utilisation characteristics (Table 2a), there is warrant in investigating this strain further as more comparative evidence, such as DNA-DNA hybridisation is required to make a definitive decision to whether UTAA053 is a novel species of the genus *Arthrobacter*.

2.3.4 Arthrobacter isolate UTAA124

UTAA124 was originally isolated from Gardner Island, approximately 4km west of Davis Station in the Vestfold Hills, Antarctica (68°35'S 77°52'E). Cells are aerobic short ovoid non-spore forming rods that are Gram-positive and catalase positive. Colonies on NA are bright yellow in colour. UTAA124 displayed metabolism in the presence of: acetic acid, n-acetyl L-glutamic acid, adenosine, D-alanine, L-alanine, L-alaninamide, L-alanyl-glycine, L-asparagine, 2,3-butanediol, 2'-deoxy adenosine, dextrin, d-galacturonic acid, d-gluconic acid, L-glutamic acid, glycyl-L-glutamic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-

phenylacetic acid, inosine, α-keto glutaric acid, α-keto valeric acid, L-lactic acid, lactulose, D-mallic acid, L-mallic acid, maltotriose, mono-methyl succinate, α-methyl-D-galactoside, β-methyl-D-galactoside, α-methyl-D-glucoside, 3-methyl glucose, α-methyl-D-mannoside, palatinose, propionic acid, D-psicose, putrescine, L-pyroglutamic acid, pyruvic acid, L-serine, stachyose, succinamic acid, succinic acid, and thymidine.

Growth occurs between 2°C and 30°C and up to 10% (w/v) NaCl, much the same as *A. rhombi* that is reported to grow between 4°C and 30°C, and up to 10% (w/v) NaCl. *Arthrobacter* UTAA124 exhibits growth between pH 4.5-9.3 and possesses a dominant $C_{15\ 0}$iso fatty acid and a major quinone of MK-8(H_0), however none of this information is currently available for *A. rhombi*. The cell walls for both strains are of A4α type and the DNA G+C content of UTAA124 and *A. rhombi* are 66.3 and 61 mol% respectively (Osorio et al., 1999). The primary cell wall sugar was identified as being galactose. There is no conclusive evidence into whether UTAA124 belongs to *A. rhombi*, so further investigation is required including DNA-DNA hybridisation, and further phenotypic comparisons.

2.3.5 Promicromonospora isolate *UTAA143*

UTAA143 was isolated from Lichen Valley, Antarctica (69°23'S 78°19'E). This bacterium is an aerobic, Gram-positive, catalase positive, bacterium that is filamentous and produces substrate mycelia that are able to penetrate agar. After long periods of incubation the mycelia fragment into ovoid rod shaped cells (Figure 2ii). Colonies are a dull yellow in colour on NA. UTAA143 metabolised in the presence of: acetic acid, adenosine, adenosine-5'-monophosphate, l-alaninamide, amygdalin, D-arabitol, D-arabinose, L-arabinose, arbutin, α-cyclodextrin, β-cyclodextrin, D-cellobiose, 2'-deoxy adenosine, dextrin, esculin, D-fructose, fructose-6-phosphate, D-galactose, gentiobiose, D-gluconic acid, D-glucose, glycogen, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, inosine, L-lactic acid, D-lactose, D-maltose, maltotriose, D-mannitol, D-mannose, D-melbiose, D-melezitose, β-methyl-D-galactoside, α-methyl-D-glucoside, β-methyl-D-glucoside, α-methyl-D-mannoside, methyl pyruvate, methyl-αD-glycopyranoside, methyl-αD-mannopyranoside, methyl-βD-xylopryanoside, mono-methyl succinate, propionic acid, pyruvic acid, D-ribose, salicin, L-serine, starch, sorbitol, D-sucrose, thymidine, thymidine-5'-monophosphate, D-trehalose, D-turanose, Tween 40, Tween 80, uridine, uridine-5'-monophosphate, and D-xylose.

The closest phylogenetic neighbour to UTAA143 is *P. sukumoe*, bearing a 98.4% 16S rRNA gene similarity, just below the aforementioned suggestion of a 98.6% level of similarity required for novel speciation within the genus *Promicromonospora*. Growth of UTAA143 occurs between 2°C and 37°C, pH ranges of 6.2-9.3, and can tolerate up to 10% (w/v) NaCl. *P. sukumoe* grows

between 10-35°C, between pH 5-10, and up to 7% NaCl (w/v). UTAA143 has a fatty acid profile dominated by $C_{15.0}$ anteiso and $C_{15\,0}$ iso respectively that is consistent with all available data known for *Promicromonospora*. Both UTAA143 and *P. sukumoe* contain a VI cell wall chemotype containing lysine and MK-9 (H_4) as the major quinone (Takahashi et al., 1988). The main cell wall sugar identified was galactose. The total DNA G+C content of UTAA143 is 70.3 mol%. *Promicromonospora* UTAA143 and *P. sukumoe* were submitted to DSMZ for DNA-DNA hybridisation. This pairing gave a 30.7-31.4% DNA-DNA similarity, showing that these two strains are not of the same species, according to the 70% DNA-DNA similarity level considered to be the benchmark for the definition of a species (Wayne et al., 1987). As phenotypic characteristics and phylogenetic data place UTAA143 in the genus *Promicromonospora*, and 16S rRNA gene sequencing showing *P. sukumoe* as the closest relative, it is proposed that *Promicromonospora* UTAA143 be submitted as a novel species. Subject to approval of novel speciation by peer review, the name to be given to this novel species will be *Promicromonospora lichenicola* (latin for "lichen dweller").

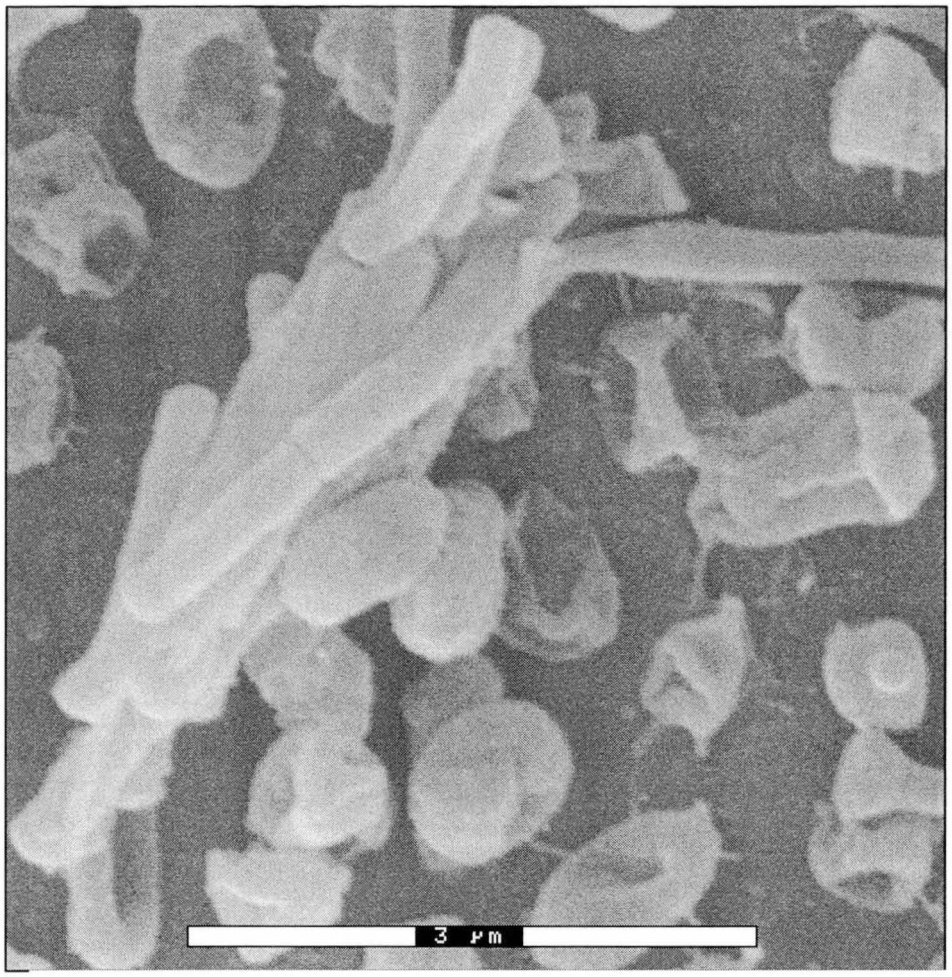


Figure 2ii

SEM micrograph of stationary *Promicromonospora* UTAA143 cells

2.3.6 Rhodococcus isolate UTAA121

Also from Gardner Island, near a penguin colony in Antarctica (68°35'S 77°52'E), is a Gram positive, catalase positive, aerobic, non-spore forming ovoid rod shaped organism (Figure 2iii). Colonies are cream coloured on NA. UTAA121 metabolised in the presence of: acetic acid, n-acetyl-D-glucosamine, adenosine, adenosine-5'-monophosphate, D-alanine, L-alanine, L-alaninamide, L-alanyl-glycine, D-arabitol, α-cyclodextrin, 2'-deoxy adenosine, dextrin, erythritol, esculin, D-fructose, D-gluconic acid, glucose-L-phosphate, glucose-6-phosphate, L-glutamic acid, glycerol, D-L-α-glycerol phosphate, glycyl-L-glutamic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-phenylacetic acid, inosine, inositol, α-keto glutaric acid, α-keto valeric acid, lactamide, D-lactic acid, L-lactic acid, maltotriose, methyl ester, D-mallic acid, L-mallic acid, D-mannitol, α-methyl-D-glucoside, β-methyl-D-glucoside, 3-methyl glucose, mono-methyl succinate, palatinose, propionic acid, D-ribose, sedoheptulosan, L-serine, thymidine, thymidine-5'-monophosphate, D-trehalose, Tween 40, Tween 80, uridine, and uridine-5'-monophosphate.

Growth of this organism occurs between 2°C and 30°C, within the pH ranges of 6.2-9.0, and in the presence of up to 10% NaCl. Its closest phylogenetic neighbour was found to be *R. coprophilus*, with a 95.7% 16S rRNA gene similarity, well below the 97% cutoff limit suggested for species level discrimination for 16S rRNA gene sequences showing no need to conduct DNA-DNA hybridisation studies (Stackebrandt & Goebel, 1994). Both strains possess a major quinone content of MK-8(H_2) and cell wall type A1γ, typical of *Rhodococcus spp.* (Collins et al., 1985,

Rowbotham & Cross, 1977). Galactose followed by arabinose were the primary sugars present in the cell wall of UTAA121. UTAA121 and *R. coprophilus* possess a DNA G+C content of 74.4 and 60-69.3 respectively (Mordarski et al., 1980a, Mordarski et al., 1980b, Mordarski et al., 1981, Rowbotham & Cross, 1977). Given the vast differences in phenotypic traits it appears that UTAA121 may be a novel species of the genus Rhodococcus. Additional work required before publication could be considered would include additional analysis of cellular components including fatty acid analysis that unfortunately were not able to be completed during the course of this study.

2.3.7 Rhodococcus isolate UTAA127

UTAA127 was isolated from a sample taken from the old science laboratory entrance area at Davis Base, Antarctica (68°35'S 77°58'E). This isolate is aerobic, Gram-positive, catalase positive and forms small ovoid rods. Colonies are orange on NA. UTAA127 metabolised in the presence of: acetic acid, L-alanine, L-alaninamide, L-arabinose, D-arabitol, L-asparagine, 2,3-butanediol, D-fructose, D-galactose, D-glucose, D-gluconic acid, L-glutamic acid, glycerol, α-keto valeric acid, D-lactose, L-mallic acid, D-mannitol, D-mannose, D-melbiose, D-melezitose, mono-methyl succinate, D-psicose, L-serine, D-sorbitol, succinic acid, D-sucrose, D-trehalose, Tween 40, and Tween 80.

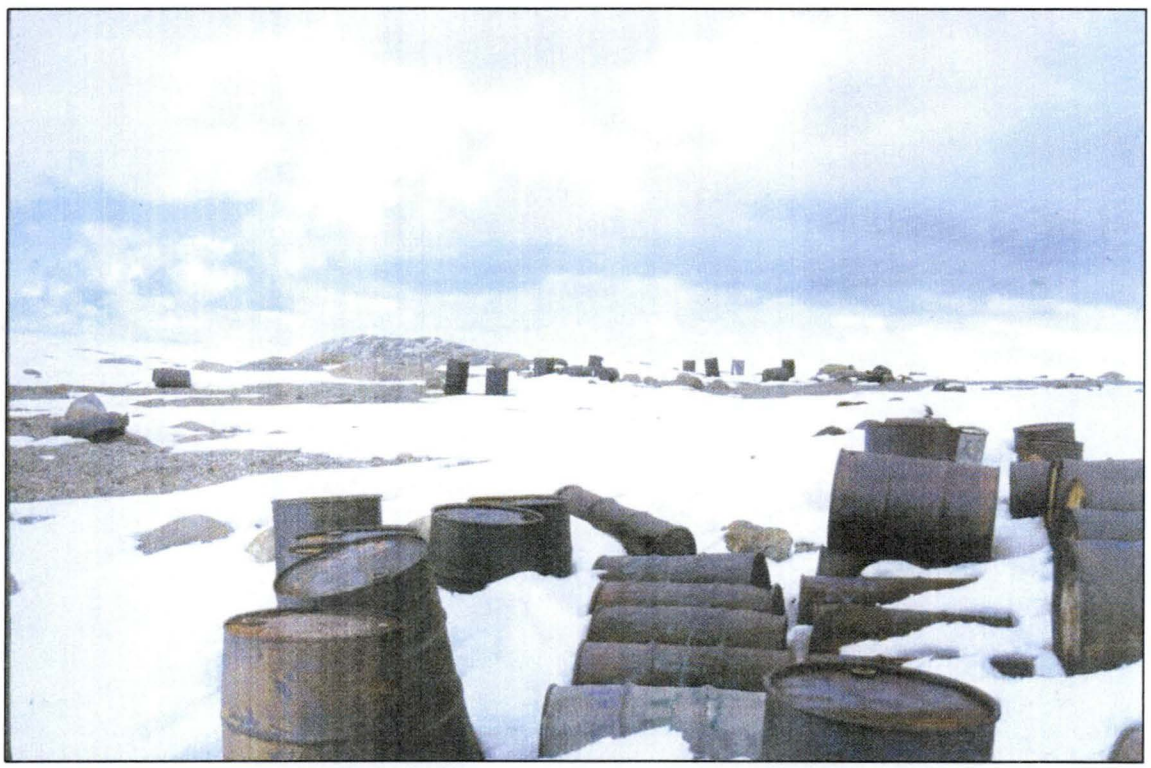
Growth occurred between 2°C and 30°C, pH 5.1-10.2 and up to and including 10% (w/v) NaCl and is comparable to that of UTAA127's closest relatives *R. fascians* and *R. yunnanensis*. All strains share a major quinone of MK-8(H_2) and are of the cell type A1γ. The total DNA G+C content of UTAA127, R. fascians, and R.

yunnanensis are 73.7, 64,1, and 63.5mol% respectively (Nesterenko et al., 1982, Zhang et al., 2005). Arabinose followed by galactose were the primary cell wall sugars detected in the cell wall hydrolysate of UTAA127. The information obtained from this polyphasic study does not clearly indicate whether UTAA127 is a strain of *R. fascians* or *R. yunnanensis*, so further investigation such as DNA-DNA hybridisation is required. Studies between *R. fascians* and *R. yunnanensis* revealed a similarity of 40.1% (Zhang et al., 2005), effectively eliminating the possibility that these three strains are of the same species. The major difference of G+C contents of UTAA127 to the other strains suggests there is a chance this isolate is a novel species and warrants further investigation.

2.3.8 Conclusions

This chapter focussed on 5 isolates from a 1600 strain collection of Antarctic Actinobacteria. Of these isolates, one was clearly shown to be a novel species of the genus *Promicromonospora*, with the other four warranting further investigation. To give five potentially novel species, only 150 isolates were examined through a relatively crude selection process, where 112 were excluded from the analysis as they were of the genus *Streptomyces*. This shows great promise for the rest of the UTAA collection as there are still 1450 isolates in the UTAA unanalysed. In addition to this, this is the first instance of a *Promicromonospora* isolate being isolated from Antarctica.

Chapter 3 The utilisation of hydrocarbons by Antarctic *Actinobacteria*



Oil drums

Despite a proactive approach to preserving the pristine condition of the Antarctic continent, many such dumping sites still exist

Photo courtesy of Dr Shane Powell

3.1 Introduction

3.1.1 Hydrocarbon pollutants in Antarctica

A driving force in the examination of the microbiota of an environment is to uncover useful physiological attributes that can solve current problems. The cleanup of polluted sites is an ongoing occurrence in the world, and many bioremediation companies today promote the use of microorganisms to speed up the breakdown of many toxic pollutants, including hydrocarbons. Hydrocarbons are molecules consisting of hydrogen and carbon that are sourced primarily from crude oil, and make up the oils and fuels in use today. Both research activities and tourism use vast amounts of diesel-based fuels in Antarctica and despite care being taken to avoid spills, accidents do occur, especially around the stations (Aislabie et al., 2004).

The main strategy when spills occur in Antarctica is for the physical removal of the pollutants, as the introduction of non-indigenous organisms is prohibited through the Madrid Protocol, an international agreement created in 1991 to protect the Antarctic environment. It is impossible to completely remove hydrocarbon contamination via physical means, and the natural microbial bioremediation process is not effective at low temperatures, despite a significant spike in bacterial concentrations after a contamination event (Delille et al., 1997). Australia's current contribution to Antarctic research involves work into understanding the processes that limit the bioremediation of petroleum contaminated soils (Ferguson et al., 2003). With careful management of chemical and environmental parameters, the natural bioremediation process can be sped up. An important factor in this is to learn more

about the identity and physiology of the microorganisms capable of degrading hydrocarbons.

3.1.2 Hydrocarbon degrading Antarctic bacteria

Several bacteria have been identified as being key players in hydrocarbon degradation in Antarctica. A study conducted on hydrocarbon-contaminated soil found the dominant bacterial members to be of the phylum *Proteobacteria* – namely *Pseudomonas*, *Sphingomonas*, and *Variovorax*. *Actinobacteria* were also identified belonging to the genera *Arthrobacter, Microbacterium, Nocarioides, Rhodococcus, Rubrobacter*, and *Streptomyces* (Saul et al., 2005). *Pseudomonas* and *Sphingomonas spp.* are known hydrocarbon degraders in Antarctica (Aislabie et al., 1998, Baraniecki et al., 2002), and *Pseudomonas* in particular has been implicated in possessing useful catabolic activity at low temperatures (Stallwood et al., 2005). Another member of the *Proteobacteria*, *Acinetobacter,* also has demonstrated hydrocarbon degrading ability in Antarctic conditions (Mac Cormack & Fraile, 1997). Studies conducted detecting *alkB* genes involved in alkane hydroxylation found the predominant bacteria responsible for alkane degradation in Antarctic soils to be *Rhodococcus*, a member of the phylum *Actinobacteria* (Luz et al., 2004, Whyte et al., 2002a). *Rhodococcus* isolates have been identified from Antarctica capable of growing on straight chain alkanes from hexane (C_6) to eicosane (C_{20}), and the branched alkane pristane (Bej et al., 2000). The potential does exist for the Antarctic bacteria identified here being used in bioremediation projects elsewhere on Earth, especially in cold environments such as Arctic or alpine locations.

3.1.3 UTAA collection

In the previous chapter, the UTAA collection was screened for novel bacterial species. Five such strains of interest were identified belonging to the genera *Arthrobacter*, *Promicromonospora*, and *Rhodococcus*. It has been well documented that strains of both *Arthrobacter* and *Rhodococcus* can utilise a wide range of carbon sources, including those that are toxic to many other organisms, and *Rhodococcus* was just mentioned as being the major force in alkane degradation in Antarctica. There is no data available on the ability of *Promicromonospora spp.* to utilise hydrocarbons, so this study will also shed light on the potential of this genus to do so.

3.1.4 Methodology to assess microbial hydrocarbon utilisation

Many methods exist for evaluating the growth of a bacterium on a hydrocarbon substrate. These methods range from most probable number techniques (Wrenn & Venosa, 1996), to the use of respiration indicators, such as WST-1 (Johnsen et al., 2002). A rapid method for evaluating hydrocarbon utilisation of bacteria is to coat a minimal media agar plate with the hydrocarbon of interest and observe zones of clearance and growth indicative of hydrocarbon degrading bacteria (Alley & Brown, 2000, Kiyohara et al., 1982, Tongpim & Pickard, 1996). This method does have downfalls, as artificial media may not accurately mimic a real life scenario. There may be problems ensuring a complete even covering of hydrocarbon over the agar plate, and oligotrophic bacteria may be able to utilise the agar or trace carbon giving rise to false positives. It must also be noted that Antarctic soil temperatures commonly exceed 20°C (Ferguson et al., 2003), so it is reasonable to assume that any hydrocarbon degradation activity shown *in vitro* could also occur in Antarctic

soils using temperatures as high as this. In fact, it has been demonstrated in Arctic tundra soil where hydrocarbons are able to be readily degraded at or above 0°C, that the effects are increased with a freeze-thaw cycle with temperatures comparable to that of Antarctic soils (Eriksson et al., 2001).

However, it is wise to include confirmatory tests on any bacterial isolates that grow on a hydrocarbon substrate that utilisation is indeed occurring. Numerous molecular studies have been conducted on detecting genes involved in the microbial degradation pathway of straight chain alkanes and polycyclic aromatic hydrocarbons based on *Rhodococcus* and *Pseudomonas* genomes (Andreoni et al., 2000, Luz et al., 2004, Nyyssonen et al., 2006). These studies have identified primer sets for alkane hydroxylase genes and naphthalene-hydroxylating dioxygenase genes that serve as useful indicators of whether a bacterium exhibited the potential for bioremediation studies.

3.1.5 Aims of this study

Five Actinobacteria isolates were scrutinised for their abilities to utilise hydrocarbons through traditional culturing experiments and via molecular screening for catabolic gene sequences. As four out of the five isolates in this Chapter are from genera with known biotechnological potential in biodegradation, it was hoped to identify any strains that could be used as future environmental remediation markers in Antarctica or as direct biological agents to clean hydrocarbon pollution elsewhere on Earth.

3.2 Materials and methods

3.2.1 Growth of bacterial isolates using volatile vapours as a sole carbon source

Testing was conducted for the utilisation of volatile vapours from kerosene, Special Antarctic Blend diesel (SAB), and methanol. 10ml of each substance was sealed within a preserving jar containing bacteria streaked onto Bushnell Haas agar (BH agar). BH agar is comprised of BH broth (0.2g/L $MgSO_4$, 0.02g/L $CaCl_2$, 1.0g/L KH_2PO_4, 1g/L $(NH_4)_2HPO_4$, 1g/L KNO_3, 0.05g/L $FeCl_3$. BD Diagnostics; Cat. #257820) supplemented with either 1.5% Bacteriological Agar No. 1 (Oxoid; Cat. #L11) or 1.5% Agarose I (Amresco; Cat. #0710-500g). Growth was compared to that of bacteria streaked onto BH agar in an identical preserving jar containing no volatile substances. Each strain in this assay was tested on triplicate plates. All glassware used in the preparation of the media were washed with chloroform to reduce any organic impurities, and the dH_2O was filtered using a 0.2μM filter to remove any large particulate matter that may be present.

3.2.2 Growth of bacterial isolates using solid phase hydrocarbons as a sole carbon source

Bacterial isolates were grown to pure culture then streaked onto BH agar. In a fume cabinet, 10mg of crystallised hydrocarbon dissolved in 1mL acetone was overlayed, leaving a thin film of the hydrocarbon to be evaluated once the acetone had evaporated. For alkanes with higher melting points, the alkane was heated in the

acetone to ensure it has dissolved (Sigma-Aldrich Pty Ltd; Cat. #298506, #300276). A negative control with no hydrocarbon overlay was also implemented. Plates were incubated at 20°C until growth was exhibited, with the exception of n-tetradecane and n-pentadecane where plates were incubated at 4°C to prevent them volatilising. Each hydrocarbon was tested for each bacterial strain in triplicate.

3.2.3 PCR amplification of hydrocarbon degradation gene sequences

The purified DNA used for the PCR amplification of hydrocarbon degradation gene sequences were obtained from the study conducted in section 2.2.3. The PCR was carried out using the conditions and primer pairs designed to target alkane monooxygenase and naphthalene dioxygenase gene sequences as conducted by Luz *et al* (Luz et al., 2004). The primer set *alkB1*-F2 (5'-ATCTGGGCGCGTTGGGATTTGAGCG-3') and *alkB1*-R1 (5'-CGCATGGTGATCGCTGTGCCGCTG C-3') amplify a 642bp fragment from the alkane monooxygenase gene of *Rhodococcus sp.* Q15 (AF388181), as does the primer set *alkB2*-F1 (5'-ACTCTGGCGCAGTCGTTTTACGGCC-3') and *alkB2*-R1 (5'-CCCACTGGGCAGGTTGGGCGCACCG-3'), which amplify a 552bp fragment (AF388182) (Whyte et al., 2002b). The primer set *ndoB*-F (5'-CACTCATGATAGCCTGATTCCTGCCCCCGGCG-3') and *ndoB*-R (5'-CCGTCCCACAACACACCCATGCCGCTGCCG-3') amplify a 642bp product corresponding to the naphthalene dioxygenease gene from *Pseudomonas putida* ATCC 17484 (M23914) (Kurkela et al., 1988). The PCR cycling conditions were as follows: 30 cycles each of 1 minute denaturation at 94°C, 1 minute annealing at 60°C, and 1 minute extension at 72°C, and a final extension of

3 minutes at 72°C (Luz et al., 2004). PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen Pty Ltd Cat. # 28704) and visualised on a 1.5% agarose gel stained with 500ng/ml Ethidium bromide.

3.2.4 Cloning and DNA sequencing of PCR products

Purified PCR products were cloned using a TOPO TA Cloning Kit for Sequencing (Invitrogen Corporation Cat. # K4575-J10) following the manufacturer's instructions. Clones were screened using the vector primers M13 Forward -20 (5'-GTAAAACGACGGCCAG-3') and M13 Reverse (5'-CAGGAAACAGCTATGAC-3'), and sequences were generated using the CEQ 8000 Genetic analysis system (Beckman Coulter Inc.) and its associated GenomeLab DTCS Quick Start Kit reagents (Cat. #608120).

3.2.5 Sequence analyses

Raw sequence files (*.scf) were imported into Sequencher version 4.5 (Gene Codes Corporation) where chromatograms were analysed and sequence fragments were aligned. Consensus sequences were then compared against other sequences on the Genbank database (Benson et al., 2003) using the BLAST function (Altschul et al., 1990). Catabolic sequences were also obtained from Genbank that were identified from publications of previous studies for comparative purposes (Heiss-Blanquet et al., 2005, Nyyssonen et al., 2006). The closest matches of each clone, as well as the closest cultured match were imported into the program SeaView where all sequences were aligned using the ClustalW function, and neighbour joining trees calculated using the Jukes-Cantor distance model and 500 bootstraps were then constructed

with the program PHYLO_WIN (Galtier et al., 1996). The resulting tree output was made into a publishable quality using Adobe Photoshop CS Version 8.0 (Adobe Systems Incorporated).

3.2.6 Nucleotide sequences

Nucleotide sequences derived from this study are included in the Appendix CD, and will be submitted to Genbank upon publication of said data.

3.3 Results

3.3.1 Growth of Actinobacteria isolates on oligotrophic media using hydrocarbon volatiles as a sole carbon source

Arthrobacter isolates UTAA053 and UTAA124, *Promicromonospora* isolate UTAA143, and *Rhodococcus* isolates UTAA121 and UTAA127 were screened for their ability to utilise kerosene, methanol, or SAB as a sole carbon source. UTAA053 showed little growth when exposed to each substrate, whilst UTAA124 grew well on each. Both *Rhodococcus* isolates UTAA121 and UTAA127 grew well with each carbon source and *Promicromonospora* UTAA143 grew well when exposed to kerosene and SAB, but exhibited little or no growth when exposed to methanol. For each bacterium, the negative control plates showed growth after 2 weeks at 20°C, with the *Promicromonospora* isolate UTAA143 softening the agar. Substituting the Bacteriological Agar No. 1 for Agarose I in the BH agar preparation reduced this effect.

3.3.2 Growth of Actinobacteria isolates using straight chain alkanes

The five selected *Actinobacteria* isolates were screened for their ability to utilise straight chain alkanes with carbon chain lengths ranging from C_{14}-C_{30}. The results from this screen is summarised in Table 3a. A "+" score indicates growth in all of the triplicate plates with more growth exhibited than that of the negative control plates, and a clear zone of clearance around the bacterial growth. A "+/-" score indicates that growth was exhibited in all triplicate plates but growth was comparable

to that of the negative plates in one or more of the three, and no clear zone of clearance was shown. A "-" score indicates growth was not exhibited in one or more of the replicate plates. In all instances where a "-" score was observed, no growth occurred in any of the replicate plates. Of particular note was that *Rhodococcus* isolate UTAA127 grew on all alkanes tested. *Promicromonospora sp.* UTAA143 grew well only on docosane, the only compound in this screen that definitely supported growth of all the bacteria tested.

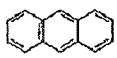
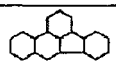
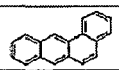
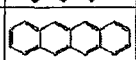
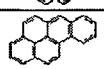
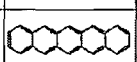
Table 3a Growth of UTAA isolates in the presence of various alkanes

Alkane	Molecular formula	Melting point	UTAA053	UTAA124	UTAA143	UTAA121	UTAA127
n-tetradecane	$C_{14}H_{30}$	5.5°C	-	+	-	-	+
n-pentadecane	$C_{15}H_{32}$	9.9°C	-	+	-	-	+
n-hexadecane	$C_{16}H_{34}$	18°C	-	+	-	+	+
n-heptadecane	$C_{17}H_{36}$	21°C	-/+	-	-	+	+
n-octadecane	$C_{18}H_{38}$	28-30°C	-/+	-	-	-	+
n-nonadecane	$C_{19}H_{40}$	32-34°C	-/+	-	-	-	+
n-eicosane	$C_{20}H_{42}$	36.7°C	-	-	-	-	+
n-heneicosane	$C_{21}H_{44}$	39°C	-/+	-	-	+	+
n-docosane	$C_{22}H_{46}$	42°C	+	+	+	+	+
n-tricosane	$C_{23}H_{48}$	48-50°C	-/+	-/+	-/+	+	-/+
n-tetracosane	$C_{24}H_{50}$	52°C	+	+	-	+	+
n-pentacosane	$C_{25}H_{52}$	53-56°C	-/+	+	-	+	+
n-hexacosane	$C_{26}H_{54}$	57°C	-/+	+	-	+	+
n-octacosane	$C_{28}H_{58}$	59°C	+	+	-	-	+
n-triacontane	$C_{30}H_{62}$	66°C	-/+	-	-	+	+

3.3.3 Growth of Actinobacteria isolates using polycyclic aromatic hydrocarbons

The testing for the utilisation of polycyclic aromatic hydrocarbons (PAHs) by the selected *Actinobacteria* isolates was carried out and scored as in the previous section. All of the strains were able to grow in the presence of azulene, benz[e]acephenanthrylene, and carbazole. Of particular note was *Rhodococcus sp.* UTAA127 that exhibited growth in the presence of 18 out of the 23 PAHs screened. A table summarising the findings is shown in Table 3b.

Table 3b Growth of UTAA isolates in the presence of various PAHs

Polycyclic Aromatic Hydrocarbon	Structure	UTAA053	UTAA124	UTAA143	UTAA121	UTAA127
acenaphthene		-	-/+	-	+	+
acenaphthylene		-	+	-	-	-
anthracene		-	-	-	+	+
azulene		-/+	-/+	-/+	+	-/+
benz[e]acephenanthrylene		+	+	-/+	+	+
1,2-benzanthracene		-	-	-	+	-/+
2,3-benzanthracene		-	-	-	-	+
benzo[ghi]perylene		-	-	-	-	-
benzo[a]pyrene		-	-	-	-	-
benzo[e]pyrene		-	-	-	-	-
carbazole		-/+	-/+	-/+	-/+	-/+
chrysene		+	-	-	-	+
coronene		-	-	-	-	+
4H-cyclopenta[def]phenanthrene		-	-	-	-	+
dibenzothiophene		-/+	-	-	+	+
fluoranthene		-	-	-	-	+
fluorene		+	+	+	-	-
naphthalene		-	-	-/+	-	+
pentacene		-	+	-	-	+
perylene		-/+	-/+	-/+	-	-/+
phenanthrene		-	-	-	-	-/+
pyrene		-	-	-	-	+
rubrene		+	+	-	+	+

3.3.4 Detection of catabolic genes involved in straight-chain alkane and naphthalene degradation

Each of the five selected *Actinobacteria* isolates were subjected to PCR screening for alkane hydroxylase gene sequences. *Rhodococcus* UTAA127 was the only isolate in which the PCR successfully amplified gene fragments the same size as cited in the literature. Figure 3i shows the similarity of this sequence between other published confirmed of putative alkane hydroxylase gene sequences. The *Rhodococcus* UTAA127 *alkB* sequence clearly clusters with that of the *Rhodococcus fascians* strain 154-S *alkB* sequence.

PCR screening using primers to detect naphthalene dioxygenase did not result in any PCR products matching the literature. In fact, the only isolate giving rise to any PCR products at all was that of *Rhodococcus* UTAA127, having a product of 493bp compared to the expected size of 642bp (data not shown). A BLASTx analysis of this sequence gave rise to only one peptide sequence giving any form of similarity, having a sequence similarity of 78% for 88% of the sequence giving an overall E-value score of 5e-82. This sequence is a putative *nusB* gene sequence for *Rhodococcus sp.* RHA1 that encodes for a protein involved in the regulation of rRNA biosynthesis by transcriptional antitermination. The phylogenetic relationship of these sequences to napththalene-hydroxylating dioxygenase gene sequences is shown in Figure 3ii. The gene sequence for *Rhododoccus* UTAA127 clustered most closely with that of the aforementioned *nusB* gene sequence, forming a clade separate to that of the majority of napththalene-hydroxylating dioxygenase gene sequences, but still lay within the whole gene family group.

Figure 3i **Neighbour Joining tree of *Rhodococcus sp.* UTAA127 *alkB* sequence with confirmed or putative alkane hydroxylase sequences obtained from Genbank. The *Pseudomonas putida xylM* sequence (AF019635) was used as an outgroup, and comparisons were made using the Jukes-Cantor model with 500 bootstrap replicates. The scale bar indicates 0.1 changes per nucleotide.**

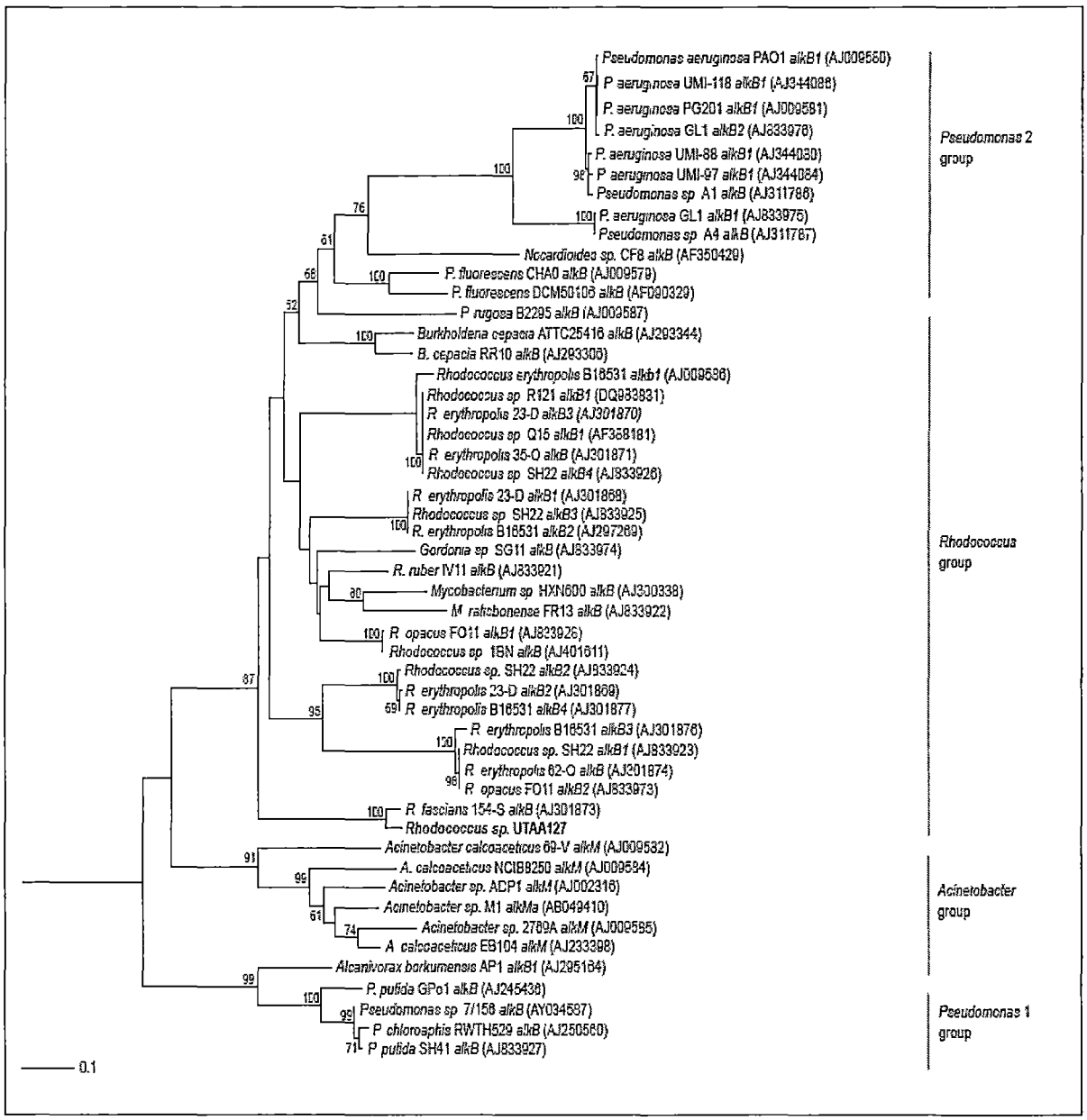
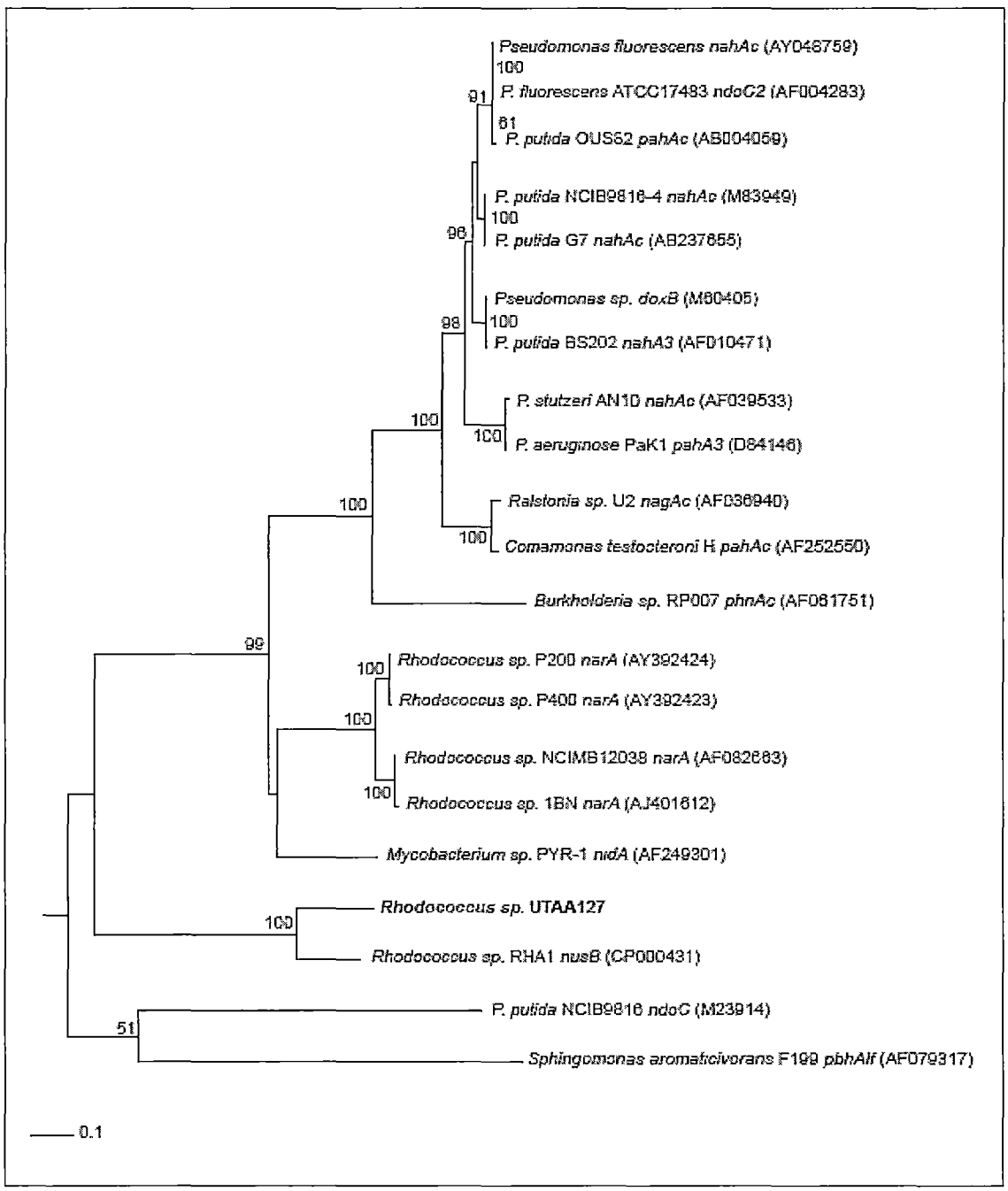

Pseudomonas aeruginosa PAO1 alkB1 (AJ009560)
P. aeruginosa UMI-118 alkB1 (AJ344086)
P. aeruginosa PG201 alkB1 (AJ009561)
P. aeruginosa GL1 alkB2 (AJ833976)
P. aeruginosa UMI-86 alkB1 (AJ344083)
P. aeruginosa UMI-97 alkB1 (AJ344084)
Pseudomonas sp. A1 alkB (AJ311786)
P. aeruginosa GL1 alkB1 (AJ833975)
Pseudomonas sp. A4 alkB (AJ311787)
Nocardioides sp. CF8 alkB (AF350429)
P. fluorescens CHA0 alkB (AJ009579)
P. fluorescens DCM50106 alkB (AF090329)
P. rugosa B2295 alkB (AJ009587)
Burkholderia cepacia ATTC25416 alkB (AJ293344)
B. cepacia RR10 alkB (AJ293306)
Rhodococcus erythropolis B16531 alkb1 (AJ009596)
Rhodococcus sp. R121 alkB1 (DQ988831)
R. erythropolis 23-D alkB3 (AJ301870)
Rhodococcus sp. Q15 alkB1 (AF358181)
R. erythropolis 35-O alkB (AJ301871)
Rhodococcus sp. SH22 alkB4 (AJ833926)
R. erythropolis 23-D alkB1 (AJ301869)
Rhodococcus sp. SH22 alkB3 (AJ833925)
R. erythropolis B16531 alkB2 (AJ297269)
Gordonia sp. SG11 alkB (AJ833974)
R. ruber IV11 alkB (AJ833921)
Mycobacterium sp. HXN600 alkB (AJ300338)
M. ratisbonense FR13 alkB (AJ833922)
R. opacus FO11 alkB1 (AJ833926)
Rhodococcus sp. 16N alkB (AJ401611)
Rhodococcus sp. SH22 alkB2 (AJ833924)
R. erythropolis 23-D alkB2 (AJ301868)
R. erythropolis B16531 alkB4 (AJ301877)
R. erythropolis B16531 alkB3 (AJ301876)
Rhodococcus sp. SH22 alkB1 (AJ833923)
R. erythropolis 62-O alkB (AJ301874)
R. opacus FO11 alkB2 (AJ833973)
R. fascians 154-S alkB (AJ301873)
Rhodococcus sp. UTAA127
Acinetobacter calcoaceticus 69-V alkM (AJ009532)
A. calcoaceticus NCIB8250 alkM (AJ009534)
Acinetobacter sp. ADP1 alkM (AJ002316)
Acinetobacter sp. M1 alkMa (AB049410)
Acinetobacter sp. 2789A alkM (AJ009535)
A. calcoaceticus EB104 alkM (AJ233398)
Alcanivorax borkumensis AP1 alkB1 (AJ295184)
P. putida GPo1 alkB (AJ245436)
Pseudomonas sp. 7/156 alkB (AY034597)
P. chloroaphis RWTH529 alkB (AJ250560)
P. putida SH41 alkB (AJ833927)
0.1
Pseudomonas 2 group
Rhodococcus group
Acinetobacter group
Pseudomonas 1 group

Figure 3ii **Neighbour Joining tree of the putative naphthalene degradation gene sequence obtained from *Rhodococcus sp.* UTAA127 and the *Rhodococcus sp.* RHA1 *nusB* gene with naphthalene-hydroxylating dioxygenase gene sequences obtained from Genbank. The *Sphingomonas yanoikuyae* B1 *bphC* sequence (U23374) was used as an outgroup, and comparisons were made using the Jukes-Cantor model with 500 bootstrap replicates. The scale bar indicates 0.1 changes per nucleotide.**


Pseudomonas fluorescens nahAc (AY048759)
100
91
P. fluorescens ATCC17483 ndoC2 (AF004283)
81
P. putida OUS82 pahAc (AB004059)
P. putida NCIB9816-4 nahAc (M83949)
100
98
P. putida G7 nahAc (AB237655)
Pseudomonas sp. doxB (M60405)
98
100
P. putida BS202 nahA3 (AF010471)
P. stutzeri AN10 nahAc (AF039533)
100
100
P. aeruginose PaK1 pahA3 (D84146)
100
Ralstonia sp. U2 nagAc (AF036940)
100
Comamonas testosteroni H pahAc (AF252550)
Burkholderia sp. RP007 phnAc (AF061751)
99
100
Rhodococcus sp. P200 narA (AY392424)
Rhodococcus sp. P400 narA (AY392423)
100
Rhodococcus sp. NCIMB12038 narA (AF082663)
100
Rhodococcus sp. 1BN narA (AJ401612)
Mycobacterium sp. PYR-1 nidA (AF249301)
Rhodococcus sp. UTAA127
100
Rhodococcus sp. RHA1 nusB (CP000431)
P. putida NCIB9816 ndoC (M23914)
51
Sphingomonas aromaticivorans F199 pbhAII (AF079317)
0.1

3.4 Discussion

3.4.1 Hydrocarbon degradation abilities of selected Actinobacteria isolates

Rhodococcus strain UTAA127 was shown to degrade the straight chain alkanes tetradecane (C_{14}) and pentadecane (C_{15}) (tested only at 4°C), and from hexadecane (C_{15}) to triacontane (C_{30}) (tested only at 20°C), with the possible exception of tricosane (C_{23}). An alkane hydroxylase gene sequence similar to that of a *Rhodococcus fascians* 154-S strain was identified to support these observations. In addition to this, growth was observed in the presence of the polycyclic aromatic hydrocarbons acenaphthene, anthracene, benz[e]acephenanthrylene, 2,3-benzanthracene, chrysene, coronene, 4H-cyclopenta[def]phenanthrene, dibenzothiophene, fluoranthene, naphthalene, pentacene, pyrene, and rubene. In the previous chapter it was revealed this isolate grew between the temperatures of 2-30°C, which provides a growth range suitable for both cold climate and temperate bioremediation.

The other *Rhodococcus* isolate, UTAA121, also exhibited the ability to grow in the presence of a wide range of straight chain alkanes, but an alkane hydroxylase gene was not detected using the PCR primers and conditions used. Each of the polycyclic aromatic hydrocarbons this strain was able to utilise, UTAA127 was able to do so also, indicative of common metabolic pathways shared by *Rhodococcus spp.*. The two *Arthrobacter* strains gave different results. *Arthrobacter* UTAA053 gave inconsistent results showing a small amount of growth with many hydrocarbons but giving no true indication of an ability to utilise these substrates. It can be concluded

that this strain is resistant to the toxic effects of many hydrocarbons and is oligotrophic. *Arthrobacter* UTAA124 on the other hand, was not able to grow as readily in comparison. The *Promicromonospora* isolate UTAA143 did not exhibit a great capacity to utilise hydrocarbons under the conditions of this assay. The only substrate that this isolate definitively exhibited catabolic activity over was fluorene. The only other hydrocarbon identified in this screening process was docosane (C_{22}). Every isolate in this study showed degradation abilities of this compound, which brings into question the process of this degradation or whether there was contamination of this compound with another readily available energy source.

3.4.2 Naphthalene-hydroxylating dioxygenase (ndoB) gene detection

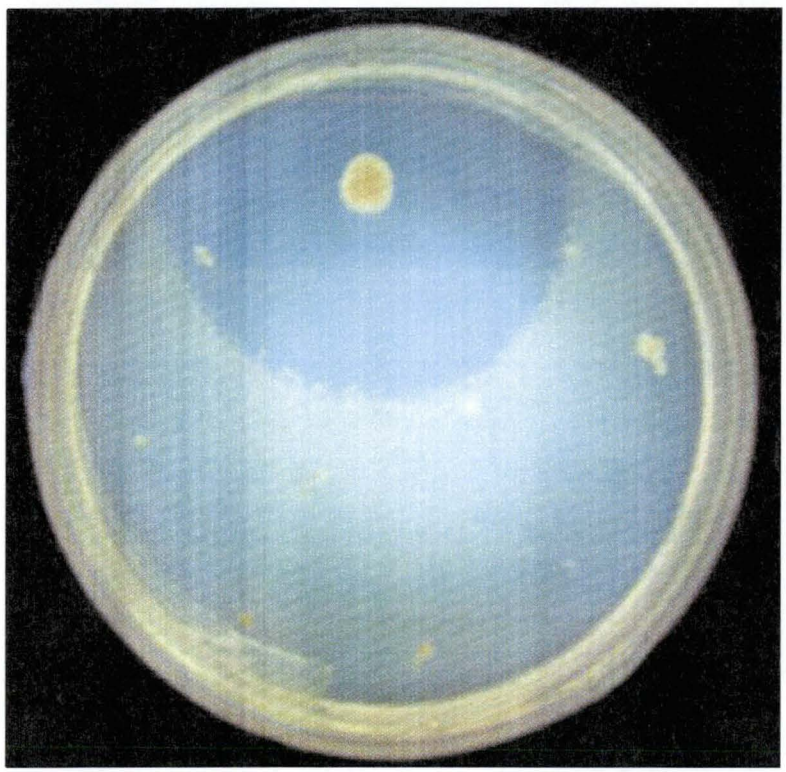
The use of PCR primers to detect catabolic gene sequences for naphthalene degradation based on the genome of *Pseudomonas* is the likely cause to why no definitive gene sequences were detected in this assay. In future experiments, it would be of interest to try *Rhodococcus* specific PAH dioxygenases PCR primers to ascertain whether other gene sequences of interest can be identified (Moser & Stahl, 2001). That being said, a related sequence of *Rhodococcus* UTAA127 was obtained, but it is uncertain whether this gene fragment is involved in the degradation pathway of naphthalene. One study using the same set of PCR primers as in this study found *ndoB*-related sequences from *Klebsiella oxytoca*, *Herbaspirillum seropedicae*, and *Bacillus megaterium* isolates, but as these bacteria were found not to catabolise naphthalene, it was suggested these genes may have been involved in trifluralin degradation (Bellinaso et al., 2003). It is also entirely possible that the gene product detected from *Rhodococcus* UTAA127 was of a novel form previously uncharacterised, as there is suggestion that ring-hydroxylating dioxygenases in

nature are extremely diverse (Yeates et al., 2000), or that the primers used are non-specific for non-*Pseudomonas* spp.

3.4.3 Conclusions

Through this investigation of the hydrocarbon degrading ability of select *Actinobacteria* strains, one isolate in particular stood out as a potentially useful biological agent to clean up environmental hydrocarbon spills. *Rhodococcus sp.* UTAA127 displayed the ability to utilise a wide range of straight chain alkanes and polycyclic aromatic hydrocarbons. While Antarctic treaties prohibiting importation of strains such as *Rhodococcus sp.* UTAA127 mean they cannot be directly implemented in environmental cleanups on the Antarctic continent, other environments exist that may benefit. Further research is needed to fully assess the feasibility of the industrial application of this strain for bioremediation.

Chapter 4 Antimicrobial screening of Antarctic *Actinobacteria*



Agar diffusion bioassay for the detection of antagonistic compounds

Inhibition of a *Listeria monocytogenes* strain by an Antarctic *Streptomyces* isolate

Photo courtesy of Mr Claudio Bittencourt

4.1 Introduction

4.1.1 The potential antimicrobial resource of the UTAA collection

In 1915 a particular soil microorganism named *Streptomyces griseus* was discovered, and later it was found to have produced the antibiotic called streptomycin. This antibiotic revolutionised medical science and brought worldwide attention to *Actinobacteria.* These remarkable bacteria are responsible for the majority of the antimicrobial products known today (Lazzarini et al., 2000). Antimicrobial products are continually being discovered, and it would seem that this would flood the market, but there also lies a highly specific demand for novel compounds able to safely combat high resistant microorganisms, both for pharmaceutical and industrial usage.

As mentioned in Chapter Two, the UTAA collection primarily consists of isolates that morphologically fit the genus *Streptomyces*. There is no doubt that this genus is one of the most important producers of antimicrobial products, but still there is evidence to suggest that only a small percent of the potential products from *Streptomyces* have been discovered (Watve et al., 2001). This leads to the idea of screening this archive of bacteria for the production of potentially novel antimicrobial compounds, and may even reveal novel *Actinobacteria* species. Previous attempts to screen bacteria isolated from Antarctica for antimicrobial compounds have been performed (Nedialkova & Naidenova, 2004-2005, O'Brien et al., 2004), and was the primary reason why the UTAA collection was created.

4.1.2 Streptomyces

The singular member of the family *Streptomycetaceae*, the genus *Streptomyces* is comprised typically of soil-borne bacteria. Members of this genus are Gram-positive aerobic microorganisms possessing a DNA G+C content of 69-78 mol%, branching substrate and aerial mycelium and a Type I cell wall peptidoglycan that is lacking characteristic cell walls amino acids instead exhibiting LL-diaminopimelic acid and glycine (Anderson & Wellington, 2001, Korn-Wendisch & Kutzner, 1992, Lechevalier & Lechevalier, 1970). A literature survey of the Pfizer Inc. Biosearch Italia Database referred to as the Antibiotic Literature Database showed that over half of the antimicrobial products listed come from *Actinobacteria*, predominantly from the genus *Streptomyces* (45.6%) (Lazzarini et al., 2000). The discovery of antibiotics produced by *Streptomyces* in the 1940s sparked a massive over-speciation of this genus, from approximately 40 to over 3000. The rules governing the taxonomy of *Streptomyces* were overhauled and currently this genus includes 533 validly described species (http://www.bacterio.cict.fr/s/streptomycesa.html).

4.1.3 Methods for screening for antimicrobial compounds

The methodology to be employed in this study is regarded as a random-screening approach to drug discovery. This type of screening can be advantageous, as it is known that any compound discovered penetrates cell membranes and the effects tend to be reproducible. However, often the active compounds found are toxic and the effects non-specific, and several mechanisms may be involved in the antagonism effect (Allsop, 1999). A commonly used and well-established method for determining antagonism is referred to as an agar diffusion assay. This method works

on the principle that the antimicrobial compound in question is able to move through the matrix of an agar plate. The distance the compound migrates before diluting itself out is directly related to the initial concentration making this method semi-quantitative (Pongtharangkul & Demirci, 2004). A multitude of alternative methods are in use today, ranging from high-throughput microtitre well testing using absorbance as an indicator of growth/no growth to nonculturing molecular methods searching for the genes directly responsible for antimicrobial compounds or enzymes that synthesise the secondary metabolites (Casey et al., 2004, Malapaka et al., 2007, Moore et al., 2005). For the purposes of this study, it was decided that a well-established method with minimum need for method development or optimisation was required, such as the agar diffusion assay.

4.1.4 Choice of a target organism

Listeria monocytogenes is a Gram-positive, facultatively anaerobic bacterium of the phylum *Firmicutes* and class *Bacilli*. It is a food borne pathogen, affecting primarily the elderly, new borne, pregnant, and immunocompromised resulting in a severe disease state referred to as listeriosis. The foods implicated in infections range largely from raw and processed foods (Rocourt & Cossart, 1997), and as with all bacterial pathogens, antimicrobial resistance is a concern (Charpentier & Courvalin, 1999, Yucel et al., 2005). To screen this microorganism against 1600 bacterial isolates for antimicrobial activity is bound to be labour intensive and it was decided that the initial screening be conducted in collaboration with Claudio Bittencourt, a fellow PhD student studying non-thermal deactivation strategies for *L. monocytogenes*. This allowed access to the multiple strains of *L. monocytogenes*, as strain-to-strain variation in *L. monocytogenes* exists in response to antagonism by

antibiotic compounds (Aarestrup et al., 2007). Five different strains were analysed to increase the chance that organisms will be isolated that produce broad-spectrum acting antimicrobials.

4.1.5 Aims of this study

This chapter will explore an alternate means of exploring the UTAA collection. The knowledge gained from this screen will primarily give insight into the antimicrobial capabilities of Antarctic Actinobacteria. In the process of doing so, potentially novel bacteria may be identified and in turn give rise to new beneficial bioactive molecules. Choosing an initial antagonistic target such as *Listeria monocytogenes* gives a unique insight also into the potential applications possible for Antarctic bacteria and the products they produce.

4.2 Materials and methods

4.2.1 Origin and maintenance of bacterial strains

The 1600 isolates belonging to the UTAA collection were cultivated as described in section 2.2.1. These were the producer strains in this study. The test *L. monocytogenes* strains FRRW2343, FRRW2345, and FRRB2542 were obtained from CSIRO Food Science Australia (NSW). FRRW2343 was isolated from salad containing pasta, cheese, and ham/bacon. FRRW2345 was isolated from ham, and FRRB2542 from salami. *L. monocytogens* strain L5-22 is a cold smoked salmon isolate obtained from the Australian Food Safety Centre of Excellence. The type strain for *L. monocytogenes*, ATCC 15313 is a clinical isolate and is referred to as the Scott A strain. The *Bacillus cereus*, *Listeria innocua*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Vibrio parahaemolyticus*, and *Yersinia enterocolitica* were obtained from the Microbiology teaching unit culture collection at the University of Tasmania.

4.2.2 Agar diffusion assay to determine anti-microbial susceptibility

A modified version of the agar diffusion assay successfully used by O'Brien *et al* was employed (Kelkessy & Piguet, 1970, O'Brien et al., 2004). Briefly, this involved growing each UTAA strain on MA for 14 days at 20°C, then stab inoculating each culture in duplicate onto MA plates. These plates were incubated at 10°C for another 14 days. The plates were then overlayed with a soft (0.7%)

Tryptone Soya Agar containing a final concentration of 10^5-10^6cfu/ml of the test organism. For each assay the test organism was cultivated directly from a frozen glycerol stock in Tryptone Soya Broth (TSB). The overlay plates were subsequently incubated at 10°C for several days until negative controls of the test organisms showed growth. Antagonism was inferred by a zone of no growth around the producer strains of greater than 2mm occurring in both of the duplicates. This was measured from the edge of the producer strain colony to the outer edge of the zone of no growth.

4.2.3 Enzymatic degradation of produced antimicrobials

5uL of a 10mg/ml solution of the selected proteolytic enzyme made up in 0.1M sodium phosphate solution was injected before the agar overlay step in the agar diffusion assay to determine whether the antimicrobial compounds present were proteinaceous. Positive results are indicated by a crescent formation displaying a lack of inhibition at the injection site. The enzymes chosen were alpha-chymotrypsin, pronase E, and proteinase K. All of these enzymes were obtained from Sigma Aldrich.

4.2.4 Molecular identification of producer strains

The DNA extraction, PCR amplification and subsequent sequencing and analyses were carried out as described in sections 2.2.3-2.2.6.

4.3 Results

4.3.1 *Inhibition of Listeria monocytogenes strains by the UTAA collection*

The UTAA collection comprising of 1600 presumed *Actinobacteria* isolates was screened for antagonism against 5 different strains of *L. monocytogenes*. In this process 337 isolates could not be resuscitated from their respective glycerol stocks for the screening process. The results of the remaining 1263 UTAA isolates are summarised in Table 4a.

Table 4a Summary of antimicrobial screening of UTAA isolates against *Listeria monocytogenes* strains

***Listeria monocytogenes* strain**	**No. of 1263 UTAA isolates displaying antagonism (% screened)**
Type strain ATCC 15313 (Scott A)	95 (7.5%)
FRRW2343	97 (7.7%)
FRRW2345	110 (8.7%)
FRRB2542	90 (7.1%)
L5-22	74 (5.9%)
All of the above	21 (1.7%)
Any of the above	199* (15.8%)

* - Represents the total number of UTAA isolates showing antagonism against *L. monocytogenes*

The 199 isolates identified in Table 4a shown to exhibit antagonism against at least one strain of *L. monocytogenes* were re-screened against the same five strains. This

assay was conducted in duplicate as the first, and there appeared to be many instances where one duplicate showed antagonism and the other did not. The results from this screen are summarised in Table 4b.

Table 4b Summary of antimicrobial screening of known producing UTAA isolates against *Listeria monocytogenes* strains

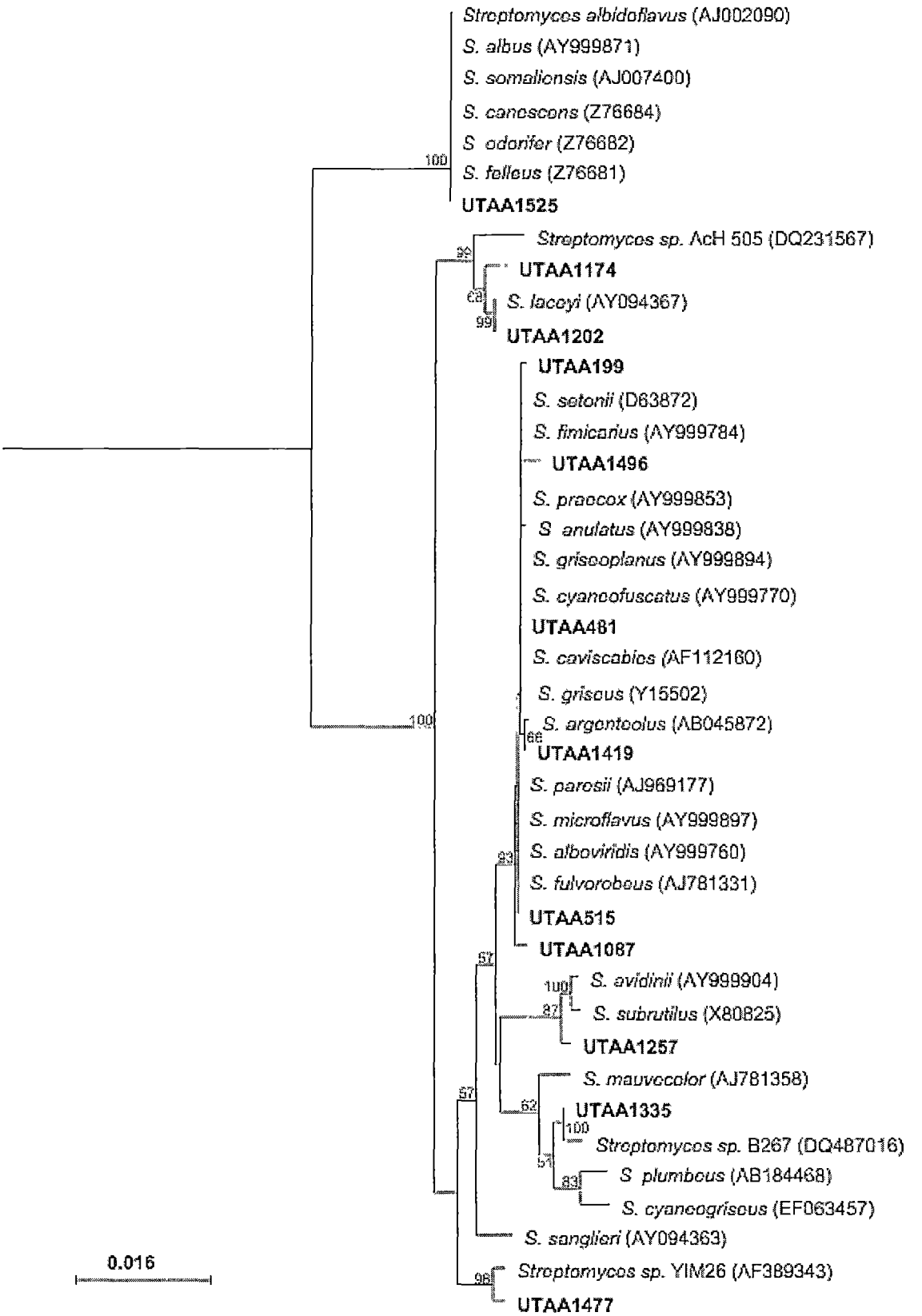
	No. of 199 UTAA isolates displaying antagonism	
Listeria monocytogenes strain	At least 1 out of 2 replicates (% screened)	2 out of 2 replicates (% screened)
Scott A	140 (70.4%)	48 (24.1%)
FRRW2343	110 (55.3%)	27 (13.6%)
FRRW2345	141 (70.9%)	54 (27.1%)
FRRB2542	135 (67.8%)	58 (29.1%)
L5-22	111 (55.8%)	45 (22.6%)
All of the above	56 (28.1%)	**20** (10.1%)

4.3.2 Identification of the antimicrobial producers from the UTAA collection

The 20 UTAA isolates identified in section 4.3.1 as reproducibly inhibiting all five strains of *L. monocytogenes* had their respective 16S rRNA genes sequenced and identities determined. Of the 20 isolates, it was determined that 12 unique sequences were present. Strains UTAA 1087, UTAA1065, UTAA1204 and UTAA1366 all shared a 100% homology, as did UTAA1139, UTAA1138, UTAA1120, and

UTAA1120; UTAA1174 and UTAA1138; UTAA515 and UTAA1478; and UTAA1496 and UTAA91. All of these strains were of the genus *Streptomyces*, and the phylogenetic relationship of these strains with other *Streptomyces spp.* are shown in Figure 4i. The isolate showing the least amount of similarity to any described *Streptomyces* species is UTAA1477, being 98.89% similar to *S. sanglieri* over a 1363bp region of their respective 16S rRNA gene sequences. It also shares a 99.57% match with *Streptomyces sp.* YIM26 over a 1406bp region.

Figure 4i **16S rRNA gene Neighbour Joining tree of *Streptomyces* isolates with closest matches from Genbank based on a total alignment length of 1317bp. *Bifidobacterium bifidum* (AY694148) and *Arthrobacter globiformis* (X80736) were used as outgroups, and comparisons were made using the Kimura two-parameter model with 1000 bootstrap replicates. Only bootstrap values greater than 50 are shown. The scale bar indicates 0.014 changes per nucleotide.**


Streptomyces albidoflavus (AJ002090)
S. albus (AY999871)
S. somaliensis (AJ007400)
S. canescens (Z76684)
S odonifer (Z76682)
S. felleus (Z76681)
UTAA1525
Streptomyces sp. AcH 505 (DQ231567)
UTAA1174
S. lacoyi (AY094367)
UTAA1202
UTAA199
S. setonii (D63872)
S. fimicanus (AY999784)
UTAA1496
S. praecox (AY999853)
S anulatus (AY999838)
S. griseoplanus (AY999894)
S. cyaneofuscatus (AY999770)
UTAA481
S. caviscabies (AF112160)
S. griseus (Y15502)
S. argentoolus (AB045872)
UTAA1419
S. parosii (AJ969177)
S. microflavus (AY999897)
S. alboviridis (AY999760)
S. fulvorobeus (AJ781331)
UTAA515
UTAA1087
S. avidinii (AY999904)
S. subrutilus (X80825)
UTAA1257
S. mauvecolor (AJ781358)
UTAA1335
Streptomyces sp. B267 (DQ487016)
S plumbeus (AB184468)
S. cyaneogriseus (EF063457)
S. sanglieri (AY094363)
Streptomyces sp. YIM26 (AF389343)
UTAA1477
0.016

4.3.3 Enzymatic degradation of anti-microbial compounds

The anti-microbial compounds produced by *Streptomyces* isolates UTAA199, UTAA481, UTAA1138, and UTAA1496 were found to be proteinaceous in nature as they were digested using alpha-chymotrypsin. Pronase A and proteinase K were only able to digest the compounds produced by UTAA199 and UTAA481, and UTAA1496.

4.3.4 Antagonistic specificity of anti-microbial compounds

Table 4c shows the antagonism of the selected UTAA isolates against a range of Gram-positive and Gram-negative bacteria. UTAA1477 displayed varying degrees of inhibition against both Gram-positive and Gram-negative bacteria, but was unable to inhibit *Staphylococcus aureus*. None of the UTAA strains were able to inhibit the *Pseudomonas aeruginosa* strain tested.

4.3.5 Phenotypic characterisation of Streptomyces sp. UTAA1477

The phenotypic properties of *Streptomyces sp.* UTAA1477 and *Streptomyces sanglieri* were examined to further investigation the possibility of shared speciation. Table 4d shows the pigmentation exhibited by UTAA1477 and *Streptomyces sanglieri* on ISP medium 2 and 3. Under microscopic examination UTAA1477 exhibited the spiral and open looped of smooth surfaced sporeson aerial hyphae as described for *S. sanglieri* (Manfio et al., 2003).

Table 4c Summary of antimicrobial screening of select UTAA isolates against various bacterial strains

	Gram Positive Bacteria				*Gram Negative Bacteria*				
	Bacillus cereus	*Listeria innocua*	*Staphylococcus aureus*	*Streptococcus faecalis*	*Escherichia coli*	*Klebsiela pneumoniae*	*Salmonella typhimurium*	*Vibrio parahaemolyticus*	*Yersinia enterocolitica*
UTAA199	-	-	-	-	-	-	-	-	-
UTAA481	-	-	-	-	-	-	-	+	-
UTAA515	++	+	+++	+++	-	-	-	-	-
UTAA1087	-	-	-	++	-	-	-	-	-
UTAA1174	++	++	++	++	-	-	-	-	-
UTAA1202	+++	++	+++	+++	-	-	-	-	-
UTAA1257	-	-	-	-	-	-	-	-	-
UTAA1335	+	+	+	+	-	-	-	-	-
UTAA1419	+++	+	+++	+++	-	-	-	-	-
UTAA1477	+++	+	-	+	+	+	+	+++	+
UTAA1496	+++	+++	+++	+++	-	-	-	-	-
UTAA1525	+	-	+	-	-	-	-	-	-

+++ = large zone of inhibition (≥1mm zone of clearance radius); ++ = moderate inhibition (6-10mm), + = slight inhibition (1-5mm); - = no effect observed NB. All strains tested negative for *Pseudomonas aeruginosa*.

Table 4d Pigmentation of UTAA1477 and *Streptomyces sanglieri*

	Mycelium type	**ISP Medium 2 (Yeast/malt extract agar)**	**ISP Medium 3 (Oatmeal Agar)**
UTAA1477	Aerial	White	White
	Substrate	Orange	Deep yellow (pigmentation)
Streptomyces sanglieri	Aerial	Grey*	Grey*
	Substrate	Red/orange*	Red/orange (pigmentation)*

* - Sourced from published data (Manfio et al., 2003)

4.4 Discussion

4.4.1 *The outcomes of the antimicrobial screening against Listeria monocytogenes*

Bearing in mind the antimicrobial screening discussed in this chapter is base purely on *Actinobacteria*, the primary "hit rate" of the antimicrobial screen described in this chapter of 15.8% was much higher than that of other studies by two orders of magnitude (Coventry et al., 1997, O'Brien et al., 2004, Vaughan et al., 1994). This is comparable to that of screens focussed on environments where antimicrobial activity is already known, such as a study carried out on bacteria isolated from Mediterranean sponges with a 11.3% hit rate (Hentschel et al., 2001). *Actinobacteria* were also found to be highly effective against *Listeria monocytogenes* and confirms *Actinobacteria* as being an important source of antimicrobial products. Focussing on the suspected "broad spectrum" antimicrobial producers, all were identified as being of the genus *Streptomyces*. Four of the identified organisms were found to produce proteinaceous compounds. This is not surprising, as *Streptomyces* are known producers of type A and B lantibiotics, antimicrobial peptides capable of creating pores in cell membranes (Chen & Hoover, 2003, Sahl & Bierbaum, 1998).

4.4.2 Antimicrobial screening as a taxonomic tool

Based on 16S rRNA gene sequencing alone, a definitive classification of *Streptomyces* isolates to the species level is not possible. It is not uncommon for *Streptomyces* species to be nearly identical in their 16S rRNA gene sequences (Anderson & Wellington, 2001). *Streptomyces sp.* UTAA1525 shared the same level of homology with six different species, and it is nigh impossible to distinguish *Streptomyces* isolates UTAA199, UTAA1496, UTAA481, UTAA1419, UTAA515, and UTAA1087 from the other 13 isolates in the represented cluster without further investigation. The only isolate identified in this study as not closely grouping with any described *Streptomyces* species was UTAA1477.

4.4.3 Streptomyces sp. UTAA1477

Streptomyces sp. UTAA1477 was first isolated from rabbit droppings on Macquarie Island. Through 16S rRNA gene comparisons this isolate is most closely related to *Streptomyces sp.* YIM26, an uncharacterised psychrotolerant *Streptomyces sp.* isolated from soil in China. The next closest relative according to 16S rRNA gene sequence identity is *Streptomyces sanglieri*. The first strain of this species isolated was from soil associated with meadow hay plots in the United Kingdom. *S. sanglieri* produces the antibiotic and antitumour compound Lactonamycin Z (Holtzel et al., 2003). This compound inhibits a range of Gram-positive bacteria including *Staphylococcus aureus* (ATCC 12600). *Streptomyces sp.* UTAA1477 was unable to inhibit this organism, suggesting these may be two separate compounds. Antibiotic production may be a result of horizontal gene transfer however (Anderson et al.,

2000), and a novel or different compound is not necessarily an indication of novel speciation.

Both UTAA1477 and *S. sanglieri* look comparable in cell morphology, but differ in pigmentation as shown in Table 4d. This in itself is not enough to justify these two isolates are separate species, but it does warrant that *Streptomyces* isolate UTAA1477 be studied in more detail, examining defining attributes such as DNA-DNA hybridisation comparisons with *S. sanglieri*, utilisation of different carbon sources, and analysis of its genomic G+C content (Manfio et al., 2003). In any event the antimicrobial compound produced by UTAA1477 needs to be purified and characterised, as it clearly exhibits potential as a broad-spectrum antimicrobial compound, and any comparisons made should be made in parallel with *Streptomyces sp.* YIM26 as it is probable they are both strains of the same species.

4.4.4 Future work

Aside from a more focussed study on *Streptomyces sp.* UTAA1477, more information can be gleamed out of the rest of the UTAA collection. Despite the high hit rate of this antimicrobial screening, alternative methods exist that can be employed to complement this work. Knowing after the fact that the UTAA collection is primarily comprised of the genus *Streptomyces* including those with the most potential as antimicrobial producers it makes sense to suggest a more focussed approach on this genus for any future studies on the UTAA collection. Antibiotics tend to be secondary metabolites, and even though *Streptomyces* secondary metabolite production may be strain specific, rather than at the species level (Anderson & Wellington, 2001), it would be worth investigating this process at a more detailed level.

A range of enzymes known in secondary metabolite production, polyketide synthases, are known for being produced by *Actinobacteria* (Wawrick et al., 2005), particularly *Streptomyces* (Gonzalez et al., 2005). The value of this information is that studies have shown that polyketide synthases from Actinobacteria have an evolutionary relationship to each other and could possibly be a tool to determine *Streptomyces* speciation (Jenke-Kodama et al., 2005). The obvious limit of this idea is that the polyketide synthase genes from all currently described *Streptomyces* species needs to be sequenced and evaluated first.

Nonetheless this chapter has reaffirmed the great antimicrobial potential of Antarctic *Actinobacteria*, in particular of the genus *Streptomyces* and has identified a strain that may be of biotechnological interest.

Chapter 5 Bacterial diversity of epiglacial lakes of the Framnes Mountains

Lake Bicuspid

An epiglacial lake associated with the Framnes Mountains, Antarctica.

Photo courtesy of Dr Thomas Pickard

5.1 Introduction

5.1.1 Epiglacial lakes

Epiglacial lakes are freshwater lakes that are perennially covered in ice. They form on the downward ice flow side of mountains below glacial ice. During summer, heat is stored in the rock and melt water accumulates below the glacial ice, and over the years more water is added as more heat is stored in the rock. The glacial ice that caps this type of lake eventually lessens through ablation and summer melt, with ice underneath being formed from refreezing lake water, eventually replacing all of the glacial ice. They are moulded and subsequently enclosed by rock and ice, and differ from ephemeral lakes in that these lakes are generally permanent features in their respective geological landscape. These lakes occur throughout Antarctica where mountains break through the ice cap and considering the wide distribution of mountain ranges on Antarctica; epiglacial lakes may be the most common lake type on the continent. Little is known about epiglacial lakes however, so this chapter will endeavour to counter this by taking a preliminary look at the types of microbiota that inhabit certain Antarctic epiglacial lakes.

5.1.2 The epiglacial lake network of the Framnes Mountains

Located approximately 30 kilometres from Mawson Station are the Framnes Mountains (67°50'S 62°35'E), a group of mountains on the Antarctic continent that include Mt Henderson, and the Casey, Masson, and David Ranges. On the down wind facing side of the nunataks and ranges are at least 15 epiglacial lakes, varying

in area from 0.02-0.85km^2. A survey was conducted in the summer of 2004/2005 that looked at eight of these lakes in an attempt to elucidate their previously unknown limnology (Gibson, 2005). The lakes included in this investigation were Lake Bicuspid, Lake Burnett, Lake Henderson, Lake Linear, Northdoodle Lake, Painted Lake, Patterned Lake, and Sonic Lake. The Framnes Mountains and associated lakes are depicted in Figure 5i.

Figure 5i **Location of Framnes Mountains Lakes (Pickard, 1982)**

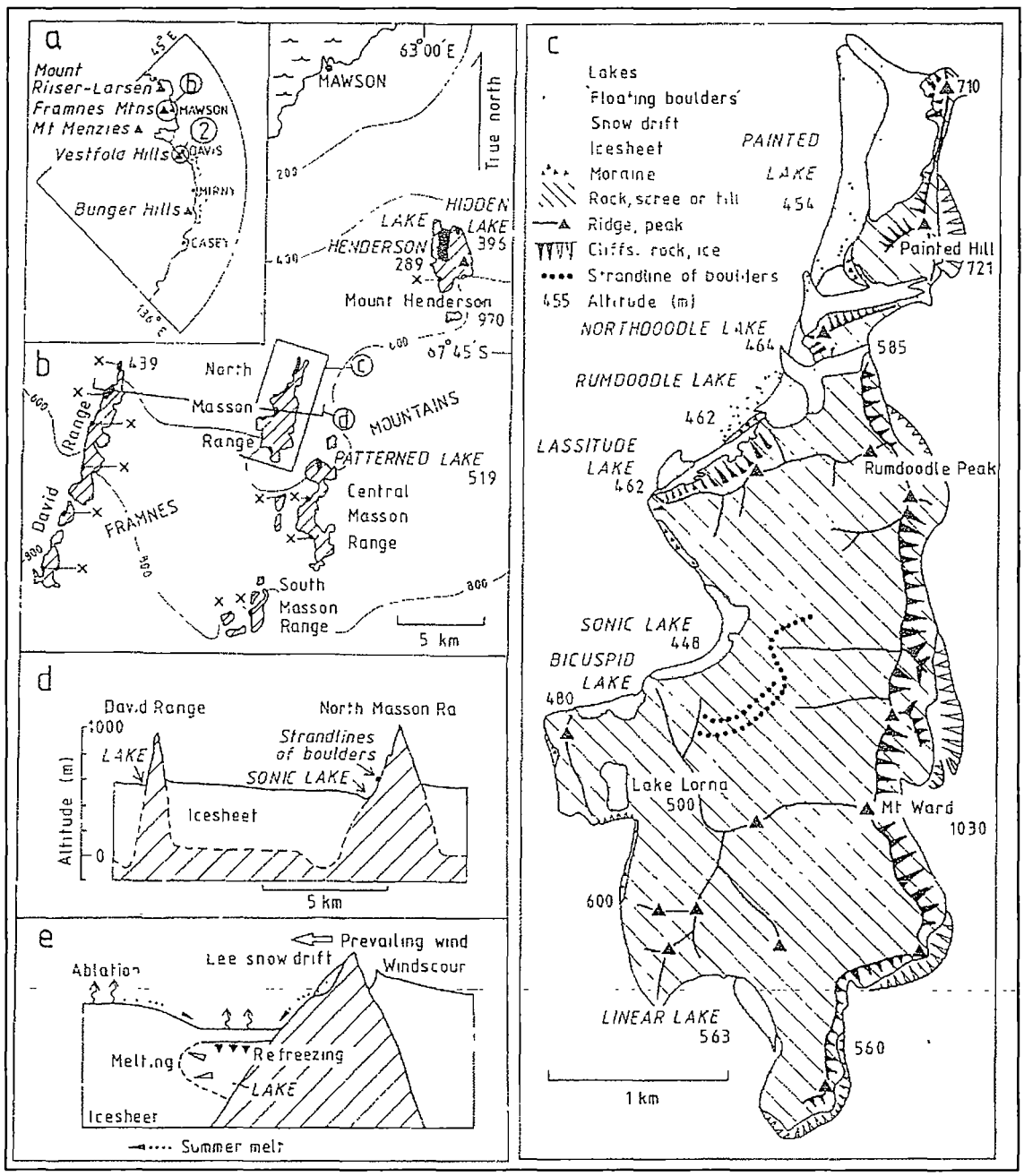
(a) Location map of East Antarctica.

(b) Location map of Framnes Mountains.

(c) North Masson Range showing lakes.

(d) Section from David Range to North Masson Range showing location of lakes.

(e) Diagrammatic section of lake showing lake and ice hydrodynamics.


a
Mount Riiser-Larsen
Framnes Mtns
Mt Menzies
Vestfold Hills
Bunger Hills
MAWSON
DAVIS
MIRNY
CASEY
45°E
135°E
63°00'E
MAWSON
True north
200
HIDDEN LAKE
HENDERSON LAKE
289
396
Mount Henderson
970
400
67°45'S
b
439
North Masson Range
MOUNTAINS
PATTERNED LAKE
519
Central Masson Range
South Masson Range
David Range
FRAMNES
300
500
800
5 km
c
Lakes
Floating boulders
Snow drift
Icesheet
Moraine
Rock, scree or hill
Ridge, peak
Cliffs, rock, ice
Strandline of boulders
455 Altitude (m)
PAINTED LAKE
454
710
Painted Hill
721
NORTHOODLE LAKE
464
585
RUMDOODLE LAKE
462
LASSITUDE LAKE
462
Rumdoodle Peak
SONIC LAKE
448
BICUSPID LAKE
480
Lake Lorna
500
Mt Ward
1030
600
LINEAR LAKE
563
560
1 km
d
David Range
North Masson Ra
1000
LAKE
Strandlines of boulders
SONIC LAKE
Icesheet
Altitude (m)
0
5 km
e
Prevailing wind
Lee snow drift
Windscour
Ablation
Melting
Refreezing
LAKE
Icesheet
Summer melt

Little is known about these lakes. Lake Bicuspid (67°46'S 62°47'E) is named so due to its shape, as is Lake Linear (67°47'S 62°48'E). Lake Henderson (67°42'S 63°01'E), the largest of the epiglacial lakes in the Framnes Mountains, Lake Burnett (67°53'S 62°44'E), and Painted Lake (67°45'S 62°50'E) are named after the mountain peaks they are associated with. Northdoodle Lake (67°46'S 62°32E) is named due to the fact of it being north of Rumdoodle Lake (not represented in this study). The ice cover of Patterned Lake (67°48'S 62°51'E) contains large crystals that form patterns and is a characteristic unique to the lakes in the Framnes Mountains. Finally Sonic Lake (67°48'S 62°48'E) derives its name from the sound heard whilst standing on the frozen surface that is likened to sonic booms (Gibson, 2005, Pickard, 1982). Large boulders appear to float on the surface of many of the Framnes Mountains epiglacial lakes with about 40 boulders on Lake Henderson and 20 on Painted Lake. All of the lakes in this study were found to be permanently covered in 4 to 5.66 metres of ice, effectively cutting the water beneath off from the atmosphere. In a previous survey of these epiglacial lakes in the 1980/01 austral summer season, there was evidence that at some stage Bicuspid Lake flowed into Sonic Lake (Pickard, 1982). The evidence of water flow was not seen by the study by Gibson (2005), indicating this may have been a relatively recent change.

Water was sampled from each lake at the point below the ice at the lake interface all the way to the bottom. Little sediment was found in any of the epiglacial lakes. The analysis conducted on the samples from each lake included chemical analyses, estimations of chlorophyll abundance, and light microscopy looking primarily for metazoa. Metazoa identified as members of the genera *Philodina*, *Ptygura*, *Habrotrocha* (rotifers), *Plectus* (nematode), *Acutuncus* (tardigrade), *Daphnia* and

harpacticoid copepods (crustacean) were found in various lakes with no metazoans being identified from Bicuspid Lake. A cyanobacterium of the genus *Leptolyngbya* was identified in samples from Patterned Lake, and *Phormidium* spp. was identified in Linear Lake. All of the lakes were found to contain water of a low salinity that was extremely alkaline, ranging from pH 10 to 11.4. Figure 5ii shows the pH of the water column of each of the epiglacial lakes in this study. Of particular note is Sonic Lake, where there is a pronounced pH change from alkaline waters to a relatively neutral pH, indicating that Sonic Lake was once a lake of neutral pH before receiving waters from Bicuspid Lake. Samples were chosen from each of these lakes for examination of their microbial content to be described in this chapter, as depicted by triangles. Samples were taken from just below the ice/lake water interface at each lake, plus at the bottom of Sonic Lake. Table 5a shows the samples obtained for this study.

Figure 5ii Plot showing the pH of the water along the entire depth of each epiglacial lake in this study. The pH values obtained within the lake ice were due to mixing of water from the ice and/or surface. Samples were obtained from within the ice cored region of each lake and from the bottom of Sonic Lake (depth of 45 metres).

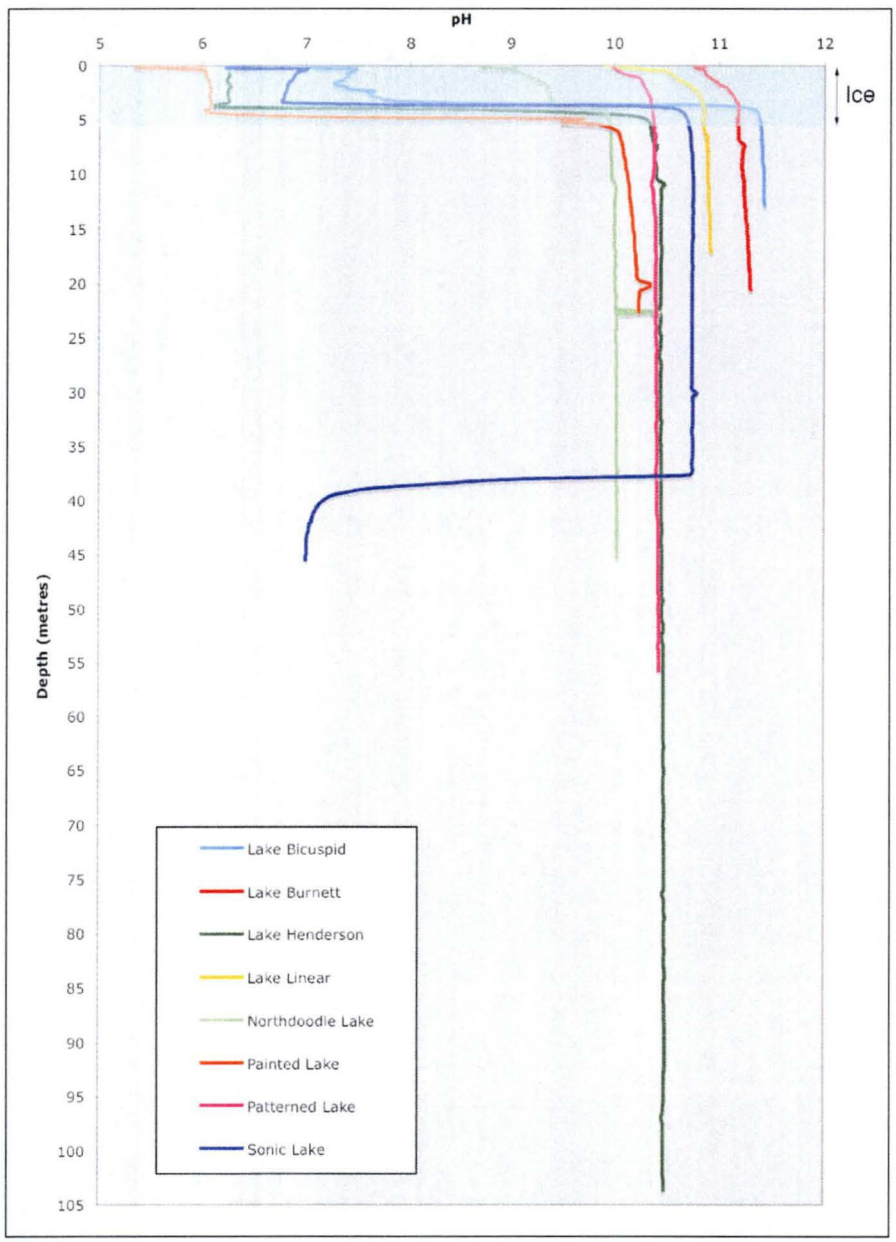

pH
5
6
7
8
9
10
11
12
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
Depth (metres)
Ice
Lake Bicuspid
Lake Burnett
Lake Henderson
Lake Linear
Northdoodle Lake
Painted Lake
Patterned Lake
Sonic Lake

Table 5a **Physicochemical characteristics of lake samples obtained for this study**

	Ice Thickness	Temperature	pH	Salinity (PSS)	DO mg/L (% Saturation)
Lake Bicuspid	4m	-0.1°C	7.72	0.000569	6.45 (33.5)
Lake Burnett	4.17m	-0.05°C	11.15	0.076	7.37 (38.4)
Lake Henderson	4.3m	-0.05°C	8.55	0.014	8.34 (43.4)
Lake Linear	5.66m	-0.02°C	10.84	1.004	11.14 (58.3)
Northdoodle Lake	4.9m	-0.02°C	9.88	0.022	8.73 (45.5)
Painted Lake	4.51m	-0.05°C	6.08	0.001	6.82 (35.5)
Patterned Lake	4.54m	0.01°C	10.36	0.075	11.74 (61.2)
Sonic Lake - Top	4.1m	0.11°C	10.61	0.682	9.18 (48.1)
Sonic Lake – Bottom*	4.1m	-0.18°C	7	1.214	5.43 (28.3)

* - Sampled at the bottom of Sonic Lake at a depth of 45 metres.

5.1.3 Fresh water Antarctic lakes

A great variety of lakes exist on the Antarctic continent, ranging from those with freshwater to extremely hypersaline lakes, such as Don Juan Pond in the McMurdo Dry Valleys with a salinity of 33% (Cameron et al., 1972). As stated previously, the epiglacial lakes in this study consist of fresh to brackish water, ranging from a neutral pH to extremely alkaline. Although several epiglacial lakes have been discovered across the Antarctic continent, this is the first time they have been examined for their microbial composition. There are no other types of lake yet discovered on the Antarctic continent that consist of both fresh/brackish water and alkaline conditions, but many exist that are of a relatively neutral pH (Cowan & Tow,

2004). Countless freshwater lakes have been identified, ranging from small ephemeral ponds to large water bodies (Gillieson et al., 1990). Most freshwater Antarctic lakes are covered in ice for at least 8 months of the year (Vincent, 1988). Some, such as those found in the Taylor Valley in southern Victoria Land also have a perennial ice cover. An example of one such lake is located on the eastern end of the Taylor Valley. Lake Fryxell (77°37'S 163°07'E) is a 18.5m deep brackish and meromictic lake with a perennial 3-4.5m ice cover that is prone to a peripheral moat each summer (Spigel & Priscu, 1998). The microbial diversity of this lake was found to be very diverse, being dominated by an array of *Cyanobacteria* (Taton et al., 2003). Microbial mats obtained from the lake contained *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*, and members of *Clostridium/Bacillus* and of the *Cytophaga-Flavobacterium-Bactereroides* (CFB) group (Brambilla et al., 2001).

5.1.4 Alkaline environments around the world

Alkaline environments rich in alkaliphilic microorganisms are becoming increasingly more common as a result of human activity leading to leeching of contaminants into natural wetlands and groundwater (Fahy et al., 2006, Roadcap et al., 2006). Naturally occurring alkaline environments are scarce, but those that have been discovered are well documented and are continued to be studied with the prospect of finding novel bacteria and alkaliphilic enzymes. Soda lakes from temperate and subtropical environments are the best described, possessing a pH of 8-12, containing high concentrations of sodium carbonates with low levels of Ca^{2+} and Mg^{2}, being dominated by microorganisms that are both alkaliphilic and halophilic or extremely halotolerant (Duckworth et al., 1996, Jones et al., 1998, Sorokin et al., 2003). Stable low saline alkaline environments with pH values over 10 containing alkaliphilic

bacteria have also been discovered in the 40-93°C waters at the Lost City Hydrothermal Field in the Atlantic Ocean (Kelley et al., 2001, Schrenk et al., 2004), and in the 20.5°C ground water of Cabeço de Vide in Southern Portugal (Schmidt et al., 2006a, Tiago et al., 2004). The microorganisms identified and isolated from the above alkaline environments often represent separate lineages within accepted taxa or show no strong relationship to any other known species.

Until this study, there was only one environment on Earth where an alkaline environment was described for its microbiology and that had both low salinity and a low temperature. Ikaite is a rare hexahydrate of calcium carbonate and precipitates of this are able to form long columns in a saturated environment of calcium and carbonate ions while in the presence of high levels of phosphate. The ikaite tufa columns from the Ikka Fjord in southwest Greenland possess novel ecological spring water niches that possess a pH of 10.4 and a salinity of 0.9% (Schmidt et al., 2006a). Additionally, the nature of the ikaite columns is that they are only stable at temperatures below 6°C. The microbial content of these columns were shown to be primarily *Proteobacteria* and *Cyanobacteria*, with a notable presence of several uncultured CFB group bacteria clones. Several bacteria identified in this study also showed similarity to isolates and phylotypes from the Arctic and Antarctica.

5.1.5 Molecular methods in determining microbial diversity

A multitude of methods currently exists for examining a pool of DNA to ascertain the microbial diversity of a sample. These methods include nucleic acid hybridisation techniques, the cloning and sequencing of community rRNA genes, and community finger printing using techniques such as ARDRA, RAPD, REP-PCR,

and DGGE (Theron & Cloete, 2000). Still to this day, clone library construction using universal bacterial primers remains the gold standard in microbial community analysis. The choice of primers for this study will utilise a similar region to that of the study of the ikaite columns in Greenland (Schmidt et al., 2006a) for comparability looking at a region of the 16S rRNA gene from position 519-1513 (based on *E. coli* numbering), containing the most conserved region of the 16S rRNA gene (Stackebrandt & Goebel, 1994).

Another method becoming increasingly popular in microbial ecology that will be employed in this study is terminal-restriction fragment length polymorphism (t-RFLP) bacterial community profiling. This technique modifies the existing technology of PCR, with either one or both of the primers used possessing a fluorescent dye label on its 5' end. These labelled PCR products are then cut using a restriction enzyme, and injected into a standard sequencer. As sequencers employ size separation, a variety of peaks will be generated, each corresponding to a different sized labelled fragment. Each of these peaks is regarded as an individual phylotype in an environmental sample, and can be used in determining the level of similarity between samples. The t-RFLP data can also be correlated with clone library data provided the same set of PCR primers are used (Avaniss-Aghajani et al., 1994, Liu et al., 1997).

No technique however is without pitfalls, and as t-RFLP is a relatively new technique, there is currently no consensus on the appropriate approach to take in choosing restriction enzymes or analysing the resulting raw data, and so is regarded as being quite subjective (Abdo et al., 2006). It appears that using multiple

restriction enzymes will overcome bias that may occur when using only one particular enzyme system (Osborne et al., 2006). Numerous software packages have been developed to analyse raw t-RFLP data to eliminate lengthy manual handling of the data, one being T-ALIGN (Smith et al., 2005). With this particular online software package, replicate t-RFLP profiles are used to generate a single profile where peaks that do not appear in both of the profiles are eliminated. This in effect removes the bias of manually handling the raw data and the interference of any non-specific PCR products that may occur in any individual t-RFLP run. In choosing the statistical methodology for representing the final data, the Jaccard measure of similarity (Milligan, 1981) has been proved to be an effective method of clustering binary data when using a 1bp range of similarity when binning fragments (Abdo et al., 2006). When dealing with a weighted analysis to avoid the overpowering effect of rare phylotypes distorting the data, non-transformed Bray-curtis similarity (Bray & Curtis, 1957) has been used (Schmidt et al., 2006a). In either instance the final data is most effectively portrayed using non-metric Multi Dimensional Scaling (MDS) (Kruskal, 1964).

5.1.6 Aims of this study

To keep with the theme concerning the biodiscovery and biodiversity of Antarctic bacteria, a molecular analysis study focussing on the bacterial content of eight Framnes Mountains lakes was carried out. This was undertaken using a combination of 16S rRNA gene clone library analyses and t-RFLP bacterial community based profiling. From this information the dominant bacterial members of these lakes can be identified as well as different the bacterial diversities are between lake samples.

Comparisons are able to be made also to other fresh and alkaline lakes discussed previously.

5.2 Materials and methods

5.2.1 Sample origin and collection

Lake water samples were taken from eight permanently frozen lakes located around the Framnes Mountains in Antarctica as part of a limnological study into the Framnes Mountains lakes (Gibson, 2005), with the results of this study summarised in section 5.1.2. To obtain the samples for that study, lake water was filtered through GF/F glass fibre filters (0.7μM nominal pore size). Water was sampled from each lake just below the ice at the lake water interface. A total of 1945mL of water was filtered from Lake Bicuspid; 1965mL from Lake Burnett; 1990mL from Lake Henderson; 1980mL from Lake Linear, 1925mL from Northdoodle Lake; 1985mL from Painted Lake; 1240mL from Patterned Lake; and 1865mL from Sonic Lake. 1970mL of water was also filtered from the bottom of Sonic Lake at a depth of 45 metres and so the two Sonic Lake samples will be referred to as Sonic (top) and Sonic (bottom) respectively. Unfiltered lake water from Patterned Lake was also obtained from the same sampling site. These filter samples and lake water were then stored at -20°C and were graciously donated by Dr John Gibson to form the basis of this study.

5.2.2 DNA extraction

The frozen filter papers were aseptically transferred to PowerBead tubes from the Powersoil DNA Kit (MoBio Cat. # 12888-100), where DNA was extracted from each sample following the manufacturer's instructions. For the Patterned Lake water

sample, 1mL was used. An additional recommended step of heating at 70°C for 10 minutes after the addition of reagent C1 to help lyse cells was employed. Extracted DNA was stored at -20°C.

5.2.3 PCR amplification of 16S rRNA gene

The 16S rRNA gene sequences of the DNA extracted from Sonic-Top were PCR amplified as described in section 2.2.4. The primers used were the universal bacterial primers 519f (5'-CAGCMGCCGCGGTAATAC-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') (Lane, 1991). Amplicons were generated using a MJ Research PTC-200 peltier thermal cycler using the following program: one cycle of 15 minutes at 95°C; 35 cycles of 1 minute at 94°C, 1 minute at 50°C, 1.5 minutes at 72°C; and a final extension step of 10 minutes at 72°C. The resulting PCR product was then purified using a MinElute Reaction Cleanup Kit (Qiagen Pty Ltd Cat. # 28204), and visualised on a 1.5% agarose gel stained with 500ng/ml Ethidium bromide.

5.2.4 Clone library construction

Clone libraries were constructed as described in section 3.2.4.

5.2.5 Sequence analysis

Raw sequence files (*.scf) were imported into Sequencher version 4.5 (Gene Codes Corporation) where chromatograms were analysed and sequence fragments were aligned. Consensus sequences were then compared against other sequences on the

Genbank database (Benson et al., 2003) using the BLAST function (Altschul et al., 1990). The closest matches of each clone, as well as the closest cultured match were imported into the program SEAVIEW (Galtier et al., 1996), where all sequences were aligned using the ClustalW function. Neighbour joining trees calculated using the Kimura two-parameter DNA distance model and 500 bootstraps were then constructed using PHYLO_WIN with the resulting tree output made into a publishable quality using Adobe Photoshop CS Version 8.0 (Adobe Systems Incorporated).

5.2.6 Estimation of species richness

An estimation of species richness corresponding to the Sonic-Top and Patterned Lake water clone library was obtained by using the ChaoI (Chao, 1984) statistical output computed by the EstimateS software, version 7.5 (Colwell, 2005). A 3% cut off was used to determine differing phylotypes according to the 16S rRNA gene sequences obtained.

5.2.7 Generation of terminal restriction fragment length polymorphism (t-RFLP) amplicons

PCR amplicons from the 16S rRNA gene sequences of each lake filter DNA sample were generated as described previously in section 5.2.3 with the 519f and 1492r primers possessing dyeD3 and dyeD4 tags respectively at the 5' end (Sigma-Proligo). Each PCR was performed in duplicate and subsequently combined using a QIAquick Spin column (Qiagen Pty Ltd Cat. # 28106). The labelled PCR products then underwent restriction enzyme digestion using *HaeIII*, *MspI*, or *RsaI* (New England

BioLabs) at 37°C for 17 hours with each digest being carried out in duplicate. The digested labelled fragments were then cleaned using ethanol precipitation using 3M NaOAc (pH 5.2) with glycogen as a carrier molecule. Cleaned fragments were eluted in 30μL formamide (Beckman Coulter Inc. Sample Loading Solution) containing 0.25μL of GenomeLab DNA Size Standard Kit-600 (Beckman Coulter Inc.; Cat. # 608095). Fragments were then obtained using the CEQ 8000 Genetic analysis system (Beckman Coulter Inc.), using the Frag-4 method that involved an injection of 2.0kV for 30 seconds, and was run at a capillary temperature of 50°C at 4.8kV for 60 minutes.

5.2.8 t-RFLP analysis

Raw fragment data was filtered initially using the fragment analysis software included with the Beckman Coulter CEQ8000 Genetic Analysis System whereby a peak height lower threshold was set to 5% and any peaks with an area or height less than 1000 DFU (Dye Fluorescent Units) were removed from the data set. In addition to this peak clusters were converted to a single peak according to the highest peak in the cluster. The peak data was then imported into Microsoft Excel (Microsoft Corporation) where peak areas less than 5% of the total peak area in each respective t-RFLP were removed from the study. To obtain a single fragment data set from each set of duplicates, T-Align (http://inismor.ucd.ie/~talign/index.html) was used where fragments were binned with a 1.0 base confidence that also culled any fragments not present in duplicate samples (Smith et al., 2005). The data obtained was then analysed using the statistical software for ecological studies, Primer 6 (Primer-E Ltd.) using both the Jaccard and Bray-curtis measures of similarity.

Similarity profile permutation testing incorporated in the clustering analysis determines whether any groups formed can be statistically differentiated. This was carried out using 1000 permutations with a significance level of 5%. Data was then expressed as a non-metric MDS plot. Stress values given for each plot are interpreted as follows: values greater than 0.2 indicate that the plot was randomly generated, less than 0.2 indicates a 2-dimensional relationship is present, and less than 0.1 indicates the plot may show relationships less influenced by artifactual data (Clarke & Warwick, 2001).

5.2.9 Nucleotide sequences

Nucleotide sequences derived from this study are included in the Appendix CD, and will be submitted to Genbank upon publication of said data.

5.3 Results

5.3.1 Bacterial diversity of Patterned Lake

The lake water sample from Patterned Lake was subjected to 16S rRNA gene clone library analysis. As Figure 5iii shows, Patterned Lake is dominated by Cyanobacteria species closely related to the genera *Pseudanabaena* and *Leptolyngbya*, corresponding to the visible Cyanobacterial mat segments in the original lake water sample. Bacteria found to be associated with this mat vary with members of *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria*, *Verrucomicrobia*, and *Planctomycetes* being detected. In total 88 clones were screened to comprise this show of diversity. The ChaoI derived index of species richness according to this data is 323.

5.3.2 Bacterial diversity of Sonic (Top)

One filtered lake sample, Sonic (Top) was chosen at random for 16S rRNA gene clone library analysis. As shown in Figure 5iv, Sonic-Top is dominated by algal plastids with one cluster being related to a *Nannochloropsis*-like sequence, a genus of small algae recently described in fresh and brackish environments and in cold and ice-covered waters (Fawley & Fawley, 2007). This cluster also contained uncultured *Cyanobacterium* clones. Both *Betaproteobacteria* and *Gammaproteobacteria* were identified that belonged to the genera *Polaromonas* and *Pseudomonas* respectively. Identified was also a significant clade containing members of the CFB group with similarities to genera such as *Flavobacterium*, *Arcicella*, and *Hymenobacter*.

Various Cyanobacteria were also detected. The ChaoI-derived predicted number of lineages for Sonic-Top was 139.

5.3.3 Comparative bacterial diversities of Framnes Mountain Lakes

The t-RFLP derived phylotypes of each lake sample were converted to binary presence/absence data and analysed using Jaccard similarity. Figure 5v part A shows a MDS plot of the relationships given with a stress level well below 0.1. The level of similarity between lakes ranged between ~15-25%, with no clustering of significance observed when viewed as a dendrogram (data not shown). This indicates that even though some of the lakes clearly group together, such as Northdoodle Lake and Sonic Lake (bottom) in one grouping, and Painted Lake, Patterned Lake, and Lake Henderson in another, the level of similarities were primarily shared across all of the lakes. There was no difference to this when the t-RFLP data was analysed using a weighted Bray-Curtis similarity (Figure 5v part B), although Painted Lake was replaced by Sonic Lake (top) with its pairing with Patterned Lake and Lake Henderson. The Bray-Curtis analysis did result in a higher stress level of 0.12 however.

Figure 5iii **16S rRNA gene Neighbour Joining tree of Patterned Lake clones with closest matches obtained from Genbank. Numbers in brackets denote number of clones sharing >97% similarity with representative clone. *Thermotoga elfii* (X80790) and *Aquifex pyrophilus* (M83548) were used as outgroups, and comparisons were made using the Kimura two-parameter model with 500 bootstrap replicates. The scale bar indicates 0.029 changes per nucleotide.**

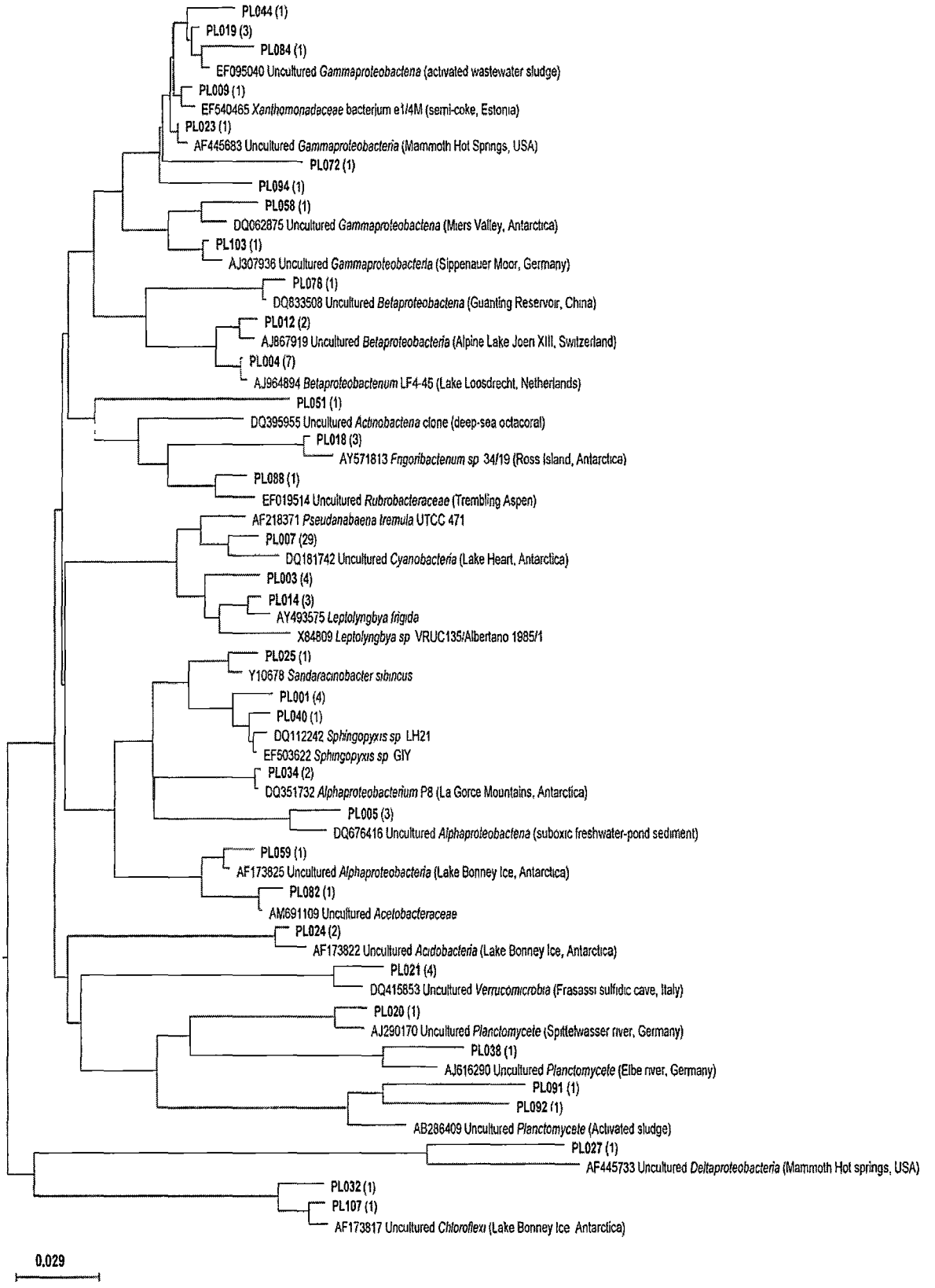

PL044 (1)
PL019 (3)
PL084 (1)
EF095040 Uncultured Gammaproteobactena (activated wastewater sludge)
PL009 (1)
EF540465 Xanthomonadaceae bacterium e1/4M (semi-coke, Estonia)
PL023 (1)
AF445683 Uncultured Gammaproteobacteria (Mammoth Hot Springs, USA)
PL072 (1)
PL094 (1)
PL058 (1)
DQ062875 Uncultured Gammaproteobactena (Miers Valley, Antarctica)
PL103 (1)
AJ307936 Uncultured Gammaproteobacteria (Sippenauer Moor, Germany)
PL078 (1)
DQ833508 Uncultured Betaproteobactena (Guanting Reservoir, China)
PL012 (2)
AJ867919 Uncultured Betaproteobacteria (Alpine Lake Joen XIII, Switzerland)
PL004 (7)
AJ964894 Betaproteobactenum LF4-45 (Lake Loosdrecht, Netherlands)
PL051 (1)
DQ395955 Uncultured Actinobactena clone (deep-sea octacoral)
PL018 (3)
AY571813 Fngoribactenum sp 34/19 (Ross Island, Antarctica)
PL088 (1)
EF019514 Uncultured Rubrobacteraceae (Trembling Aspen)
AF218371 Pseudanabaena tremula UTCC 471
PL007 (29)
DQ181742 Uncultured Cyanobacteria (Lake Heart, Antarctica)
PL003 (4)
PL014 (3)
AY493575 Leptolyngbya frigida
X84809 Leptolyngbya sp VRUC135/Albertano 1985/1
PL025 (1)
Y10678 Sandaracinobacter sibiricus
PL001 (4)
PL040 (1)
DQ112242 Sphingopyxis sp LH21
EF503622 Sphingopyxis sp GIY
PL034 (2)
DQ351732 Alphaproteobacterium P8 (La Gorce Mountains, Antarctica)
PL005 (3)
DQ676416 Uncultured Alphaproteobactena (suboxic freshwater-pond sediment)
PL059 (1)
AF173825 Uncultured Alphaproteobacteria (Lake Bonney Ice, Antarctica)
PL082 (1)
AM691109 Uncultured Acetobacteraceae
PL024 (2)
AF173822 Uncultured Acidobacteria (Lake Bonney Ice, Antarctica)
PL021 (4)
DQ415853 Uncultured Verrucomicrobia (Frasassi sulfidic cave, Italy)
PL020 (1)
AJ290170 Uncultured Planctomycete (Spittelwasser river, Germany)
PL038 (1)
AJ616290 Uncultured Planctomycete (Elbe river, Germany)
PL091 (1)
PL092 (1)
AB286409 Uncultured Planctomycete (Activated sludge)
PL027 (1)
AF445733 Uncultured Deltaproteobacteria (Mammoth Hot springs, USA)
PL032 (1)
PL107 (1)
AF173817 Uncultured Chloroflexi (Lake Bonney Ice Antarctica)
0.029

Figure 5iv **16S rRNA gene Neighbour Joining tree of Sonic-Top clones with closest cultured and clone matches from Genbank. Numbers in brackets denote number of clones sharing >99% with representative clone. *Thermotoga elfii* (X80790) and *Aquifex pyrophilus* (M83548) were used as outgroups, and comparisons were made using the Kimura two-parameter model with 500 bootstrap replicates. The scale bar indicates 0.1 changes per nucleotide.**

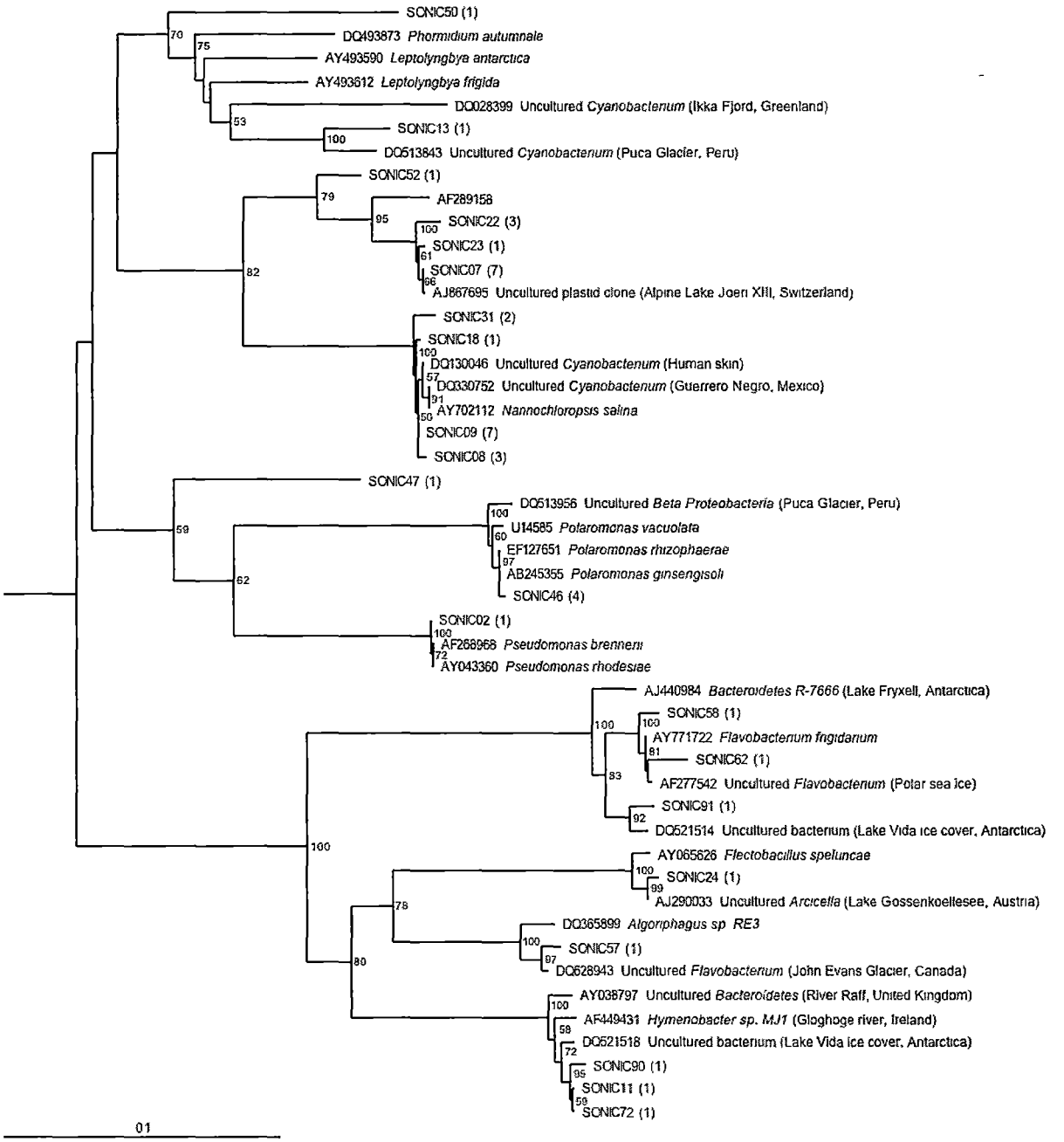
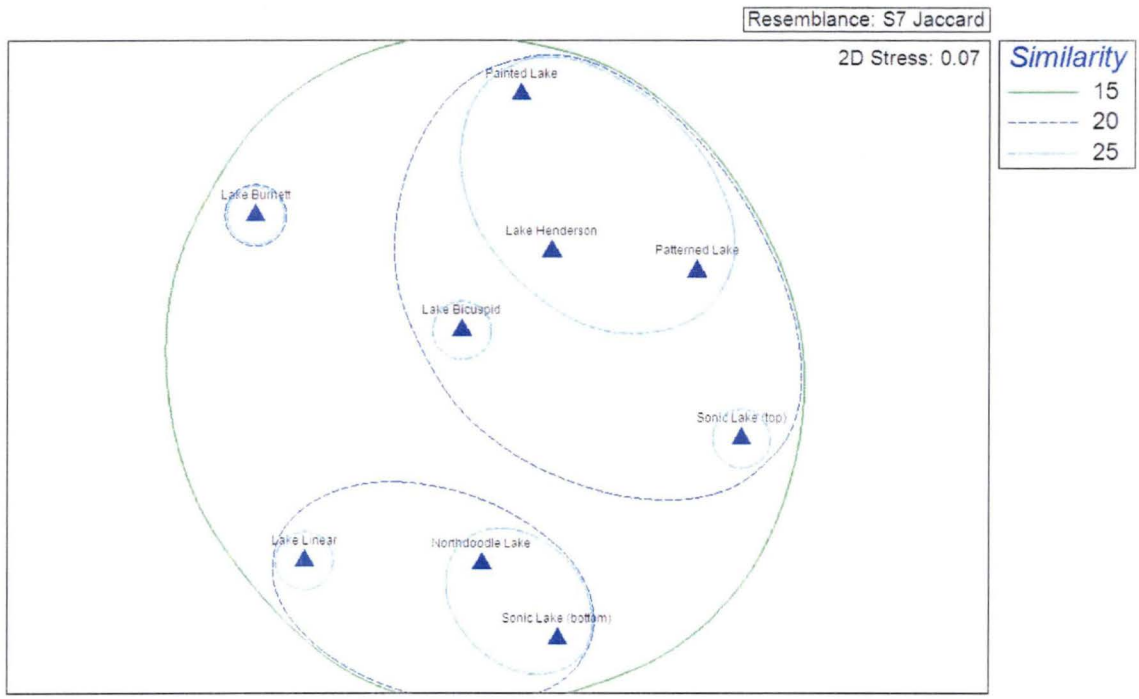

SONIC50 (1)
DQ493873 Phormidium autumnale
AY493590 Leptolyngbya antarctica
AY493612 Leptolyngbya frigida
DQ028399 Uncultured Cyanobacterium (Ikka Fjord, Greenland)
SONIC13 (1)
DQ513843 Uncultured Cyanobacterium (Puca Glacier, Peru)
SONIC52 (1)
AF289158
SONIC22 (3)
SONIC23 (1)
SONIC07 (7)
AJ867695 Uncultured plastid clone (Alpine Lake Joen XIII, Switzerland)
SONIC31 (2)
SONIC18 (1)
DQ130046 Uncultured Cyanobacterium (Human skin)
DQ330752 Uncultured Cyanobacterium (Guerrero Negro, Mexico)
AY702112 Nannochloropsis salina
SONIC09 (7)
SONIC08 (3)
SONIC47 (1)
DQ513956 Uncultured Beta Proteobacteria (Puca Glacier, Peru)
U14585 Polaromonas vacuolata
EF127651 Polaromonas rhizophaerae
AB245355 Polaromonas ginsengisoli
SONIC46 (4)
SONIC02 (1)
AF268968 Pseudomonas brennerii
AY043360 Pseudomonas rhodesiae
AJ440984 Bacteroidetes R-7666 (Lake Fryxell, Antarctica)
SONIC58 (1)
AY771722 Flavobacterium frigidarium
SONIC62 (1)
AF277542 Uncultured Flavobacterium (Polar sea ice)
SONIC91 (1)
DQ521514 Uncultured bacterium (Lake Vida ice cover, Antarctica)
AY065826 Flectobacillus speluncae
SONIC24 (1)
AJ290033 Uncultured Arcicella (Lake Gossenkoellesee, Austria)
DQ365899 Algoriphagus sp RE3
SONIC57 (1)
DQ528943 Uncultured Flavobacterium (John Evans Glacier, Canada)
AY038797 Uncultured Bacteroidetes (River Raff, United Kingdom)
AF449431 Hymenobacter sp. MJ1 (Gloghoge river, Ireland)
DQ521518 Uncultured bacterium (Lake Vida ice cover, Antarctica)
SONIC90 (1)
SONIC11 (1)
SONIC72 (1)
01

Figure 5v t-RFLP analysis of 16S rRNA gene sequences obtained from lake filter samples from epiglacial lakes in the Framnes Mountains.

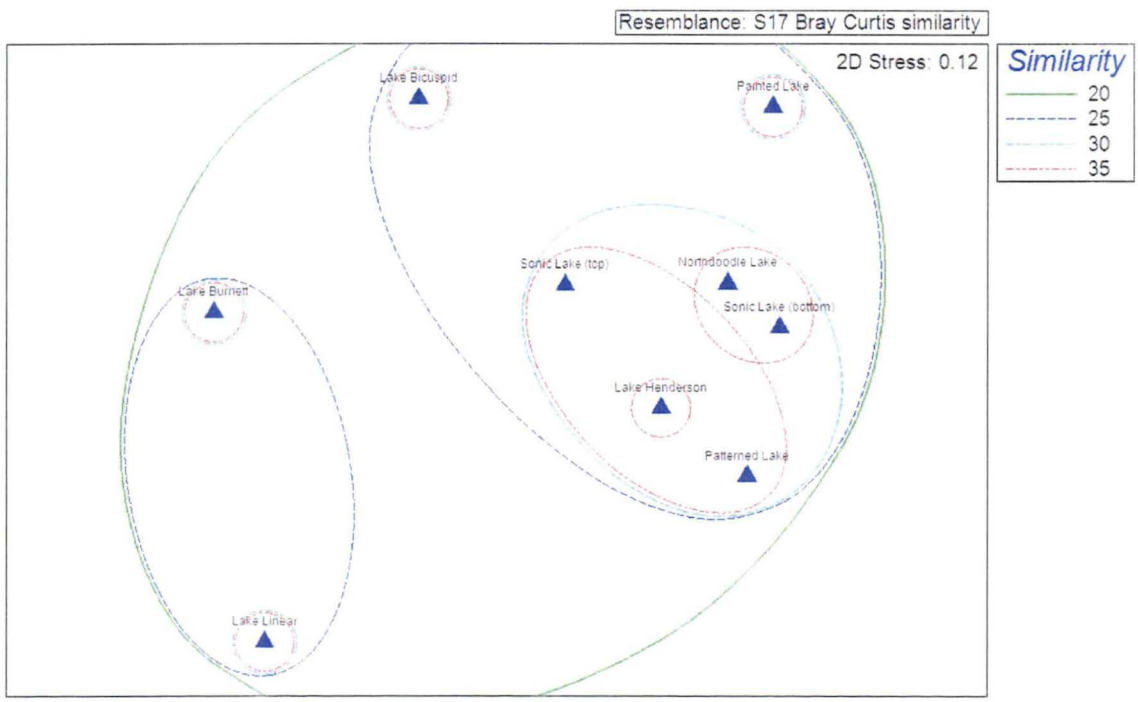
A. Jaccard transformed data represented as a 2D MDS plot overlayed with clustering analysis

B. Bray-Curtis transformed data

A.


Resemblance: S7 Jaccard
2D Stress: 0.07
Similarity
15
20
25
Painted Lake
Lake Burnett
Lake Henderson
Patterned Lake
Lake Bicuspid
Sonic Lake (top)
Lake Linear
Northdoodle Lake
Sonic Lake (bottom)

B.


Resemblance: S17 Bray Curtis similarity
2D Stress: 0.12
Similarity
20
25
30
35
Lake Bicuspid
Painted Lake
Sonic Lake (top)
Northdoodle Lake
Lake Burnett
Sonic Lake (bottom)
Lake Henderson
Patterned Lake
Lake Linear

5.4 Discussion

5.4.1 *The microbial diversity of the Framnes Mountains epiglacial lakes*

Patterned Lake was shown to be dominated by Cyanobacteria associated with various bacteria (*Actinobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, *Gammaproteobacteria*, *Chloroflexi*, *Plantomycetes*, and *Verrucomicrobia*) related to members found in other Antarctic lake environments and alkaline waters found world-wide. Sonic Lake on the other hand appears to be predominately algal driven associated with members of *Betaproteobacteria* but also contained somewhat different bacterial members than Patterned Lake such as *Flavobacteria* and *Sphingobacteria*. In addition, the microbiota of Sonic Lake appears to be primarily associated with more fresh water ecosystems world-wide. It is not surprising that the clones screened showed closest matches to isolates from typically pH neutral environments, as epiglacial lakes are formed from the melting of glacial ice at a rock interface. It is fair to assume that the development of an alkaline environment in these lakes was a gradual occurrence and selection took place for the most robust biota. Little similarity was apparent between these two lakes and an analogue environment, the permanently cold alkaline ikaite columns from the Ikka Fjord Greenland, which is both freshwater and seawater derived (Schmidt 2006a).

5.4.2 t-RFLP results

The results gained from the t-RFLP study indicate there is great variation between the bacterial diversity of the epiglacial lakes of the Framnes Mountains which correlates with the clone library results. This may be true of the samples analysed, but this is only indicative of a snapshot of a single replicate from each lake. To ascertain the true relationship between these lakes, more replicates would need to be taken, and at differing depths, to elucidate the heterogeneity of each individual lake. Obviously this was unavoidable from the samples obtained for this study, but future studies would most certainly need to take this into account. Nonetheless this is further evidence to suggest the microbiota comprising each lake may be dependant upon the native microbiota of the rock surface that lake forms on, as well as those trapped within the glacial ice. Unlike other Antarctic lakes, the life that forms in an epiglacial lake would be dependent upon the native life found on the mountain prior to glaciation, biota trapped within the ice itself, in addition to possible transfer of material to the lake is established, such as by a stream, or by particulates traveling through the ice cover via solar heating and localised melting (Priscu et al., 1998).

5.4.3 Future work

This is the first time the bacterial diversity of Antarctic epiglacial lakes has been investigated, and the results will most certainly form the basis of many avenues of exciting research to come. Having both permanently cold waters and being highly alkaline presents a strong case for bioprospecting (Horikoshi, 1999), and the ikaite columns of Greenland have shown that a similar environment was home to novel microbiota (Schmidt et al., 2006b, Schmidt et al., 2007). Little still is known about

the total biota of Antarctic epiglacial lakes, as only one element of the lakes from the Framnes Mountains was investigated. Archaea have been shown to play an important part in alkaline ecosystems (Whitman et al., 1999), although the study on the Greenland ikaite columns were not able to amplify any archaeal DNA (Schmidt et al., 2006a). This may not be the case for epiglacial lakes however, so future investigation would prove worthwhile. Another important recognised component of lake ecosystems are the viruses involved, particularly in Antarctica (Pearce & Wilson, 2003). As well as providing a wider picture of the dynamics of the lakes, bacteriophages are a potential source of useful biotechnological products (Marks & Sharp, 2000). As epiglacial lakes appear to be the most common lake type found in Antarctica (Gibson, 2006), they may also prove to be useful analogues to test contamination issues involved with current projects drilling into subglacial lakes such as Lake Vostok (Alekhina et al., 2007) and future work looking at the hundreds of subglacial lakes known to exist in Antarctica (Popov & Masolov, 2007, Siegert et al., 2005). As such, epiglacial lakes may prove invaluable in research concerning analogues for life on other extraterrestrial bodies such as Europa, where there is evidence to suggest subsurface ice is in contact with subsurface liquid water (Chyba & Phillips, 2002).

5.4.4 Conclusion

Antarctic epiglacial lakes are a vast and complex type of ecosystem that may hold keys into unlocking the mechanisms of adaptation to stress. In essence, each lake is an experiment into alkaline adaptation at low temperatures that spans over thousands of years. This study has presented data showing the varied microbiota expected to be found in such an ecosystem, and introduces another extreme Antarctic environment warranting further investigation.

Chapter 6 Analysis of bacteria trapped within Antarctic ice

Ice Cliffs near Jack's Donga, Antarctica

The Law Dome glacier terminates in ice cliffs in the Windmill Islands area.

Photo taken by Jimmy Twin

6.1 Introduction

6.1.1 Bacteria in Antarctic ice

Approximately 98% of Antarctica is covered in continental ice making it the largest body of ice on the planet and holds the majority of Earth's fresh water (CIA, 2008). In addition to this, Antarctic lakes tend to be perennially or seasonally covered in ice, and surrounding the continent is a massive sea ice sheet. Bacteria are associated with ice in a metabolically active state in veins of liquid water or brine channels, or are cryopreserved in solid ice (Mock & Thomas, 2005, Price, 2000). Bacterial DNA is prevalent in Antarctic ice, even in the oldest ice dating back 8 million years (Bidle et al., 2007). Ice is not an ideal medium for cryopreservation however, and DNA suffers from hydrolysis from acidic liquid veins running along boundaries between ice crystals; oxidative damage occurs until ice reaches 70-80 metres in thickness; as alkylation (DNA-DNA) and Maillard reaction (DNA-Protein) crosslinking also occurs (Willerslev et al., 2004b). Sensitive molecular techniques are therefore required to detect bacteria in Antarctic ice. Controversy has occurred regarding the validity of some studies of bacteria in ancient samples including ice.

6.1.2 Controversies in ancient ice research

The numbers of bacteria trapped in ice can be very low, for example analyses of ice from the Vostok site (77°S 105°E) has given estimates of bacterial numbers of 100-1000 cells/ml of melt water (Christner et al., 2001, Karl et al., 1999, Priscu et al., 1999). A low biomass increases the chance of low levels of contamination

influencing results. In addition to this, it has been found that glacial ice, despite being highly compacted, can permit liquids such as fuels to travel along grain boundaries in ice, particularly in bubble-free, non-fractured polycrystalline ice near its melting point (Jepsen et al., 2006). This is an issue as it means the drilling process itself may present itself as a source of contamination, especially as the most widely accepted form of drilling uses kerosene as a drilling fluid to prevent the drill site from refreezing. The kerosene used is generally not sterile, and using the drilling efforts towards Lake Vostok as an example again, *Sphingomonas* spp. and related bacteria were detected in the drilling fluid that is keeping the Vostok drill hole from freezing (Alekhina et al., 2007). It is a general consensus that is must be assumed all ice is covered in contaminating material as well as all reagents used in the laboratory and results must be reproducible (Cooper & Poinar, 2000, Willerslev et al., 2004b).

6.1.3 Methods for decontaminating ice samples for molecular studies

It is generally agreed that the outer surface of an ice core needs to be removed aseptically in order to reduce the chances of contamination. Numerous methods have been explored including the use of sterilised ice axes and band saws (Dancer et al., 1997, Priscu et al., 1999, Willerslev et al., 1999), devices that melt samples from the ice core interior (Abyzov et al., 2001, Christner et al., 2003), and melting the outside layer with various washes such as 95% ethanol, filtered milliQ or HPLC H_2O, sodium hydroxide, hydrochloric acid, hydrogen peroxide, hexane, as well as the use of UV-irradiation (Bidle et al., 2007, Karl et al., 1999, Lavire et al., 2006, Rogers et al., 2004). Melting the outside layer of an ice core with 5.25% sodium hypochlorite (undiluted Clorox bleach product) has been found to be the most effective, and a study conducted by Rogers *et al.* also included deliberate spiking of the outside of

the ice with a known "contaminant" (Rogers et al., 2004). The added benefit of this treatment is that the hypochlorite in theory should also penetrate any fractures in the ice that may have gone unnoticed. For the purposes of this study a combination of hypochlorite and filtered milliQ water has been used.

6.1.4 Culturing of bacteria from ice samples

Whilst there are claims of detecting metabolic activity from culturing studies involving 8 million year old ice (Bidle et al., 2007), bacteria can confidently be cultured from ice hundreds of thousands of years old, providing the bacteria are well preserved and buffered against environmental stresses, such as being frozen in association with high levels of organic material. The media chosen to cultivate bacteria from ice is dependent upon the environment, and it is agreed that bacterial cells require time in which to recover from cellular damage, environmental stresses, and deal with toxic compounds such as hydrogen peroxide, superoxide, and free radicals (Dodd et al., 1997). A dilute broth or agar media is preferred over a highly nutrient rich media. Agar types such as R2a and diluted TSA or NA tend to yield the most colonies based on previous studies (Christner et al., 2000, Miteva et al., 2004).

6.1.5 Chinese Amery Ice Shelf drilling project

The Amery Ice Shelf is the largest ice shelf in East Antarctica, approximately 60000km^2 in area (Figure 6i). The ice that forms this shelf originates from the Lambert Glacier and calves off into Prydz Bay. Marine ice was found to have accreted beneath this ice shelf, and later remote sensing reported that this ice can be up to 200m thick (Fricker et al., 2001, Morgan, 1972). Borehole imagery was obtained from a site 100km from the calving front of the Amery Ice Shelf (69°26'S, 71°27 E) that identified small brine cells and veins throughout the marine ice, leading to a more honeycomb structure approximately 175m below the marine ice/continental ice interface. This honeycomb structure would make this last ~100m of marine ice porous to seawater (Craven et al., 2005). Protists have been found to be associated with samples taken from this core such as diatoms, crysophytes, silicoflagellates, and dinoflagellates (Roberts et al., 2007).

In the 2002/2003 season, a collaborative ice drilling project was carried out between the ACE CRC and the Polar Research Institute of China (PRIC). This resulted in a 296m long core that penetrated approximately 20m into the accreted marine ice (69°26'S 71°26'E). Despite previous work noting the presence of microbiota, the work carried out by Cai *et al.* (2006) found no living biological material from DNA staining nor they did note any high level of particular matter when melt water was examined by flow cytometry. Also at a simple inspection, no visible brine channels were noted in these 20m of cored ice. Five ice samples along this core were kindly donated to this study for analysis of any possible bacterial constituents.

Figure 6i **Map of East Antarctica showing the locations of the Amery Ice Shelf and Law Dome glacier with respect to the Australian Government Antarctic Division's bases. Map obtained from the Australian Antarctic Data Centre (http://data.aad.gov.au/aadc/mapcat/display_map.cfm?map_id=13394).**


0°
SYOWA
MOLODEZHNAYA
MAWSON
HEARD ISLAND
AMERY ICE SHELF
ZHONGSHAN
LAW-RACOVITA
DAVIS
AMUNDSEN - SCOTT
South Pole
AUSTRALIAN ANTARCTIC TERRITORY (AAT)
90°E
MIRNY
VOSTOK
80°S
CONCORDIA
Law Dome
CASEY
ROSS ICE SHELF
McMURDO
MARIO ZUCCHELLI
AAT
COMMONWEALTH BAY
180°
Produced by the Australian Antarctic Data Centre
Map Catalogue No 13394
© Commonwealth of Australia

6.1.6 Law Dome glacier

The Law Dome glacier (66°44'S, 112°50'E), as shown in Figure 6i, is situated approximately 125km East-Southeast of Casey Station, Antarctica. This glacier dates back more than 80000 years, with the combination of high accumulation and elevation, low temperatures during summer, and lack of katabatic winds ensuring the ice from this glacier is well preserved (Curran et al., 2003, Morgan et al., 1997). The deposition on Law Dome is marine derived and as such ice cores gained from this glacier have proven useful into many studies into global climate change and volcanic events, sea ice decline, and heavy metal concentrations in the Antarctic atmosphere (Curran et al., 2003, Hong et al., 1998, Morgan et al., 2002, Palmer et al., 2001). No prior microbiology has been carried out on ice obtained from this glacier.

6.1.7 Aims of this study

This chapter will provide evidence of the bacteria detected from two different types of Antarctic ice. Sampling of sites such as marine ice accreted beneath the Amery Ice Shelf is rare, and provides a unique opportunity to examine the micro-organisms associated with this ice either trapped in solid ice or metabolically active in brine pockets. This also provides an opportunity to evaluate the methodology chosen. Law Dome has been the focus of many studies into global climate change, but this is the first attempt to extract microbial DNA from its marine influenced ice. This study assesses the use of this glacier as a possible site for studying bacteria from the past.

6.2 Materials and methods

6.2.1 Origin of ice core samples

All ice samples used in this study were kindly donated by the Antarctic Climate and Ecosystem CRC (ACE CRC). Five marine ice samples, CAIS394, CAIS399, CAIS404, CAIS409, and CAIS414 were obtained from a single core of a depth between 278.29-294.86m from the top of the Amery Ice Shelf, corresponding to a distance of 1.51-18.1m from the interface between the marine ice and the ice shelf. Each core sample was evenly spaced 2.7-3.3 metres from the next sample. No visible cracks or fractures were observed in the samples received. The originating ice core was obtained via hot water drilling.

Three sections of ice cores drilled from the Law Dome glacier, DSS99-72, DSS395, and DSS883 were used in this study with ages of 115, 755±3, and 2467±50 years (before 2008) respectively. DSS99-72 was obtained by dry mechanical drilling; whereas DSS395 and DSS883 were drilled using kerosene based drilling fluid. As before, no cracks or fractures were observed. All ice samples were held at -20°C until use. More detailed information on the ice samples obtained from the ACE CRC is included in the Appendix CD.

6.2.2 Cleaning and melting of ice

While the ice samples were still frozen, a 100µL "test contaminant" was spiked onto the surface of each sample. The "test contaminant" consisted of a 1:1 mix of *Promicromonospora* UTAA143 cells (48 hours @ 25°C; ~1 x 10^7 cfu/ml) in TSB and 25µg/ml purified DNA. If this particular isolate was detected in subsequent analysis, it could be assumed proper cleaning of the ice did not take place. The laminar flow hood used for the cleaning of the ice was first wiped down with 6% sodium hypochlorite (industrial strength bleach with 6% available hypochlorite) then UV irradiated for 1 hour as was all equipment used. Sterile gloves were worn throughout that were cleaned regularly in 6% sodium hypochlorite. Sterilised stainless steel tongs were used at all times in handling the ice. Ice samples were immersed in 6% sodium hypochlorite until approximately 10-30% of the outer core piece was removed. The ice was then transferred immediately to ice cold sterile filtered milliQ H2O for 10 seconds to dilute any remaining bleach, and then the ice was placed in gamma sterilised PET containers (Labserv Cat. # LBS351000WG). The cleaned ice samples were melted in the dark at 4°C for 24 hours. The melt water was then filtered with a 0.22µM filter and aseptically cut into half for DNA extraction and culturing studies. Aliquots of the bleach solutions and water used were included as contamination controls in subsequent analysis. The laminar flow and all equipment were also swabbed for culturing studies. The volume of melt water filtered was 10ml for each CAIS sample, 100ml for DSS99-72 and DSS395, and 5ml for DSS883. As an additional control, mock ice cores were included comprised of 0.22µM filtered milliQ H_2O.

6.2.3 DNA extraction

DNA was extracted from the filter paper using the method described in 5.2.2. All work was carried out in a laminar flow hood that had been cleaned with 6% sodium hypochlorite. The hood was also UV irradiated for at least one hour every time a new potential contaminant entered the hood. DNA extraction controls testing each reagent were also used. Swabs were also taken of every surface to later test for contamination. A beaker containing 6% sodium hypochlorite was also placed in the hood for cleaning sterile gloves prior to handling any tubes or reagents.

6.2.4 16S rRNA gene clone library generation and t-RFLP analyses

16S rRNA gene clone libraries were generated as described in sections 5.2.3-5.2.5, and the procedure for t-RFLP analyses was carried out as in sections 5.2.7 and 5.2.8.

6.2.5 Culturing of bacteria filtered from ice melt water

Filter paper from each ice sample was resuspended in 5ml sterile 0.1% peptone by gentle mixing and 100µl aliquots were placed in 20 different 10ml broths. The broths used were Nutrient Broth (NB), Tryptone Soya Broth (TSB), Marine Broth (MB) and R2b (recipes found in Appendix CD), at full strength and 1/100 dilution, and with and without 5g/L pyruvate. The broths were then incubated in the dark at 4°C for up to 12 months. Each month during this time 100µL aliquots were spread plated onto their respective agar equivalents, the only exception being the 1/100 dilution broths being plated onto their full strength equivalent. This was all done in duplicate, with one lot of plates incubated at 4°C, and the other at 20°C. All plates were checked weekly for growth.

6.2.6 Nucleotide sequences

Nucleotide sequences derived from this study are included in the Appendix CD, and will be submitted to Genbank upon publication of said data.

6.3 Results

6.3.1 Bacteria trapped in CAIS399

A 16S rRNA gene clone library was conducted on one of the CAIS ice samples, CAIS399, and the results are summarised in Table 6a. Members of the classes *Actinobacteria*, *Bacilli*, *Chlamydiae*, *Alphaproteobacteria*, *Betaprotebacteria*, *Deltaproteobacteria*, and *Gammaproteobacteria* were detected from this ice sample. The *Chylamydiae* clone bore a similarity species of the genus *Parachlamydia sp.*, which are endosymbiotes of amoebae. 7 clones bore similarity to uncultured clones PE31 and S1-10-CL6, which cluster with the species *Methylobacterium aquaticum* and *Methylobacterium lusitanum* respectively (data not shown). The clones themselves are of plant (*Polygala sp.*) and mushroom associated bacteria whereas the two *Methylobacterium* species are associated with drinking water and a sewage station (Doronina et al., 2002, Gallego et al., 2005). The other 53 clones screened were directedly related to microorganisms that have been detected in marine or aquatic ecosystems. The "test contaminant" *Promicromonospora* UTAA143 was not detected in the DNA extracts of the ice or any of the controls implemented. In addition, the PCR no template controls used appeared to contain no PCR product on an agarose gel was after subjection to cloning, direct sequencing, and t-RFLP analysis failed to produce any clones with inserts, sequence data or fragments above the background noise.

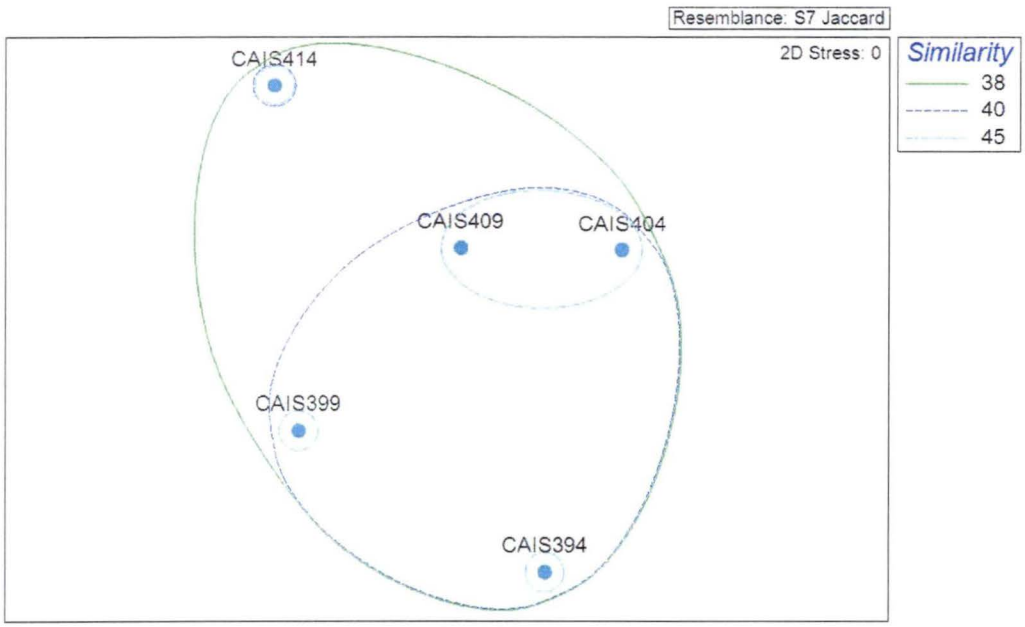
6.3.2 t-RFLP results for CAIS core samples

Analysis of the CAIS t-RFLP data using the Jaccard measure of similarity gave rise to the MDS plot shown in Figure 6iii. This plot had a stress level of zero, indicating a near perfect representation of the relationship between samples based on the data given. The levels of similarity between samples ranged from 38-45% with no distinct clustering able to be significantly formed.

Table 6a BLAST matches of CAIS399 16S rRNA gene clones

Class	Closest BLAST match	Accession Number	Location	Similarity	Number of clones
Actinobacteria	*Dietzia sp* CNJ898	DQ448696	-	99%	3
	Propionibacterium acnes	AB108482	-	99%	1
Bacilli	*Bacillus sp* BWDY-12	DQ314537	-	99%	9
Chlamydiae	*Parachlamydia acanthamoebae*	DQ309029	-	94%	1
Alphaproteobacteria	*Brevundimonas aurantiaca*	BAJ227787	-	99%	2
	Methylobacterium fujisawaense	AB175634	-	99%	3
	Methylobacterium hispanicum	AJ785570	-	99%	3
	Methylobacterium podarium	AY468363	-	98%	1
	Methylobacterium sp K611B	DQ507203	-	92%	1
	Sphingobium yanoikuyae	AB109749	-	100%	3
	Sphingomonas sp D31C2	AY162145	-	92%	1
	Uncultured clone PE31	AY838487	King trumpet (oyster) mushroom	99%	5
	Uncultured clone S1-10-CL6	AY728074	Decayed velvetleaf seed	99%	2
Betaproteobacteria	*Curvibacter gracilis*	AB109889	-	99%	2
	Delftia tsuruhatensis	AY684785	-	99%	2
	Uncultured clone BG a7	DQ228361	Bench Glacier, Alaska	99%	7
	Uncultured clone Bg g4	DQ228381	Bench Glacier, Alaska	100%	3
	Uncultured clone ctg NISA211	DQ396042	Deep-sea octacoral	99%	6
Deltaproteobacteria	Uncultured clone C16T12	AY822280	Fish-farm sediment	98%	3
Gammaproteobacteria	*Pantoea ananatis*	DQ133546	-	97%	1
	Uncultured clone Bioluz K32	AF324537	Activated sludge	98%	1
			Total clones screened		**60**

Figure 6ii **2D MDS plot showing Jaccard transformed data overlayed with clustering analysis. Analysis was conducted on 16S rRNA gene t-RFLP data obtained from filter samples from CAIS ice melt water**



6.3.3 CAIS culturing trial

Three organisms were successfully cultured from the CAIS ice. An isolate with a 100% 16S rRNA gene match to *Williamsia maris* was isolated from core sample CAIS399 after a 32 day incubation at 4°C in TSB followed by approximately 30 days at 20°C on TSA. *Williamsia maris* is an orange pigmented member of the *Actinobacteria* and was originally isolated from the Sea of Japan (Stach et al., 2004). An isolate bearing a 99% 16S rRNA similarity to *Bacillus cereus* strain HS-MP13 (EF100616) was cultured from CAIS404 after 120 day incubation at 4°C on TSB (with pyruvate) followed by approximately 90 days at 20°C. A budding yeast culture morphologically resembling a member of the *Saccharomycetales* was isolated from CAIS399 after 32 days incubating at 4°C on 1/100 strength TSB followed by approximately 270 days at 20°C.

6.3.4 Bacteria trapped in Law Dome ice

The clone data of 16S rRNA gene sequences obtained from the Law Dome ice samples are summarised in Table 6b. The most abundant clones detected in DSS99-72 were related to the Algal family *Eustigmatophyceae* and to various *Gammaproteobacteria*. *Gammaproteobacteria* were also the most abundant type of microorganism detected in sample DSS395. DSS99-72 and DSS395 possessed clone identity similarities of 59.6% and 47.8% respectively to each other. The bacterial constituents of DSS883 were distinctly different to the other samples with clones primarily detected related to chloroplast sequences. No bacteria were successfully cultured from any of the Law Dome samples after 12 months of incubation.

Table 6b BLAST matches of Law Dome 16S rRNA gene clones

Closest BLAST match	Accession Number	Location	Class	Number of clones		
				DSS99-72	DSS395	DSS883
Methylobacterium rhodoseanum	AB175643	-	*Alphaproteobacteria*	-	-	1
Methylobacterium sp MM4	AY468370	-	*Alphaproteobacteria*	-	-	2
Uncultured bacterium MP104-0927-b68*	DQ088806	Crustal biotome	*Alphaproteobacteria*	-	-	3
Uncultured bacterium 1-5B	EU289439	Trewia nudiflora	*Alphaproteobacteria*	-	-	1
Uncultured bacterium BacA_011	EU335238	Soil	*Alphaproteobacteria*	1	-	-
Uncultured beta proteobacterium	AM849430	Piburger See Lake, Austria	*Betaproteobacteria*	-	2	-
Delftia sp ZM-1	EF061135	-	*Betaproteobacteria*	-	-	5
Polaromonas sp BAC74	EU130993	-	*Betaproteobacteria*	2	1	-
Delftia sp AP1-2	EU310441	-	*Betaproteobacteria*	1	-	-
Burkholderia sp JB1	X92188	-	*Betaproteobacteria*	-	-	2
Proteus vulgaris	AJ233425	-	*Gammaproteobacteria*	7	-	-
Proteus myxofaciens NCIMB 13273	DQ885259	-	*Gammaproteobacteria*	-	13	-
Proteus myxofaciens NCIMB 13273	DQ885259	-	*Gammaproteobacteria*	1	-	-
Pseudomonas mephitica	AM748811	-	*Betaproteobacteria*	1	3	-
Pseudomonas sp RD9SR1	AM911646	-	*Gammaproteobacteria*	1	-	-
Stenotrophomonas sp 7-3	EU054384	-	*Gammaproteobacteria*	2	-	-
Serratia sp DAP7	EU302832	Adult male southern corn rootworm	*Gammaproteobacteria*	3	7	-
Bacillus thuringiensis	AM747225	-	*Bacilli*	-	-	2
Uncultured bacterium clone F2G	DQ860043	Anchovy intestinal microflora	*Bacilli*	1	-	-
Uncultured *Bacteroidetes* OM1_F	DQ513858	Puca Glacier, Peru	*Bacteroidetes*	1	-	-
Uncultured *Bacteroidetes* RBEIC	EF111092	Bogota River, Columbia	*Bacteroidetes*	2	-	-
Flavobacterium frigidarium S3-9	AY771722	-	*Flavobacteria*	-	4	-
Uncultured soil bacterium TIIB5	DQ297953	Hydrocarbon contaminated soil	*Flavobacteria*	-	-	1
Uncultured bacterium ANTLB1_B03	DQ521472	Lake Vida ice, Antarctica	*Flavobacteria*	1	4	-
Uncultured bacterium GKS2-216	AJ290033	Austria Lake Gossenkoellesee	*Sphingobacteria*	2	4	-
Uncultured *Hymenobacter* SH2B-3C	EU073784	Coal	*Sphingobacteria*	-	1	-
Uncultured Cyanobacterium LPR90	DQ130046	Human skin	*Eustigmatophyceae*	12	5	-
Lactuca sativa cultivar Salinas chloroplast	DQ383816	-	*Magnoliopsida*	-	-	31
Uncultured phototrophic eukaryote	AJ867695	Switzerland Alpine Lake Joen XIII	-	9	2	-
			Total clones screened	**47**	**46**	**48**

* - Closest BLAST match to *Sphingomonas sp*

6.4 Discussion

6.4.1 *Bacteria associated with Amery Ice Shelf accreted marine ice*

The bacteria identified in CAIS399 are associated with aquatic ecosystems and predominately of the classes *Alphaproteobacteria* and *Betaproteobacteri*a. The most abundant genus detected was *Methylobacterium*, comprising 25% of the 60 clones screened (inclusive of uncultured clones PE31 and S1-10-CL6). It is difficult to say whether these two uncultured clones are possible contaminants as they are related to plant associated *Methylobacterium* spp.. Diversity analysis using t-RFLP revealed a similarity between all 5 ice core samples of at least 38%. The differences between CAIS samples could be due to low biomass detected in the work carried out by Cai *et al.* (2006), or the presence of sporadic brine pockets. The melt water of each ice core sample also was only 10ml, and future work should endeavour to examine a larger volume of ice if possible. The *Williamsia maris* isolate cultured in this study is a known marine organism and may prove to possess anti-freezing properties to survive in marine ice if no brine pockets are present. As *Bacillus* and *Candida* spp. are known laboratory contaminants, there is a question of whether these isolates truly were cultured from the ice.

6.4.2 Bacteria associated with Law Dome ice

The younger two Law Dome core samples analysed, DSS99-72 (115 years) and DSS395 (755 years), are similar in terms of bacterial composition. Apart from age, the major difference between these two ice core samples is that DSS99-72 was drilled using a dry process and DSS395 a kerosene based system. It cannot be said if there is drilling fluid contamination associated with DSS395 as both core samples contain bacteria that have been found able to persist in kerosene, namely *Pseudomonas spp.* (Wokoma, 2001). The major classes of bacteria detected in these ice samples were *Betaproteobacteria* and *Gammaproteobacteria*. *Proteobacteria* are among the more persistent bacteria found in glacial ice worldwide, with *Clostridia* and the Actinomycete *Arthrobacter* (Willerslev et al., 2004a), although neither of the latter were detected in this study. *Eustigmatophyte* were also detected, and have been documented to exist in Antarctic marine ecosystems, particularly *Nannochloropsis* spp. that acts as photosynthetic autotrophic producers (Andreoli et al., 1999).

The Law Dome ice core sample DSS883 (2467 years), however, clearly contained contaminating DNA. Research is regularly being carried out at the University of Tasmania on lettuce varieties and another 2 suspect clones bear a close similarity to *Bacillus thuringiensis*, which is used on lettuce leafs as a pesticide (Roh et al., 2007). As the lettuce clones dominated those screened, no data concerning the contents of DSS883 can be showcased. This was an obvious case of contaminating DNA, and the results were included in this study to highlight the need to query the origin of the other clones obtained in this part of the study.

6.4.3 Contamination issues and future work

The results gleamed from this study raise questions into whether the samples, particularly Law Dome ice core sample DSS883, were at some stage contaminated. All of the controls put into place in this study showed no signs of contamination however, such as the mock ice core controls, the *Promicromonospora* "test contaminant" not being detected in any samples, and no obvious contamination arising from the culturing studies. This suggests the contamination may have arisen through the PCR amplification steps, or through airborne contamination of PCR product in latter stages of cloning. Nonetheless the next obvious step in this process is to repeat the experiment in a separate laboratory. The Australian Centre for Ancient DNA (ACAD) is a recently built facility dedicated to the study of ancient and degraded DNA samples, and necessary alterations are currently being carried out for work concerning bacterial DNA. This work will then be able to be replicated.

Another approach to future work would be to take a more focussed look at specific types of bacteria, which are known to persist in ice, but are not known contaminants in a laboratory environment. *Arthrobacter* as mentioned before is one such likely candidate. The best results gained from this study were from the larger volumes of ice, so it is recommended that future work aim to gain the maximum concentration of DNA extracted. Ideally this can be done by acquiring larger volumes of ice, but as access to such samples can be limited, as it was in this study, other avenues need to be pursued. There is evidence that bacteria are able to pass through 0.2μm filters (Hahn, 2004), and that freezing stresses can lead to smaller cell morphologies (Miteva & Brenchley, 2005). A different approach to concentrating melt water samples would be to use a smaller filter pore size to reduce the potential net loss of

bacterial cells, or to use a 50000 molecular weight cut off spin filter column (Amicon Ultra; Millipore) to capture free DNA from lysed cells. Emerging on the market also are systems to amplify genomic DNA in samples such as GenomiPhi (GE Healthcare, http://www.genomiphi.com/) and are worth pursuing in future studies to minimise the effect of possible contaminating DNA.

6.4.4 Conclusion

Much work still needs to be done in ensuring the validity of findings from Antarctic ice, but the results discussed here still expand upon the exciting possibility of ice microbiology. This chapter explored the bacterial constituents of two different types of Antarctic ice. This was the first study to examine the bacterial constituents of marine ice accreted beneath the Amery Ice Shelf and showed the types of marine bacteria to become associated with this ice, and identified the actinomycete *Williamsia maris* as a potential target of future research. The initial look into the microbiology of Law Dome is promising, identifying typical bacteria that become trapped in younger glacial ice. This can lead to exciting avenues of research, such as comparing the bacterial constituents of Law Dome ice to well documented global climate events, such as major volcanic activity, to observe the potential spread of bacteria around the globe. This study also highlighted the recognised need for replication in such areas of research to avoid controversy.

Chapter 7 General discussion

7.1 Outcomes of this study

The Australian Government Antarctic Division has a vision that all research carried out on the Antarctic environment have an emphasis of the continent being valued, protected, and understood. This PhD study aimed to continue this premise through a study into the biodiscovery and biodiversity potentials of Antarctic bacteria. This dissertation explored the taxonomy of select *Actinobacteria* isolates and identified a novel *Promicromonospora* species, the first known instance of this genus of normally temperate environments being found in Antarctica. Examining the hydrocarbon degrading ability of *Actinobacteria* in greater detail, several Actinobacteria isolates, in particular a *Rhodococcus* isolate with potentially useful hydrocarbon degrading abilities were identified. An antimicrobial product screen against various strains of *Listeria monocytogenes* identified a potentially novel *Streptomyces* isolate that may produce a useful broad spectrum antimicrobial compound. A microbiological survey of several Antarctic epiglacial lakes identified the great variety of life that inhabit these lakes, showing the great potential of this ecotype for further investigation. Finally, work was carried out on both marine ice accreted beneath the Amery Ice Shelf and Law Dome glacial ice. This part of the study identified bacterial members to be focused upon in future work and highlighted both the potential of the study of Antarctic ice that with valid scrutiny can bear exciting results. This PhD study therefore touched upon the wealth of information

Antarctica has into life on Earth, and highlights that what is currently known is only the tip of the iceberg.

7.2 Future directions

The main drawback for Antarctic research is that access to the continent can be logistically difficult, relying on often slow and expensive transportation and dependent upon the correct weather conditions, limiting the majority of Antarctic research to the summer months. This normally means expeditioners are required to spend long periods in Antarctica, which in itself can cause psychological barriers to long term research (Palinkas & Suedfeld, 2008). The Australian Government Antarctic Division has commenced flights to a blue ice runway near Casey Station, Antarctica, in an attempt to allow increased access to the continent. This in itself means there will be an exponential increase in the outputs of research carried out in East Antarctica.

In addition to this, new emerging technologies such as metagenomic screening, enables shorter sampling trips to Antarctica and will result in the total genetic diversity of a particular environment being analysed. For the field of Antarctic microbiology that means in the foreseeable future, the entire picture of life in Antarctica can finally be understood. Not only will the knowledge of the biodiversity of Antarctic ecosystems give us a greater understanding of life on Earth, but as this PhD dissertation showed, there are many more biodiscoveries to be made from bacteria in Antarctica.

Chapter 8 References

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