# Antarctic Bacteria and Enzyme Temperature Optima

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submitted in partial fulfilment
of the requirements for the Degree of
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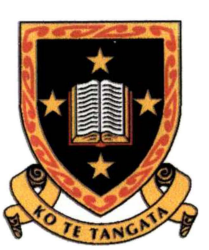
**Sarah Kate Hawkins**

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## Abstract

The temperature optimum of enzyme activity, as recently described by Daniel *et al.* (2001), is thought to play a central role in the adaptation of an enzyme to its environmental temperature. The aim of this research was to exploit this recent development in determining temperature/activity relationships by correlating the environmental temperature of Antarctic bacteria with the properties of their enzymes. To avoid the problems associated with the comparison of taxonomically distant bacteria, enzymes were sourced from a single genus, *Bacillus*.

Psychrotolerant bacteria were isolated from soils sampled at several sites (Dry Valleys, Bratina Island and Cape Crozier) in the Ross Sea region, Antarctica. At each sampling location, continuous 24 h temperature profiling of the top 2 cm of soil showed exposure to a wide temperature range (up to 20°C difference between maximum and minimum temperature) over the day. Initial characterisation revealed that 26 out of the 52 isolates obtained were Gram positive rods. Random amplified polymorphic DNA (RAPD) analysis of extracted genomic DNA and denaturing gradient gel electrophoresis (DGGE) of the 16S rRNA genes were performed on this subgroup. Five groups of closely related bacteria were identified, along with several unique isolates. 16S rDNA sequencing of eight of these isolates identified them as most likely representing the *Bacillus*, *Planococcus*, *Arthrobacter*, *Pseudomonas*, *Carnobacterium* and *Psychrobacter* genera. Isolates PR1 and MV2 (identified as species of *Bacillus*) were found to have an approximate optimum growth temperature of 27°C and >30°C, respectively.

A survey of extracellular proteases, esterases/lipases, glucosidases and galactosidases was initially performed on thermophilic Antarctic bacteria previously isolated from geothermal soils from Mount Erebus, Ross Island. The final choice of an intracellular enzyme, dihydrofolate reductase (DHFR), for temperature characterisation was preferable due to its ubiquitous expression and to allow comparison of the results with thermal properties of DHFRs from other sources.

An extrapolation of the temperature dependence of PR1 DHFR activity back to zero time revealed a 'real' temperature optima of 44°C, where at higher temperatures in the profile, the enzyme showed a decrease in catalytic rate greater than could be accounted for by irreversible denaturation. A temperature optimum determination for thermophilic DHFR from *B. stearothermophilus* also displayed a clear peak of activity at 47°C at zero time.

Data obtained for the psychrotolerant and thermophilic enzymes were insufficient to confirm the environmental significance of this intrinsic property. However, the idea that 'true' temperature optima reflects the environmental temperature was validated, being lower for the psychrotolerant enzyme compared to the thermophilic enzyme.

## Preface

The initial aim of this research was to exploit recent developments in determining temperature/activity relationships in enzymes (Daniel *et al.*, 2001) by correlating the environmental temperature of Antarctic bacteria with the molecular properties of their enzymes. This would involve a comparison of the thermal properties of homologous enzymes sourced from different environmental temperature regimes. Closely related bacteria would be isolated either from an environment of stable temperature, or an environment exposed to fluctuating temperatures. In particular, the optimal temperature of enzyme activity would be characterised, as it is thought that this newly discovered property is likely to play a central role in the adaptation of an enzyme to its environmental temperature.

This research was supported by an Antarctica New Zealand – New Zealand Post Science Scholarship, allowing me to participate in Antarctic Event K023 "Probing and exploiting DNA diversity in Antarctic biotopes", which took place in the 2001-2002 field season. As my work progressed it became more integrated with the microbial research being carried out by the other Antarctic field party members, especially Dr Craig Cary (University of Delaware, USA), who was investigating microbial diversity using molecular genetic techniques. The opportunity to expand on the ecological aspects of the research and to use current genetic technologies to characterise my bacterial isolates was considered an important learning experience within my Masters research work. The scope of this section is therefore greater than was originally intended and forms part of a larger collaborative project investigating Antarctic terrestrial biodiversity. Integration of my results with those of my field party colleagues has not been possible, since their results are not yet to hand.

As a consequence, the comparison of enzyme thermal properties is not as comprehensive as first proposed, and the work has served to lay the groundwork for future studies rather than completing them here. However, the research carried out

did support the hypothesis that these enzymes would reveal temperature optima at zero time, and that this was related to environmental temperature.

## Acknowledgements

I would like to firstly thank my supervisor, Roy Daniel, for his help and the knowledge that he has shared with me. I would like to express my gratitude to Antarctica New Zealand and New Zealand Post for funding my research and giving me the opportunity to go to Antarctica, it was a wonderful experience. Thank you also to the University of Waikato for additional financial assistance.

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Finally, special thanks to Mum, Dad, Amy and Debbie for their love and support, and especially to Dai, who has done so much for me, I really appreciate it.

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## List of abbreviations

| | |
|---|---|
| BLAST | basic local alignment search tool |
| C6 | six carbon |
| C16 | sixteen carbon |
| DGGE | denaturing gradient gel electrophoresis |
| DHFA | dihydrofolic acid |
| DHFR | dihydrofolate reductase |
| E34.A1 | thermophilic bacterium isolated from Mt Erebus soil |
| E37.A1 | thermophilic bacterium isolated from Mt Erebus soil |
| E37.A2 | thermophilic bacterium isolated from Mt Erebus soil |
| EPPS | N-2-hydroxyethylpiperazine-N′-3-propanesulphonic acid |
| MES | 2-(N-morpholino)ethanesulphonic acid |
| MOPS | 3-(N-morpholino)propane sulphonic acid |
| MV2 | psychrotolerant bacterium isolated from Miers Valley soil |
| MVS4 | psychrotolerant bacterium isolated from soil near Miers Valley seal |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NCBI | National Centre for Biotechnology Information |
| OD | Optical density |
| PCR | polymerase chain reaction |
| pI | isoelectric point |
| *p*-NP | *para*-nitrophenol |
| PR1 | psychrotolerant bacterium isolated from penguin rookery |
| RAPD | random amplified polymorphic DNA |
| r.c.f. | relative centrifugal force |
| TAE | Tris Acetate EDTA buffer |
| TBE | Tris Borate EDTA buffer |
| TCA | trichloroacetic acid |
| Tris | tris(hydroxymethyl)-aminomethane |
| TRUCC | Thermophile Research Unit Culture Collection |
| TSB | tryptic soy broth |
| 16S rRNA | small-subunit ribosomal RNA |

# Chapter 1 Literature Review

## 1.1 Overview

This thesis describes research carried out on the isolation of psychrotolerant bacteria from Antarctic soils and an initial characterisation of their enzyme properties with regard to thermal adaptation.

The aim of the research was to correlate the environmental temperature of organisms with the molecular properties of their enzymes, giving a better understanding of the biochemical and physiological adaptations of Antarctic microorganisms to the cold climate.

Bacteria were isolated from soil samples taken from four sites in the Ross Sea region of Antarctica, and identified using microbiological and molecular genetic techniques. By studying bacteria from the same genus, *Bacillus*, taxonomic complications were avoided. These result from the different evolutionary histories that more distantly related bacteria have undergone, and make comparison between enzymes difficult as differences in the structure and property result from adaptation to a complex combination of selective pressures (Arnold *et al.*, 2001)

Temperature profiling of each Antarctic site was carried out in the field, allowing a 24 hour continuous measurement of soil surface temperature to gain information on the frequency and magnitude of temperature changes during the day.

The thermal properties of dihydrofolate reductase (DHFR) were compared between a psychrotolerant *Bacillus* species isolated from a penguin rookery at Cape Crozier and *Bacillus stearothermophilus*, a thermophilic bacterium. The most important property

studied with regard to temperature adaptation was the temperature optima of enzyme activity. This may be an important measurement of the adaptation of an enzyme to its environmental temperature, as it shows that decrease in catalytic rate at temperatures above optimum are not due to irreversible thermal inactivation, but because the enzyme is working outside of its adapted, and therefore maximal, temperature range.

Preliminary work was carried out using thermophilic Antarctic bacteria isolated from Mt Erebus and stored in the Thermophile Research Unit Culture Collection (TRUCC). The objective of this work was to assay prospective enzymes and if successful, develop a method of purification and characterisation.

## 1.2 Antarctica

### 1.2.1 Introduction

The Antarctic environment offers severe challenges for biological processes. Antarctica is the coldest, highest and driest continent on Earth, and only a few percent of the continent is ice-free (Campbell *et al.*, 1997). The general climate of the continent is characterised by extreme cold, little precipitation and frequent strong winds (Weyent, 1966). Antarctica is so cold due to its high lattitude, receiving only a small amount of solar radiation. During the summer, most sunlight is re-radiated, in winter none is received and a net heat loss occurs. The warmest part of the continent is the Antarctic Peninsula, with an average temperature of $-5^{o}C$ (see Figure 1.1). Coastal regions are generally $-10^{o}C$ to $-20^{o}C$, although this quickly drops to $-40^{o}C$ a short distance from the coast. The average temperature of the central plateau is $-50^{o}C$ (Campbell and Claridge, 1987).

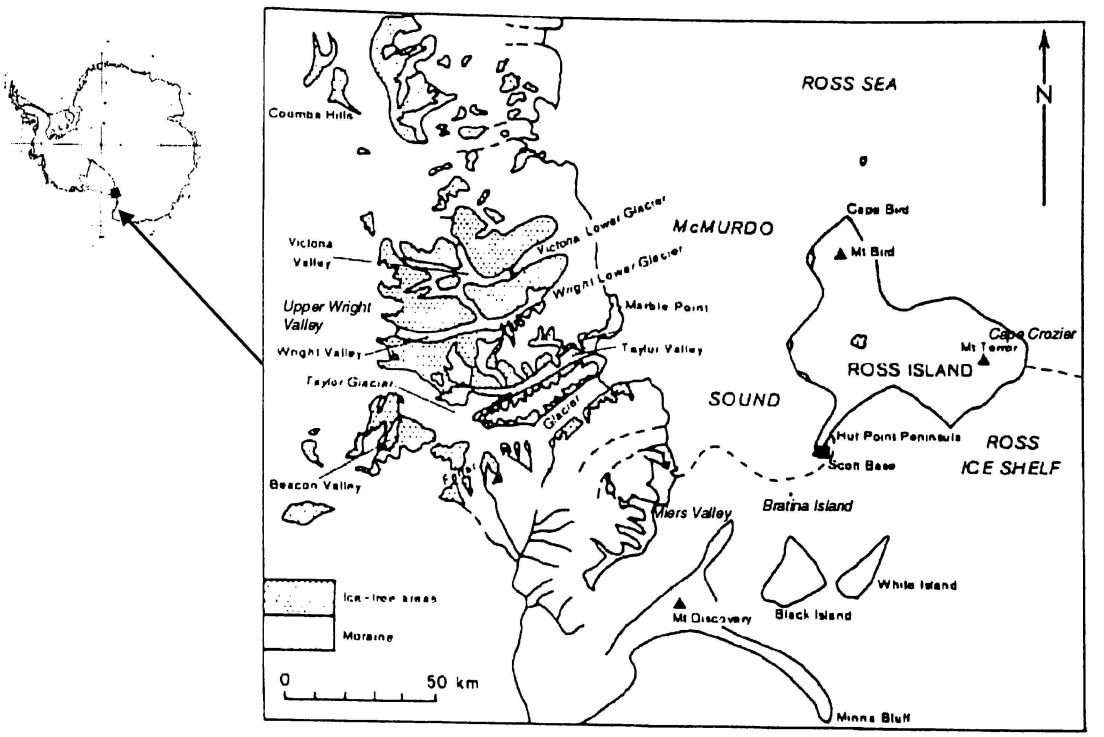



**Figure 1.1. The Ross Valley region, Antarctica, where sampling sites were located. Adapted from Campbell and Claridge (1987) and 'Ross Island and Vicinity' Map 76190-W1-RR-250, US Geological Survey**

## 1.2.2 Antarctic sampling sites

### 1.2.2.1 Dry Valleys

The Dry valley region is one of the few areas of Antarctica that remains free of ice. Three main valleys, Taylor, Wright and Victoria, run from the Transantarctic Mountains to the Ross Sea coast, and are separated by tall ranges. Glaciers draining ice from the polar plateau to the sea carved the valleys, which became dry when the land raised faster than the glaciers could move, causing them to recede (Trewby, 2002). Also, the rate of potential evaporation is much higher than the average snowfall received (Clow *et al.*, 1988), preventing the accumulation of snow on the valley floors.

#### 1.2.2.2 Cape Crozier

Cape Crozier is positioned at the eastern tip of Ross Island, at the junction of the Ross Sea and the Ross Ice Shelf. It is the site of an Adelie penguin and Emperor penguin colony, and was visited in 1911 by members of the British Antarctic Expedition for the collection of Emperor penguin eggs (Trewby, 2002).

#### 1.2.2.3 Bratina Island

This small island is located in the middle of the McMurdo Ice Shelf, south-west of Cape Armitage, Ross Island. Its position at the zone of confluence between the Ross Ice Shelf and glaciers descending from the Dry Valleys has resulted in the island being surrounded by moraines deposited into the sea ice and many meltwater ponds (Texas A & M University website, www.tamug.edu). The chemical and biological properties of these ponds have been the subject of many studies, e.g. Sheridan and Brenchley (2000), Nadeau and Castenholz (2000).

### 1.2.3 The Antarctic climate

#### 1.2.3.1 Dry Valleys

The local climate of exposed soil locations, such as the Dry Valley regions, is significantly different from snow covered sites, being warmer and drier with low precipitation (Weyent, 1966). This is mainly due to the difference in albedo (fraction of light reflected) of snow and soil. Snow has an average albedo of over 80%, i.e. less than 20% of solar radiation received is absorbed as heat. In contrast, exposed soil has an albedo of 20% at the most, so 80% of solar radiation is retained. This causes heating of the soil surface and the overlying air (Weyent, 1966).

Relative atmospheric humidity in the Dry Valleys is usually less than 10% in winter, due to strong katabatic winds. An increase in humidity occurs during summer, however precipitation is scarce and falls as snow rather than rain. An average of 5-10cm snow falls annually, but never accumulates on the valley floor due to the high potential sublimation rate of 50cm.year$^{-1}$ (Wynn-Williams, 1990).

#### 1.2.3.2 Coastal sites: Bratina Island and Cape Crozier

Coastal areas are generally warmer and have more cloud and precipitation than the central continent. They are also more strongly influenced by cyclonal activity (Weyent, 1966). A covering of snow insulates the soil from large temperature changes (Thompson, 1971). The deeper the layer of snow, the less severe winter temperatures experienced at the soil surface (Davey, 1992).

### 1.2.4 Antarctic microbiology

#### 1.2.4.1 History

Microbial research in the Antarctic began with the discovery of soil bacteria by the Swedish South Polar Expedition of 1901-1903 (Trewby, 2002). Early studies used classical medical microbiological methods to survey the bacteria present and work on the taxonomy of the species isolated (Wynn-Williams, 1990). In 1941, Darling and Siple carried out the first detailed study of species indigenous to the Antarctic (Boyd *et al.*, 1966). Sieburth (1958) investigated the gastrointestinal microbiology of Antarctic birds in an attempt to explain reports that they were "bacteriologically sterile".

Between 1962 and 1963, a survey of the Taylor and Wright Valleys was carried out by the US Antarctic Research Programme to study the types of microorganisms present and determine limiting factors towards their growth, amongst other research (Boyd *et al.*, 1966). In the 1970's, microorganisms were discovered living inside

translucent rocks in the Dry Valleys, these were termed 'cryptoendolithic communities'. This finding led to an increased level of interest in the microbiology of these areas (Trewby, 2002).

Microbiological methods, concepts and equipment for the Viking Mars program of 1976 were tested in the Ross Desert due to the similarities in the physical and chemical weathering processes of the Antarctic cold desert soils and those detected on Mars (Campbell 1998).

### 1.2.4.2 Conditions affecting microbial life

Life in the exposed soil regions of Antarctica presents many problems. Along with cold temperature, microorganisms must deal with desiccation, high salt concentration, cycles of freezing and thawing, low solar radiation, high winds, low humidity, short duration of water availability, plus substratum instability (Cameron, 1968; Wynn-Williams, 1990). Soil bacteria have been found more abundantly below the surface, being less vulnerable to climate conditions (Cameron, 1968). In the Dry Valleys, many microbes have been found in cryptoendolithic communities, gaining protection from the harsh conditions.

Generally, the presence of liquid water is more important than temperature in determining whether soil is habitable for microorganisms (Baross and Morita, 1978). Areas with shallow permafrost layers can undergo thawing and support microbial life, along with meltwater streams and pools (Wynn-Williams, 1990). Moisture availability is determined by frequency and length of exposure to solar radiation, air temperature, drying winds, exposure, slope and drainage of terrain and valley orientation (Cameron and Conrow, 1969).

Salts can accumulate at the soil surface as a result of upward translocation and the gradual transfer of seaspray (Wynn-Williams, 1990). High concentrations of salt limit

the microbial population that can survive in these soil types (Cameron and Conrow, 1969).

Soils in the vicinity of penguin colonies are enriched with nitrogen and phosphorus from the decomposition of guano while other areas, like the Dry Valleys, are nutrient-limited (Wynn-Williams, 1990).

## 1.3 Psychrophilic and Psychrotolerant bacteria

> *Psychrophiles have an optimum temperature of growth of 15°C or lower, a maximum temperature for growth of 20°C, and a minimum of 0°C or lower. Psychrotolerant bacteria (psychrotrophs) can grow at, or around 0°C, but have a maximum growth temperature above 20°C.*
>
> (Morita, 1975).

In 1887, Forster reported the first bacteria able to grow at 0°C. These were cultures of luminous bacteria isolated from marine fish. Fischer confirmed this finding in 1888 by culturing further strains of luminous and marine bacteria (Herbert, 1986).

As early investigators were mostly interested in whether bacteria were able to grow at or close to 0°C, confusion arose over the definition of cold-adapted bacteria (Morita, 1975). Investigators found it difficult to tell if bacteria were growing at these low temperatures or just surviving. Most bacteria found to grow around 0°C were originally defined as being psychrophilic or "cold-loving". However, this definition failed to take into account cardinal growth temperatures, often the optimum temperature for growth (i.e. that at which cell division occurred at the highest rate) was around 20-30°C (Morita, 1975).

Most investigators were unaware that bacteria truly adapted to the cold would die at temperatures above 20°C, and it is likely that their lack of success was the result of

using unsuitable source materials and handling techniques (Herbert, 1986). To isolate a psychrophilic bacterium, the environment from which the source material was sampled must have been constantly below 20°C over its history. After sampling, the material must be kept cool, and all laboratory work carried out with precooled media, pipettes and other equipment (Herbert, 1986). Many of the first investigators would not even have had the use of a refrigerated incubator (Morita, 1975).

As a result of this, most 'psychrophilic' bacteria discovered by in early studies were later found to be psychrotolerant. It was not until 1964 that the first real psychrophiles, all isolated from the marine environment, were reported (Morita, 1975).

### 1.3.1 Habitat

Polar regions cover 14% of the earths surface, while oceans cover 71%. In total, more than 90% of the earths surface is 5°C or colder (Morita, 1975). As genuinely psychrophilic bacteria are restricted to permanently cold environments, ocean water is one of the best sources of these bacteria, as it has been cold for millions of years (Morita, 1975). Other permanently cold environments include polar and alpine regions (Feller and Gerday, 1997).

In contrast, psychrotolerant microorganisms are able to grow in a wide range of environments that undergo thermal fluctuations. Even in permanently cold environments, most bacteria are psychrotolerant rather than psychrophilic (Gounot, 1991). The habitats of psychrotolerant bacteria include the Earth's atmosphere, which can reach –40°C at high altitudes; snow, ice and caves in polar regions; freshwater lakes, mountainous rivers and streams (Baross and Morita, 1978). The guts of fish, and frozen and chilled foods are other sources (Gounot, 1991).

Soils, even in polar regions, are unstable cold environments. The temperature of soils can fluctuate greatly due to heating by solar radiation, so the bacteria isolated are

predominantly psychrotolerant. However, microbial growth will only occur in soils where liquid water is present (Baross and Morita, 1978).

The role of psychrophilic and psychrotolerant bacteria in the biodegradation of organic matter and nutrient recycling is very important in colder ecosystems (Gounot, 1991; Herbert, 1986).

### 1.3.2 Species diversity

Many genera contain cold-adapted bacteria, for example *Pseudomonas*, *Vibrio*, *Flavobacterium*, *Bacillus* and *Clostridium*. (Gounot, 1991; Herbert, 1986). However, the cold-adapted species studied so far have been predominantly Gram negative.
Many mesophilic soil bacteria, such as *B. megaterium* and *B. subtilis* are capable of growth below 5°C (Baross and Morita, 1978). Apart from bacteria, cold-adapted microorganisms such as yeasts, fungi and algae have also been discovered (Herbert, 1986).

### 1.3.3 Adaptations to the cold

To live in extreme conditions, adaptations must be made at every level of an organisms function. This includes enzyme kinetics, membrane fluidity and ion channels plus entire metabolic pathways (Feller and Gerday, 1997). Specific adaptations include: an increased proportion of unsaturated fatty acids in the cell membrane to retain fluidity, synthesis of cold shock proteins (Gounot, 1991), increased efficiency of enzyme catalysis and effective active transport of nutrients (Brock and Madigan, 1991).

## 1.4 The Genus *Bacillus*

### 1.4.1 Introduction

The genus *Bacillus* consists of aerobic, rod-shaped, Gram positive, endospore-forming bacteria most commonly found in soils, and was one of the first to be described. Most bacteria are saprophytic, playing an important role in the cycling of nutrients by breaking down complex polysaccharides and by proteolysis (Norris *et al.*, 1981). Due to the heat resistance of its spores, and ability to break down organic compounds, the genus is an important group of spoilage organisms (Norris *et al.*, 1981).

Members of the genus display a range of nutritional requirements, growth conditions, metabolic diversity and genetic composition presenting a taxonomical challenge (Ash *et al.*, 1991). Reclassification of the species began in 1991 using morphological and physiological criteria (Goto *et al.*, 2000).

Classification of bacteria based on morphology is difficult because they are small and simple looking, while using physiological features is not feasible for bacteria that cannot be isolated in pure culture (Muyzer, 1999). Molecular genetic techniques, such as Random Amplified Polymorphic DNA (RAPD) analysis, DNA-DNA hybridisation, restriction mapping and 16S rRNA sequencing, have done much to elucidate phylogenetic relationships between species. Currently, at least thirteen genera have been revealed: *Alicyclobacillus*, *Amphibacillus*, *Aneurinibacillus*, *Bacillus*, *Brevibacillus*, *Filobacillus*, *Geobacillus*, *Gracilibacillus*, *Jeotgalibacillus*, *Paenibacillus*, *Salibacillus, Ureibacillus* and *Virgibacillus* (Xu and Côté, 2003).

### 1.4.2 Psychrophilic and psychrotolerant *Bacillus* species

Many cold-adapted species of *Bacillus* have been isolated from environmental and food sources. Larkin and Stokes (1966) carried out one of the original studies, where

90 'psychrophilic' isolates were cultured from soil, mud and water. This led to the description of *Bacillus psychrosaccharolyticus*, *Bacillus insolitus*, *Bacillus globisporus* (now *Sporosarcina globispora*) and *Bacillus psychrophilus* (now *Sporosarcina psychrophila*). However, according to the current definition, all these bacteria are actually psychrotolerant, having an optimum temperature of growth exceeding 20°C.

Psychrotolerant *Bacillus macquariensis* (now *Paenibacillus macquariensis*) was isolated from Antarctic Macquarie Island soil by Marshall and Ohye (1965). *Bacillus marinus* (now *Marinibacillus marinus*), initially described as a subspecies of *B.globisporus* (Rüger, 1983) has had both psychrotolerant and psychrophilic strains isolated from deep-sea sediments (Rüger *et al.*, 2000). Two recently described psychrotolerant species are *Bacillus psychrotolerans* and *Bacillus psychrodurans* from soil and water samples (Abd-El-Rahman *et al.*, 2002).

Psychrotolerant *Bacillus* species are the primary cause of spoilage of pasteurised milk and milk products. *Bacilli* capable of growth at 6.5°C included *B. cereus*, *B. lentus*, *B. polymyxa*, *B. thuringiensis*, *B. circulans*, *B. pumilis*, *B. mycoides*, *B. pasteurii* in a study carried out by Garcia-Armesto and Sutherland (1997).

## 1.5 Temperature adaptation of enzymes

### 1.5.1 Temperature optima

Previous studies of the temperature adaptation of enzymes have focused on two properties, the Arrhenius activation energy, which is the energy barrier of the reaction (Feller and Gerday, 1997), and the thermal stability of enzymes, i.e. the ability of the enzyme to retain a functional conformation against increasing temperature. A combination of these two properties has sometimes been presented as the temperature optima of the enzyme, but this is not a biochemically valid parameter, being a result

of assay duration as well as these properties. As Daniel *et al.* (2001) point out, in a graph of "enzyme activity vs. temperature", the ascending limb results from the temperature coefficient of catalytic rate (i.e. the Arrhenius activation energy) until denaturation becomes significant, then activity decreases from a combination of denaturation and thermal stability. Because the contribution of denaturation is depend on assay duration as well as temperature, the apparent optimum is shifted to lower temperatures for longer assays. Thus the temperature optimum determined is not a true biochemical parameter. To describe a true temperature optimum, a new model of thermal inactivation has been described by Daniel *et al.* (2001) where the enzyme can be in three states: active, inactive and thermally denatured,

$$\text{i.e. } N \leftrightarrow I \rightarrow D$$

This means that temperature also affects the equilibrium position between the inactive and active protein forms, in addition to its other effects on enzyme activity. Therefore, even at time zero in an "enzyme activity vs. temperature" plot, when no denaturation could have taken place, the enzyme will show a temperature optimum (Daniel *et al.*, 2001). This 'Equilibrium Model' contrasts with the 'Classical Model', which shows no optimum temperature of activity at zero time but an infinite increase in enzyme activity (Figure 1.2).

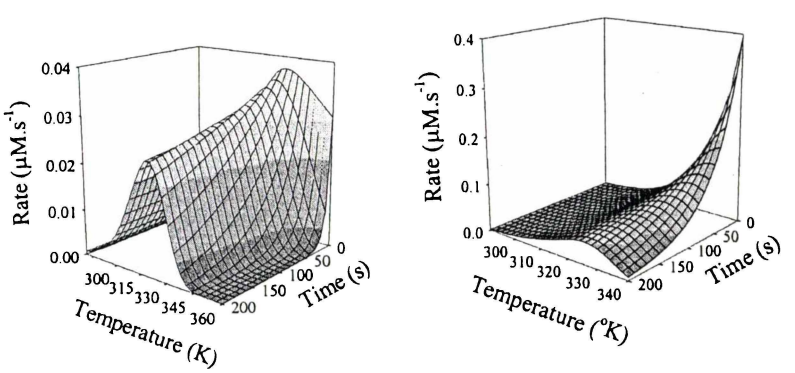



**Figure 1.2. A, The Equilibrium Model of enzyme activity; B, the Classical Model**

Studies which have taken these factors into account show that some enzymes have real temperature optima, for example, Thomas and Scopes (1998) showed that $K_{cat}$ values for a mesophilic and thermophilic 3-phosphoglycerate kinase reached a maximum, and then decreased with higher temperature before irreversible denaturation had any significance.

### 1.5.2 Cold-adapted enzymes

Decreasing temperature has many effects on the aqueous systems in which enzymes operate. These include the increased viscosity of water, reduced diffusion of solutes, reduced salt solubility, increased pH in biological buffers and an exponential drop in reaction rate due to the reduced thermal motion of the enzyme resulting from cold temperature (Marshall, 1997).

Cold-adaptation of enzymes is reached through improved turnover number and catalytic efficiency. This is normally due to enzyme structure becoming more flexible compared with its mesophilic counterpart, allowing greater movement. However, this means a reduced stability of the enzyme at higher temperatures (Zecchinon *et al.*, 2001).

Laboratory evolution of proteins has demonstrated that activity at low temperature and stability at high temperature can be improved simultaneously. However, as there is no requirement for psychrophilic enzymes to be stable at high temperature, this property (not fixed by selection) will eventually vanish due to random genetic drift (Miyazaki *et al.*, 2000). Some enzymes, however, have been found to retain thermal stability, e.g. an aspartate transcarbamylase from a psychrophilic bacterium was shown to be stable at 60°C by Sun *et al.* (1998).

Previous studies have given insights into the adaptation of enzymes to cold temperatures. For example, a psychrophilic DNA ligase from *Pseudoalteromonas*

*haloplanktis*, an Antarctic seawater bacterium was found to have an increased catalytic turnover ($K_{cat}$) which compensated for the reduction in metabolic rate caused by low temperature (Georlette *et al.*, 2000).

Decreasing $K_m$ (which generally reflects the affinity of the enzyme for the substrate), can increase catalytic efficiency ($K_{cat}/K_m$) and is another adaptive strategy to cold temperature (Zecchinon *et al.*, 2001).

# Chapter 2 Survey of Thermophilic Antarctic Bacteria for Enzyme Production

## 2.1 Introduction

Initial experimental work was carried out to look for an enzyme that was easy to assay and purify, and present in relatively high concentration from Antarctic bacteria already held in the TRUCC. It was hoped that the methods used in this research could be applied to Antarctic isolates collected in later parts of the study. Extracellular enzymes are easy to screen as they are secreted into the culture medium. This also makes them simple to purify as they can be separated from a bacterial culture with a minimal amount of cell lysis. This prevents the target enzyme from becoming mixed up with the intracellular proteins, reducing the number of purification steps required. *Bacilli* are well known to produce extracellular proteases and lipases, so these enzymes seemed like a good choice for initial screening.

## 2.2 Materials and Methods

### 2.2.1 Bacterial cultures

The Antarctic isolates used in this section were collected at Mt Erebus, Ross Island, Antarctica by R.M. Daniel in 1996. Sampled from geothermal soils at temperatures of 45-65°C and pH 4.5-5.0, they were characterised as thermophilic, rod-shaped, aerobic bacteria with an optimum growth temperature of 55°C. The three isolates were assigned sample names E34.A1 (TRUCC TG793), E37.A1 (TRUCC TG794) and E37.A2 (TRUCC TG795). The ability to clear casein agar plates indicated that the isolates could produce extracellular proteases.

### 2.2.2 Protease Production

#### 2.2.2.1 Growth of cultures in casein media

Freeze-dried ampoules of cultures E34.A1, E37.A1 and E37.A2 were aseptically opened by rinsing with 70% ethanol, then wrapping with a tissue and snapping the end containing cotton wool. This was removed and a small volume of casein broth (Appendix 1) pipetted into the ampoule, then drawn up and down several times to resuspend the cells. The cell suspension was transferred to a flask of casein broth and incubated overnight at 55°C in a shaking incubator. Protease activity in the whole culture was assessed using the azocasein assay.

The paper label was removed from the culture ampoule and placed onto a casein agar (Appendix 1) plate. Streaks were made from the wet paper across the surface of the agar and the plate incubated at 55°C overnight.

#### 2.2.2.2 Azocasein assay

| | | |
|---|---|---|
| Assay contained: | 50mM MOPS, pH 7.2 | 400µL |
| | 0.4% azocasein in dist. water | 500µL |
| | [100mM $CaCl_2$ | 50µL] |
| | Enzyme solution (whole culture) | 100µL |

Reactions were incubated at 70°C for 30 minutes, then stopped with 500µL 15% trichloroacetic acid (TCA) and cooled on ice for 10 minutes. Next, tubes were centrifuged at 16.1 r.c.f. for 5 minutes using an Eppendorf Centrifuge 5415 D. Assays were performed with or without $CaCl_2$ and consisted of two replicates plus one control, in which the enzyme solution was not added until after termination of the reaction. Sample absorbance was measured at a wavelength of 420nm.

One unit of protease activity was defined as producing an $OD_{420}$ change of one per minute.

### 2.2.2.3 Modification of azocasein assay

The composition of assay reagents was altered to try to increase detection of protease activity. Two modified assays were tested, one with less substrate present (in case of substrate inhibition), the other with a greater amount of enzyme.

| | | |
|---|---|---|
| Less substrate assay: | 50mM MOPS, pH 7.0 | 350µL |
| | 0.4% azocasein in dist. water | 100µL |
| | 100mM $CaCl_2$ | 50µL |
| | Distilled water | 400µL |
| | Enzyme solution (whole culture) | 100µL |
| More culture assay: | 200mM MOPS, pH 7.0 | 100µL |
| | 0.4% azocasein in dist. water | 100µL |
| | 100mM $CaCl_2$ | 50µL |
| Enzyme solution (whole culture) | 750µL | |

These assays were incubated for 30 minutes or 2 hours at 70°C and were carried out with all three cultures grown in casein broth (Appendix 1) plus a positive control, *B. subtilis*.

### 2.2.2.4 Growth of E34.A1 in casein media of varying pH

Casein broth was prepared with pH altered to either pH 4, 5 or 6. A 1mL volume of culture was inoculated into each flask and incubated overnight as previously described (Section 2.2.2.1). Cell morphology was assessed and an azocasein assay performed to determine protease activity.

### 2.2.2.5 pH profile of protease activity

The effect of pH on enzyme activity for all three cultures was tested using buffers of varying pH. Buffers were prepared at room temperature but pH was adjusted to be the correct value at 70°C.

Buffers used were:

- 50mM sodium acetate, pH 5
- 50mM MES, pH 6
- 50mM MOPS, pH 7
- 50mM EPPS, pH 8 and 8.5

Protease activity was measured using the azocasein assay (Section 2.2.2.2), reactions were incubated for 30 minutes and 2 hours at 70°C.

### 2.2.2.6 Growth of cultures E34.A1 and E37.A1 in Modified R medium

Modified R medium (Appendix 1) was used to try to increase cell growth and extracellular protease production.

A 1mL volume of E34.A1 or E37.A1 grown in casein broth was inoculated and incubated shaking at 55°C overnight. Protease production was assayed using the azocasein method.

### 2.2.2.7 Kunitz assay

Culture E37.A1 was assayed for protease activity using an alternative method, the Kunitz assay, to confirm the azocasein assay was giving the correct results.

| | | |
|---|---|---|
| Assay consisted of: | 0.5% Hammersten casein in 50mM Tris, pH 7.0 | 500µL |
| | Enzyme solution (whole culture/supernatant*) | 100µL |

*Culture supernatant had been concentrated by a factor of two using a Centricon filter tube (3000 MW cut-off, Millipore corporation, USA).

Reactions were incubated at 70°C for 30 minutes and stopped with 750µL 15% TCA. Tubes were cooled on ice for 10 minutes, then centrifuged at 16100 r.c.f. Absorbance was measured at 280nm. Assays were performed in duplicate with one control, in which the enzyme solution was not added until after termination of the reaction.

One unit of protease activity was defined as producing an $OD_{280}$ change of one per minute.

The Kunitz assay was also performed with E34.A1, E37.A1 and E37.A2 cultures that had been grown in a modified casein broth to try to increase protease production. This was made as described in Appendix 1, however, trypticase peptone was not added and the amount of yeast extract was decreased from 1g to 0.5g.

Cultures were grown overnight as described previously. The Kunitz assay was carried out with and without $Ca^{2+}$, ie:

| | |
|---|---|
| 0.5% Hammersten casein in 50mM Tris, pH 7.0 | 500µL |
| [100mM $CaCl_2$ | 50µL] |
| Enzyme solution (whole culture/supernatant*) | 200µL |

*Culture supernatant was concentrated to 5x its original concentration using a Centricon filter tube (3000 MW cut-off, Millipore Corporation, USA).

The reaction was stopped using 750µL 15% TCA.

### 2.2.2.8 Growth of culture E37.A1 in basic media

Five types of basic media were prepared:

| | | |
|---|---|---|
| 1. Salts + low N | $(NH_4)_2SO_4$ | 0.2 g |
| | $MgSO_4.7H_2O$ | 0.5g |
| | $CaCl_2.2H_2O$ | 0.25g |
| | $KH_2PO_4$ | 1.0g |
| | Yeast extract | 0.2g |
| 2. Salts + protein | Salts as above, 5g gelatin | |
| 3. Salts + N | Salts as above, 2g yeast extract, 2g trypticase peptone | |
| 4. Salts + glucose | Salts as above, 1mg glucose | |
| 5. Salts + N + glucose | Medium 3 + 1mg glucose | |

Each medium was prepared at pH 5.5 and 7.5, made up to 1L with distilled water and autoclaved. A 1mL volume of E37.A1 was inoculated into each medium, plus all three cultures were inoculated into casein broth and incubated as described in Section 2.2.2.1 for approximately 3 days. Protease activity was determined at 7-18 hour intervals using the azocasein assay.

### 2.2.2.9 Enzyme production over time

E34.A1, E37.A1 and E37.A2 plus *B. subtilis* were inoculated into casein broth, pH 6.0, and placed in the shaking incubator at 55°C. Samples of approximately 1mL were removed at 9, 11, 25, 28, 31 and 34 hours. Protease activity was measured using the azocasein assay, with an incubation period of 30 minutes at 62°C.

### 2.2.2.10 Growth of culture E37.A2 in modified basic media

Culture E37.A1, having been found to produce the greatest amount of protease activity in the previous growth experiment (Section 2.2.2.9) was tested for an increased level of protease production in basic media. The media used were changed slightly from those used in Section 2.2.2.8, based on the results of this initial attempt. *B. subtilis* was included in this growth and enzyme production experiment as a positive control.

Media:

1. 2% casein broth (Appendix 1), pH 6.0
2. Salts*, N (2g/L yeast extract, 2g/L trypticase peptone), 1mg/L glucose, pH 7.5
3. Salts, N (2g/L yeast extract, 0.5g/L trypticase peptone), 1mg/L glucose, pH 7.5
4. Salts, N (0.5g/L yeast extract, 2g/L trypticase peptone), 1mg/L glucose, pH 7.5

*Salts were the same as used in Section 2.2.2.8

E37.A2 was inoculated as previously described. Culture growth was monitored by absorbance at 650nm and protease activity measured using the azocasein assay (Section 2.2.2.2).

### 2.2.2.11 Casein agar plate method for determination of protease activity

Proteolytic activity is visualised by formation of a white *para*-casein precipitate ring around sample wells cut into the casein agar plate. The diameter of the rings is measured to determine protease activity.

Casein agar plates were prepared by dissolving 10g Hammersten casein and 17.5g agar into 500mL distilled water, then mixing with an equal volume of 100mM sodium acetate pH 5.0, MES pH 6.0 or MOPS pH 7.0. The medium was autoclaved, allowed to cool and poured into plates. When agar had set, 8mm holes were cut into

the agar and 100μL whole culture (E34.A1, E37.A1 and E37.A2) added. Plates were sealed with tape and incubated overnight at 55°C. Diameter of *para*-casein ring was measured using a ruler.

### 2.2.3 Esterase and lipase production

#### 2.2.3.1 Growth of bacterial cultures in Claudia medium and Tryptic Soy Broth

Flasks containing Claudia medium (see Appendix 1) or TSB (30g/L; Becton, Dickinson and Co., USA) were inoculated with E34.A1, E37.A1 or E37.A2 cultures and incubated overnight shaking at 55°C. Presence of lipase or esterase activity in the culture supernatant was detected using the *p*-nitrophenyl assay, which measures the release of yellow *p*-nitrophenol (*p*-NP) from *p*-nitrophenyl esters of fatty acids.

#### 2.2.3.2 *p*-Nitrophenyl assay for detection of esterase or lipase activity

Substrate emulsion was prepared as follows: 1mg/mL *p*-NP substrate (*p*-NP caproate, C6, for esterase activity or *p*-NP palmitate, C16, for lipase activity) dissolved in 1mL ethanol was dispersed into 8mL 0.1M MOPS, pH 7.2, containing 5mM $CaCl_2$ and 0.1% Triton X-100, by vigorous mixing.

| | | |
|---|---|---|
| Assay consisted of: | Substrate emulsion | 900μL |
| | Enzyme solution (bacterial culture) | 100μL |

The reaction was thoroughly mixed and incubated at 55°C for 30 minutes or 2 hours. The assay was terminated by the addition of 500μL 15% TCA. The tubes were cooled on ice for 10 minutes, centrifuged for 5 minutes at 16100 r.c.f. then absorbance of the supernatant measured at 310nm wavelength. The extinction coefficient for *p*-NP used to calculate activity was 10.26 $mM^{-1}cm^{-1}$ under the assay conditions (L. Chen, personal communication).

### 2.2.3.3 Test of *p*-Nitrophenyl assay method

The *p*-NP assay was carried out as previously described (Section 2.2.3.2) except that the concentration of enzyme in the assay was altered, ie assay was carried out with 100μL bacterial culture and 900μL substrate emulsion, or 200μL culture and 800μL emulsion. Assays were incubated for 30 minutes, 1 hour or 2 hours at 60°C using the *p*-NP caproate substrate. The reaction was stopped by the addition of 500μL of ice cold buffer, the tubes were cooled on ice for 5 minutes then centrifuged for 5 minutes at 16100 r.c.f. Sample absorbance was measured at 400nm.

### 2.2.3.4 Location of esterase in bacterial cells

Cultures of E34.A1 and E37.A2 were grown in nutrient broth (8g/L) to an optical density of at least one (650nm wavelength). A 1 mL sample of each culture was centrifuged at 4500 r.c.f. for 10 minutes, the supernatant transferred to a new tube, and the cell pellet resuspended in the same volume of fresh nutrient broth. Supernatant, resuspended pellet and whole cultures were assayed for esterase activity using *p*-NP caproate for 30 minutes at 60°C (Section 2.2.3.2). A 1:100 dilution of 1μg/mL porcine liver esterase solution was included as a positive control for the assay.

### 2.2.3.5 Release of esterase activity by washing bacterial cells

A sample of E37.A2 culture grown in nutrient broth (8g/L) to an optical density of one (at 650nm wavelength) was centrifuged at 4500 r.c.f. for 10 minutes. The supernatant transferred to a new tube and cells resuspended in one of the following solutions:

- 100mM KCl
- 2% (w/v) sodium citrate
- 2% (w/v) lysine
- 0.1% Triton X-100
- 1% Sodium dodecyl sulphate (SDS)
- 8g/L nutrient broth

The suspensions were re-centrifuged and the supernatant assayed for esterase activity (Section 2.2.3.2).

### 2.2.4 Glucosidase and galactosidase production

#### 2.2.4.1 *p*-NP assay method

Assay consisted of:

| | |
|---|---|
| 1mg/mL *p*-NP substrate in 100mM MOPS, pH 6.3 | 100µL |
| Enzyme solution (whole culture) | 5µL |

Incubated at 60°C for 10 minutes and stopped with 100µL 1M $Na_2CO_3$. Tubes were cooled on ice for 5 minutes, spun at 16100 r.c.f for 5 minutes then absorbance was read at 420nm.

*p*-NP substrates used were: *p*-NP α-D-glucopyranoside
*p*-NP β-D-glucopyranoside
*p*-NP β-D-galactopyranoside

The extinction coefficient for *p*-NP under the assay conditions was 18.3 $mM^{-1}cm^{-1}$ (J. Bragger, personal communication).

### 2.2.4.2 Location of glucosidase and galactosidase activity in bacterial cells

Cultures of E37.A1 and E37.A2 were grown in nutrient broth to an absorbance of 0.903 and 0.485, respectively. Samples of 1mL were sonicated on ice for two cycles of one minute and one cycle of 30 seconds, with cooling in between. The samples were centrifuged at 16100 r.c.f. for 10 minutes and the supernatant transferred to a new tube. The pellet was resuspended in 1mL nutrient broth. The supernatant, resuspended pellet and whole culture samples were assayed for glucosidase and galactosidase activity (Section 2.2.4.1).

### 2.2.4.3 Release of α-glucosidase or β-glucosidase activity from cell membranes

E37.A1 was grown in nutrient broth to an optical density of one. The culture was centrifuged and cell pellet washed with solutions as described in Section 2.2.3.5. Additional washing solutions used were 0.001% Tween-80, 4mM deoxycholic acid and distilled water. The supernatant was assayed for α-glucosidase and β-glucosidase activity (Section 2.2.4.1).

### 2.2.4.4 Induction of α-glucosidase or β-glucosidase using modified media

Nutrient broth (8g/L) was prepared with 0.2% (w/v) maltose, a substrate for α-glucosidase, or 0.2% (w/v) cellobiose or carboxymethyl cellulose, both substrates for β-glucosidase. Media were inoculated with a 1mL volume of culture E37.A1 and incubated shaking at 55°C. Cultures were assayed after 24 and 48 hours using the following *p*-NP substrates:

*p*-NP α-D-glucopyranoside
*p*-NP α-D-maltoside
*p*-NP β-D-glucopyranoside
*p*-NP β-D-maltoside
*p*-NP β-D-cellobioside

### 2.2.4.5 Growth in minimal media

A Salts + N medium was made (Section 2.2.2.8) with 0.2% (w/v) maltose and 0.5g/L or 2g/L yeast extract. Half (4g/L) and full strength (8g/L) nutrient broth with 0.2% maltose was also used. The three cultures were inoculated into each medium and incubated shaking at 55°C. α-glucosidase activity was assayed between 39-45 hours growth using *p*-NP α-D-maltoside as a substrate.

### 2.2.4.6 Growth of cultures E34.A1 and E37.A2 for enzyme production

Seven 500mL flasks were each filled with 100mL nutrient broth and inoculated with 500μL of actively growing E34.A1 or E37.A2 culture. They were incubated at 55°C for about 48 hours in the shaking incubator. Samples of 1mL culture were taken at regular intervals, the cells spun down for 5 minutes at 16100 r.c.f and the supernatant assayed for α-glucosidase and β-glucosidase activity (Section 2.2.4.1). When enzyme activity in the culture supernatant was high enough, the total volume of culture was spun down at 6166 r.c.f. for 15 minutes, then the supernatant ultrafiltrated at 4°C using an Amicon ultrafiltration system (Millipore Corporation, USA) with a YM10 membrane. The eluate from the ultrafiltration cell was assayed to ensure enzyme activity was retained in the cell.

### 2.2.4.7 Isoelectric focussing and staining of bands for α-glucosidase and protein

Concentrated culture supernatants for E34.A1 and E37.A2, plus a marker protein ladder (Broad pI callibration kit, Pharmacia, Sweden), were electrophoresed on a Phast System (Pharmacia, Sweden) isoelectric focussing gel between pH 3 and 9. The gel was stained by spreading a 1mg/mL solution of *p*-NP α-D-maltoside in 100mM MOPS over the gel with a hockey stick spreader, then incubating the gel at 60°C until colour development occurred. After enzyme activity staining, the gel was fixed and stained for protein according to the Phastgel Coomassie Blue staining instructions.

### 2.2.4.8 Protein determination of enzyme extracts using Bradfords method

Culture E37.A2 concentrated extract was diluted by 1/10 with distilled water and E34.A1 was diluted by 1/100. A 50μL volume was added to 1.5mL Bradfords reagent and incubated for 10 minutes at room temperature. Sample absorbance was read at 595nm and compared to a standard curve produced using bovine serum albumen at concentrations ranging from 10 to 1000μg/mL.

### 2.2.4.9 Assay of other *Bacillus* strains for α-glucosidase activity

Cultures of *Bacillus psychrosaccharolyticus* (TRUCC TG5) and *Bacillus subtilis* (TRUCC TG32) were also assessed for activity against *p*-NP α-D-maltoside. These were grown in nutrient broth (8g/L) at 20°C and 37°C, respectively, then assayed as previously described (Section 2.2.4.1).

### 2.2.5 Genetic analysis of cultures E34.A1, E37.A1 and E37.A2

DNA was extracted from the cultures (Section 4.2.3) and a random amplified polymorphic DNA (RAPD) analysis was carried out to determine their level of similarity at the genomic level (Section 4.2.6.1).

## 2.3 Results

### 2.3.1 Protease production

#### 2.3.1.1 Growth of cultures in casein media

E34.A1 culture grown in casein broth was found to have an activity of 0.011 U/mL when assayed with $Ca^{2+}$, and an activity of 0.008 U/mL when assayed without $Ca^{2+}$. Clearing of casein on agar plates was observed.

E37.A1 and E37.A2 grown in casein broth did not show any protease activity. However, E37.A1 grew well on agar plates and cleared all the casein, while E37.A2 also produced zones of clearing around the bacterial colonies.

Growth of all cultures in casein broth was not very good, with low cell densities (less than 0.1 OD units at 650nm) reached.

### 2.3.1.2 Modification of azocasein assay

An insignificant amount of activity was seen in assays with increased enzyme (whole culture) volume and lower substrate concentration. This is most likely to be due to the poor growth of all three Antarctic cultures in casein broth. A maximum cell density of only 0.05 absorbance units at 650nm was obtained. In contrast, over the same time period the *B. subtilis* culture reached a cell density of 0.80 and produced a relatively high level of enzyme activity (0.041 U/mL) in comparison to the other cultures that produced none.

### 2.3.1.3 Growth of E34.A1 in casein media of varying pH

Growth at different pH caused a variation in cell morphology and density, with a high number of cells grown at pH 6 (too numerous to count at 1000x magnification), smaller number at pH 5 (around 50 cells/field of view at 1000x magnification) and relatively few at pH 4 (5 cells/field of view at 1000x magnification).
Protease activity was negligible at pH 4 and 5. At pH 6, an activity of 0.010 U/mL was measured, however, it is difficult to know if this is a true activity reading as a second culture grown at pH 6.0 failed to show enzyme activity.

### 2.3.1.4 pH profile of protease activity

Protease activities at all pH values were negligible for culture E34.A1. A small level of activity was observed for pH 5 (0.008 U/mL) and pH 6 (0.006 U/mL) for cultures of E37.A1 incubated for 30 minutes, this was not reproduced in the 2 hour assays. Culture E37.A2 also produced a small amount of activity during the 30 minute assay at pH 6 (0.008 U/mL). Culture absorbances at 650nm were 0.05 OD units for E34.A1, 0.38 OD units for E37.A1 and 0.04 OD units for E37.A2.

### 2.3.1.5 Growth of cultures E34.A1 and E37.A1 in Modified R medium

Culture E34.A1 grew well in modified R medium, however, protease activity was lower than that produced in casein broth. There was no difference between the $Ca^{2+}$ and non-$Ca^{2+}$ readings. Culture absorbance of E34.A1 got to a reading of over 2 OD units at 650nm overnight. Activity was 0.008 U/mL with $Ca^{2+}$ and 0.007 U/mL without $Ca^{2+}$.

E37.A1 grew in modified R medium faster (one day) than in casein broth (two days), however, negligible enzyme activity was measured.

### 2.3.1.6 Kunitz assay

Protease activity of 0.052 U/mL was detected using a one-day-old culture of E37.A2. A smaller amount of activity, 0.008 U/mL was seen in a two-day-old culture with a greater cell density (probably because some of the protease had broken down).

Protease assay results in the presence or absence of $Ca^{2+}$ for all the cultures are presented in Table 2.1.

**Table 2.1. Protease activity of thermophilic bacteria using Kunitz assay. W, whole culture; S, 5x concentrated culture supernatant**

| Culture | Activity, U/mL $+ Ca^{2+}$ | Activity, U/mL $- Ca^{2+}$ |
|---|---|---|
| E34.A1, W | N/D | 0.010 |
| E37.A1, W | 0.078 | 0.006 |
| E37.A2, W | 0.029 | 0.010 |
| E34.A1, S | 0.026 | 0 |
| E37.A1, S | 0.014 | 0.007 |

### 2.3.1.7 Growth of culture E37.A1 in basic media

After 12 hours growth, the absorbance of E37.A1 in the different media was measured (Table 2.2). Highest culture absorbances were measured in medium 3 and 5.

**Table 2.2. Culture absorbance of E37.A1 after 12 hours growth**

| Medium | pH | Absorbance |
|---|---|---|
| 1 | 5.5 | 0.11 |
| | 7.5 | 0.12 |
| 2 | 5.5 | 0.02 |
| | 7.5 | 0.20 |
| 3 | 5.5 | 0.32 |
| | 7.5 | 0.07 |
| 4 | 5.5 | 0 |
| | 7.5 | 0.15 |
| 5 | 5.5 | 0.64 |
| | 7.5 | 0.89 |

After 41 hours incubation, culture absorbance of E34.A1 (0.42 OD units), E37.A1 (0.75 OD units) and E37.A2 (0.88 OD units) growing in casein broth was measured.

All three cultures grown in casein broth, plus cultures of E37.A1 growing in medium 2 at pH 7.5, medium 3 at pH 5.5, medium 4 at pH 7.5 and medium 5 at pH 5.5 and 7.5 were assayed for enzyme activity. However, protease activity was found to be insignificant.

These cultures were left to grow to their maximum cell density (see Table 2.3), which took approximately 35 hours, then they were re-assayed for protease production. Results were still found to be negligible.

**Table 2.3. Maximum culture absorbance obtained in five media**

| Medium | Culture | pH | Max. culture OD |
|---|---|---|---|
| 2 | E37.A1 | 7.5 | 0.20 |
| 3 | E37.A1 | 5.5 | 1.36 |
| 4 | E37.A1 | 7.5 | 0.24 |
| 5 | E37.A1 | 5.5 | 1.14 |
| 5 | E37.A1 | 7.5 | 1.22 |
| Casein | E34.A1 | 6.0 | 0.46 (67h) |
| Casein | E37.A1 | 6.0 | 0.75 (41h) |
| Casein | E37.A2 | 6.0 | 0.88 (41h) |

Growth was best in medium 3 (basic salts + N), pH 5.5 and medium 5 (basic salts + N + glucose) at both pH values. Growth was also good in casein broth after 41 hours incubation. Unfortunately, no protease activity was observed in any cultures assayed.

### 2.3.1.8 Enzyme production over time

Cultures grew to a good optical density ranging between 0.5 to 1.4 absorbance units over the 34 hours. A small amount of protease activity was detected in culture E37.A2 at 9 (0.008 U/mL) and 11 (0.005 U/mL) hours growth, none of the other cultures appeared to produce protease activity. Surprisingly, *B. subtilis* did not produce activity either, although it was used as a positive control.

### 2.3.1.9 Growth of culture E37.A2 in modified basic media

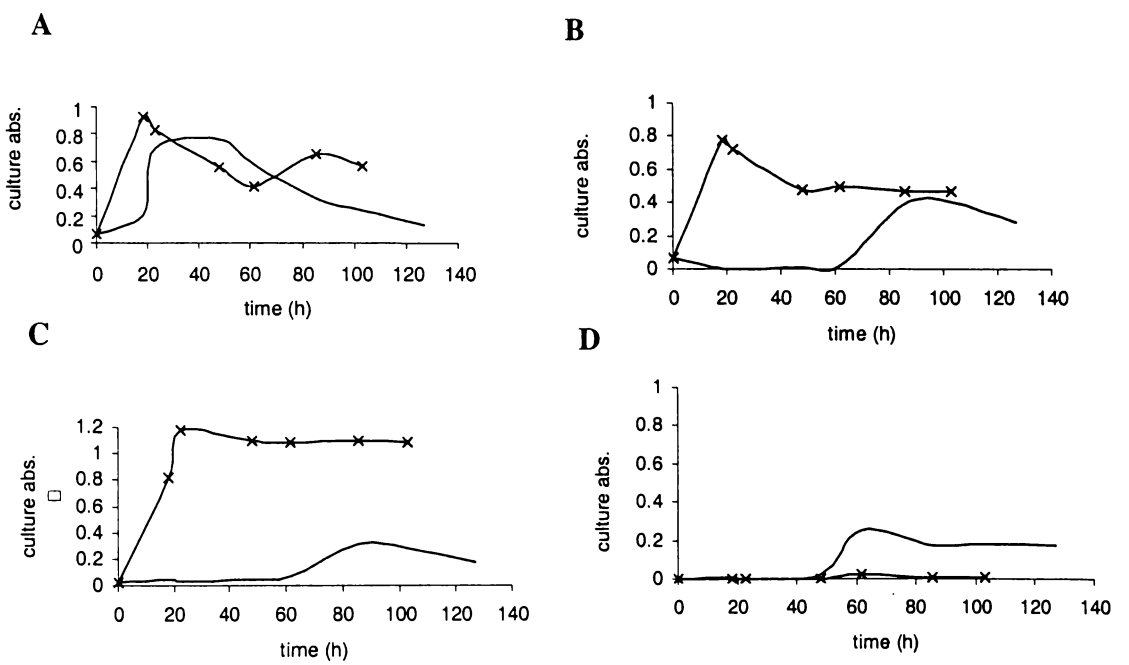
Growth in casein broth and medium 2 was better than medium 3 and 4, reaching a higher maximum absorbance of 0.76 (casein broth) and 0.40 (medium 2). Growth was fastest in casein broth, taking only 20 hours to get to near maximum density

(0.76 absorbance units). The positive control grew well in all media apart from medium 4.

At maximal culture absorbance (18h growth), protease activity in *B. subtilis* cultures was found to be: casein broth, 0.081 U/mL; medium 2, 0.033 U/mL; medium 3, 0.037 U/mL.

At maximal culture absorbance (22.5h growth), protease activity in E37.A2 cultures was found to be: casein broth, 0.015 U/mL; 5x concentrated supernatant of casein broth, 0.011 U/mL.

**Figure 2.1. Growth curves of E37.A1 in four basic media (A) casein broth; (B) medium 2; (C) medium 3; (D) medium 4**



### 2.3.1.10 Casein agar plate method for determination of protease activity

After an overnight incubation at 55°C, protease activity was detected in cultures E34.A1 and *B. subtilis*, as summarised in Table 2.4.

**Table 2.4. Diameter of *para*-casein ring produced by cultures overnight**

| Culture | pH 5 | pH 6 | pH 7 |
|---|---|---|---|
| E34.A1 | No ppte | 9mm | No ppte |
| E37.A1 | No ppte | No ppte | No ppte |
| E37.A2 | No ppte | No ppte | No ppte |
| *B. subtilis* | No ppte | 18mm | 24mm |

## 2.3.2 Esterase and Lipase production

### 2.3.2.1 Growth of bacterial cultures in Claudia medium and Tryptic Soy Broth

Growth of different cultures in Claudia broth for 48 hours produced the results summarised in Table 2.5. Esterase activity appeared to be marginally higher than lipase activity in the cultures tested.

**Table 2.5. Esterase (C6) and lipase (C16) activities produced by cultures grown in Claudia broth**

| Substrate | Culture | Activity (μmol/min/mL) |
|---|---|---|
| C6 | E34.A1 | 0.0043 |
| | E37.A1 | 0.0050 |
| | E37.A2 | 0.0030 |
| C16 | E34.A1 | 0.0031 |
| | E37.A1 | 0.0026 |
| | E37.A2 | 0.0010 |

Culture absorbance could not be measured spectrophotometrically in Claudia broth due to the presence of oil droplets in the medium. Assessment of bacterial numbers

on a microscope slide at high magnification showed between 100 and 300 bacterial cells per field of view.

E37.A2 was grown in TSB for 5 days and lipase and esterase activity measured (Table 2.6). Esterase activity was definitely higher than lipase activity, with growth in TSB causing an improvement in assayed values for this enzyme.

**Table 2.6. Esterase (C6) and lipase (C16) activities produced by E37.A2 grown in TSB**

| Substrate | No. days growth | Culture abs. (650nm) | Activity (μmol/min/mL) |
|---|---|---|---|
| C6 | 3 | 1.12 | 0.0077 |
| C16 | 3 | 1.12 | 0.0012 |
| C6 | 5 | 1.10 | 0.0130 |
| C16 | 5 | 1.10 | 0.0022 |

The best esterase production occurred using TSB , although it took longer for bacteria to grow in this compared to the Claudia medium. Esterase production was seen in E37.A1 and E37.A2 in TSB. Greater activity was seen using the C6 substrate indicating the presence of an esterase. Control values were high compared to the sample values.

### 2.3.2.2 Test of *p*-Nitrophenyl assay method

The results of the *p*-NP caproate assay are summarised in Table 2.7.

**Table 2.7. Comparison of esterase activity with varied enzyme volume or incubation period**

| Enzyme volume (μL) | Time of incubation | Activity (μmol/min/mL) | μmol/min | Mmol/mL |
|---|---|---|---|---|
| 50 | 30 min | 0.018 | 0.0009 | - |
| 100 | 30 min | 0.012 | 0.0012 | - |
| 200 | 30 min | 0.008 | 0.0015 | - |
| 100 | 1 h | 0.006 | - | 0.354 |
| 100 | 2 h | 0.003 | - | 0.300 |

A doubling of enzyme volume from 50μL to 100μL, then 100μL to 200μL did not cause a corresponding increase in activity measured (as μmol/min). Increasing

incubation period also did not cause a proportional increase in measured activity (μmol/mL).

### 2.3.2.3 Location of esterase activity in bacterial cells

The majority of enzyme activity (~70%) was found to be located in the cell membrane (Table 2.8).

**Table 2.8. Location of esterase activity in bacterial cells**

| Culture | Fraction | Activity (μmol/min/mL) |
|---|---|---|
| E34.A1 (65h) | Pellet | 0.0085 |
| | Supernatant | 0.0047 |
| | Whole culture | 0.0120 |
| E37.A2 (39h) | Pellet | 0.0110 |
| | Supernatant | 0.0033 |
| | Whole culture | 0.0140 |
| Positive control (100μL) | N/A | 0.0530 |

### 2.3.2.4 Release of esterase activity by washing bacterial cells

Washing of cells did not result in the release of enzyme activity from membrane as enzyme activity measured in all supernatants tested was negligible.

### 2.3.3 Glucosidase and galactosidase production

### 2.3.3.1 *p*-NP assay method

The greatest level of activity seemed to be generated by α-glucosidase in each of the cultures tested (Table 2.9). A relatively high activity was measured for β-galactosidase in the E37.A1 culture.

**Table 2.9. Glucosidase and galactosidase activity of thermophilic cultures**

| Culture | Enzyme measured | Activity (μmol/min/mL) |
|---|---|---|
| E34.A1 | α-glucosidase | 0.029 |
| | β-glucosidase | 0.019 |
| | β-galactosidase | 0.010 |
| E37.A1 | α-glucosidase | 0.039 |
| | β-glucosidase | 0.030 |
| | β-galactosidase | 0.096 |
| E37.A2 | α-glucosidase | 0.049 |
| | β-glucosidase | 0.026 |
| | β-galactosidase | 0.016 |

### 2.3.3.2 Location of glucosidase and galactosidase activity in bacterial cells

The results of these assays, summarised in Table 2.10, indicate that nearly all of the activity of the three enzymes is membrane associated.

**Table 2.10. Location of glucosidase and galactosidase activity in thermophilic cultures**

| Culture | Enzyme | Fraction | Activity (μmol/min/mL) |
|---|---|---|---|
| E34.A1 | α-glucosidase | Pellet | 0.030 |
| | | Supernatant | 0 |
| | | Whole culture | 0.027 |
| | β-glucosidase | Pellet | 0.027 |
| | | Supernatant | 0 |
| | | Whole culture | 0.025 |
| | β-galactosidase | Pellet | 0.020 |
| | | Supernatant | 0.001 |
| | | Whole culture | 0.020 |
| E37.A1 | α-glucosidase | Pellet | 0.036 |
| | | Supernatant | 0 |
| | | Whole culture | 0.020 |
| | β-glucosidase | Pellet | 0.020 |
| | | Supernatant | 0.001 |
| | | Whole culture | 0.022 |
| | β-galactosidase | Pellet | 0.041 |
| | | Supernatant | 0.001 |
| | | Whole culture | 0.019 |
| E37.A2 | α-glucosidase | Pellet | 0.028 |
| | | Supernatant | 0 |
| | | Whole culture | 0.034 |
| | β-glucosidase | Pellet | 0.026 |
| | | Supernatant | 0 |
| | | Whole culture | 0.016 |
| | β-galactosidase | Pellet | 0.018 |
| | | Supernatant | 0.003 |
| | | Whole culture | 0.038 |

### 2.3.3.3 Release of α-glucosidase or β-glucosidase activity from cell membranes

No enzyme activity was detected in the supernatant of cells washed with any of the solutions.

### 2.3.3.4 Induction of α-glucosidase or β-glucosidase using modified media

Cellobiose and cellulose were found to be poor inducers of β-glucosidase activity, while the use of maltose was more successful (Table 2.11). After 42 hours growth in nutrient broth containing maltose, a good level of α-glucosidase activity was measured in the culture supernatant, with the highest level of activity against *p*-NP α-D-maltoside.

**Table 2.11. Induced enzyme activity using modified media. Substrates were: (A) *p*-NP α-D-glucopyranoside; (B) *p*-NP α-D-maltoside; (C) *p*-NP β-D-glucopyranoside; (D) *p*-NP β-D-maltoside; (E) *p*-NP β-D-cellobioside**

| Medium | Substrate | Enzyme activity (24h) | | Enzyme activity (42h) | |
|---|---|---|---|---|---|
| | | WC | Spt | WC | Spt |
| Cellobiose | A | 0.015 | 0.002 | - | 0.004 |
| | B | 0.007 | - | - | - |
| | C | 0.006 | - | - | - |
| | D | 0.005 | - | - | - |
| | E | 0.005 | - | - | - |
| Cellulose | A | 0.021 | 0.002 | - | 0.002 |
| | B | 0.012 | 0.002 | - | 0.004 |
| | C | 0.011 | - | - | - |
| | D | 0.010 | - | - | - |
| Maltose | A | 0.035 | 0.003 | 0.119 | 0.037 |
| | B | 0.023 | 0.005 | 0.179 | 0.146 |
| | C | 0.008 | - | - | - |

| | | | |
|---|---|---|---|
| Culture absorbance: | Cellulose | 0.988 (24h); | 2.200 (42h) |
| | Cellobiose | 0.534 (24h); | 3.252 (42h) |
| | Maltose | 0.682 (24h); | 3.388 (42h) |

### 2.3.3.5 Growth in minimal media

No enzyme activity was released into the supernatant over 64 hours of growth of culture E37.A1 in minimal medium, however, a high level of activity was found in the supernatant of cultures growing in half strength nutrient broth with 0.2% maltose. Therefore, the amount of activity released into half strength and full strength nutrient broth with maltose was compared at 48 and 66 hours growth (Table 2.12).

**Table 2.12. α-glucosidase activity produced by E37.A1 in half and full strength nutrient broth**

| Medium | Hours growth | Enzyme Activity (μmol/min/mL) | |
|---|---|---|---|
| | | Supernatant | Whole culture |
| ½ strength NB | 48 | 0.033 | 0.084 |
| Full strength NB | 48 | 0 | 0.023 |
| ½ strength NB | 66 | 0.051 | 0.082 |
| Full strength NB | 66 | 0.039 | 0.095 |

It was found that a greater proportion of enzyme activity was located in the culture supernatant after 66 hours growth, compared with 48 hours. Half strength nutrient broth produced a higher level of enzyme activity than full strength.

### 2.3.3.6 Growth of cultures E34.A1 and E37.A2 for enzyme production

Culture E34.A1 was harvested after 48 hours growth with culture absorbance of 1.58 OD units. Enzyme activity in the supernatant was determined to be 0.088 μmol/min/mL. After increasing concentration to 104-fold, activity was 3.31 μmol/min/mL.

Culture E37.A2 was harvested after 43 hours growth with a culture absorbance of 2.11 absorbance units. Enzyme activity in the supernatant at this time was determined to be 0.175 μmol/min/mL. After reducing the volume to gain a 12.2 times concentration, an activity of 1.243 μmol/min/mL was obtained.

### 2.3.3.7 Isoelectric focussing and staining of bands for α-glucosidase and protein

Staining with *p*-NP α-D-maltoside produced an observable band of colour at the lower pH region of the gel, indicating that the enzyme has a low pI, ie the enzyme has a negative charge at neutral pH. Coomassie blue staining indicated that protein was present, a smear of bands was produced in the lower pH region of the gel.

### 2.3.3.8 Protein determination of enzyme extracts using Bradfords method

From the equation of the standard curve, the concentration of the enzyme extracts was determined to be 13.8mg/mL protein for E34.A1 and 2.6mg/mL protein for E37.A2.

### 2.3.3.9 Assay of other *Bacillus* strains for α-glucosidase activity

After 48 h growth, *B. subtilis* had produced 0.007 μmol/min/mL α-glucosidase activity, while *B. psychrosaccharolyticus* had produced 0.001 μmol/min/mL.

At 96 h growth, the level of α-glucosidase produced by *B. subtilis* decreased to 0.002 μmol/min/mL in the culture supernatant, and 0.004 μmol/min/mL in the whole culture.

After 120 h growth of *B. psychrosaccharolyticus*, the α-glucosidase activity in the supernatant was 0.001 μmol/min/mL, while the whole culture had only produced 0.002 μmol/min/mL activity.

### 2.3.4 Genetic analysis of cultures E34.A1, E37.A1 and E37.A2

The three cultures were found to have the same DNA banding pattern on a RAPD gel (see Figure 2.2).

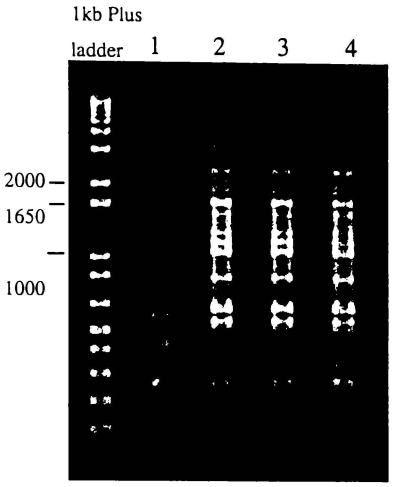



**Figure 2.2. RAPD profile of thermophilic cultures. Lanes and corresponding samples are (1) negative control; (2) E34.A1; (3) E37.A1; (4) E37.A2**

## 2.4 Discussion

### 2.4.1 Protease production

Clearing of casein agar plates occurred after freeze-dried cultures were regenerated, as it had been described at the time of isolation. However, the poor protease activity measured suggested that either the assay was not sensitive enough or the level of protease activity was higher on the casein plate due to the long incubation period, i.e. several days compared to the 30 minutes incubation of the azocasein assay. It was also possible that the secretion of acidic by-products of metabolism was the cause of the casein disappearance rather than the presence of an extracellular protease. However, the predominant cause of the poor activity was likely due to the poor growth of the cultures in liquid media. Obtaining a higher maximal cell density was an important objective for the ensuing experimental work.

Modification of the azocasein assay protocol had no effect on the level of enzyme activity measured. Therefore, it was assumed that low levels of activity were not due to substrate inhibition in the assay, as described by Peek *et al.* (1992), or insensitivity of the assay, but a result of poor enzyme production by the cultures. Inducing enzyme activity by altering the media was one method used to improve activity, monitoring the time of protease production during growth phase of the culture was another. Proteases are known to undergo autolysis (Daniel *et al.*, 1995), it is possible that some enzyme was produced by the culture but degraded over time.

Culture E34.A1 was isolated from geothermal soil with a pH of 5.0. Growth and protease production was measured in casein broth of varying pH to see if these properties would improve at a more acidic pH . However, it was found that E34.A1 grew better at pH 6.0 compared to the lower pH values. pH profiling of enzyme activity using the azocasein method indicated that the optimal pH for activity was around 5 or 6, but results were not entirely convincing as the level of activity measured was so low.

Modified R medium was inoculated with cultures E34.A1 and E37.A1 in an attempt to increase cell growth and protease production. Culture density was improved by growth in this medium, however, enzyme activity was negligible for E37.A1 and lower than that found in casein broth for E34.A1.

To confirm that the azocasein assay was giving the correct readings and to try a slightly different substrate (Hammersten casein), the Kunitz assay was used. This method was able to detect a higher amount of activity in a culture of E37.A2, which had been found to produce none using the azocasein method. The smaller amount of activity detected in the two-day-old culture compared to the one-day-old culture was probably due to the degradation of the protease over time. Calcium has previously been found to stabilise proteases against thermal denaturation and autolysis (Daniel *et al.*, 1995). This may be because calcium binding sites are vulnerable to autolysis, or that enzyme structural changes when calcium is bound prevent autolysis (Cowan and

Daniel, 1982b). When the other cultures were assayed using the Kunitz method, a higher level of activity was seen with the addition of $Ca^{2+}$.

A decreased amount of activity was observed in the 5x concentrated culture supernatant compared to the whole culture, suggesting that the protease is membrane associated or that degradation or denaturation of the enzyme occurred during ultrafiltration.

Growth in casein broth of cultures E34.A1, E37.A1 and E37.A2 had improved significantly in the 4-6 weeks since work on these had first begun, although protease production was still negligible. Possibly, the improvement in growth was due to the cultures "getting back to normal" as a bacterial culture regenerated from a freeze-dried state may need to go through several subcultures until bacteria start growing and metabolising in the usual manner (L. Parker, personal communication).

Growth was also good in some of the basic media tested, especially medium 3 (salts + N, pH 5.5) and medium 5 (salts + N + glucose, pH 5.5 and 7.5). This was probably due to their nutrient-rich composition compared with the other media. By removing freely available amino acids and nitrogen, it was hoped that the bacteria would be induced to secrete proteases into the medium. However, protease production did not occur.

Monitoring enzyme production over time found that in the culture where enzyme production did occur (E37.A2) it was early on in the growth cycle, i.e. 9-11 hours after inoculation. A previous study (Janssen *et al.*, 1991) showed that proteinase production in a *Thermus* species occurred only while the culture was metabolically active and growing, and was lost rapidly when the culture reached stationary phase. Therefore, protease production would occur during the log phase, and cultures should be tested at this time.

As E37.A2 was the only culture to produce detectable activity in the previous experiment, it was inoculated into casein broth and three basic media to attempt to increase enzyme activity produced. Casein broth was found to be the best medium for growth and results from the positive control, *B. subtilis*, showed that protease production was best in this medium. Very poor growth occurred in medium 4 (salts, 0.5g/L yeast extract, 2g/L peptone and glucose) suggesting that the low concentration of yeast extract inhibited growth.

In a final effort to detect protease activity from the three cultures, the casein agar plate assay method was performed. Incubation of plates at high temperatures, ie 45°C to 75°C, results in a relatively sensitive assay method due to the higher density of the *para*-casein ring (Cowan and Daniel, 1982a). Assay results showed that protease was produced by culture E34.A1 only. This suggests that the clearing of the casein agar plates which first indicated the possibility of protease production, was probably due to the casein becoming soluble at more acidic pH values.

### 2.4.2 Esterase and Lipase production

Although culture absorbance could not be measured in Claudia broth, it was estimated that the cell density reached was not as high as that of cultures grown in TSB. Enzyme activity was also found to be higher in TSB than Claudia broth, so it was decided to use TSB or nutrient broth, which is similar to TSB, instead. A concern with this assay was the discovery of high background absorbance levels of the control tubes. Although the difference in sample absorbance compared with absorbance of the controls appeared to be significant, the control values made up a good proportion of the activity measured. Further testing of the assay method found that the assay was not reliable, as activity did not increase linearly with increased enzyme concentration, or assay time.

The location of esterase activity in bacterial cells was investigated. When it was discovered to be membrane associated, attempts were made to wash the activity from

the cells, but this did not prove to be successful, indicating that the enzyme was more tightly bound than first thought. These results, and the problem of high control values, are the main reasons why the work into esterase and lipase production did not progress further than these initial experiments.

### 2.4.3 Glucosidase and galactosidase production

Galactosidases and glucosidases are easy to assay using *p*-NP substrates and can be produced extracellularly. Activity against these enzymes was detected, however it was also found to be membrane associated. A previous paper found that washing membranes with salt solutions and detergents was an effective way of releasing activity (Urlaub and Wober, 1978), but efforts to wash the activity from the membrane were unsuccessful. A new approach was used, which involved inducing glucosidase and galactosidase production by adding the enzyme substrates to the culture media. Addition of starch or maltose has been found to induce α-glucosidase synthesis in other studies of bacterial enzymes (Kelly and Fogarty, 1983). Of the three substrates added (cellobiose, cellulose and maltose), only cultures containing maltose released a significant amount of (α-) glucosidase activity into the culture supernatant. Further work was carried out to increase enzyme production using minimal medium, but it was found that half strength nutrient broth with maltose gave the best results.

As glucosidase activity may originate from more than one enzyme (J. Bragger, personal communication), an estimation of the purity of the enzyme, and its isoelectric point, were assessed by running an isoelectic focussing gel with concentrated culture supernatants E34.A1 and E37.A2. Activity staining revealed that several possible enzymes may have contributed to the glucosidase activity detected, and their placement on the gel suggested that they all had similar isoelectric points. This meant that it would not be possible to purify a single enzyme based on its isoelectric point, and that the purification procedure used would be more complicated. So, although a good levels of activity had been obtained, along with a

high protein concentration in the enzyme extract, the use of α-glucosidase or β-glucosidase would be complicated by the presence of several enzymes in the extract.

### 2.4.4 Genetic analysis of cultures E34.A1, E37.A1 and E37.A2

RAPD analysis showed that all three of these isolates were likely to be the same bacterial strain. This was further confirmed by analysis using DGGE (prepared by T. Niederberger), which revealed all the isolates to have the same 16s rRNA banding pattern, indicating they were the same, or similar, species. Although genetic analysis showed that isolates E34.A1, E37.A1 and E37.A2 were likely to have the same identity, enzyme analysis showed differences in enzyme activities. This demonstrates that adaptation and the accumulation of mutations in enzymes have occurred at different rates in the individual isolates, resulting in varied properties.

## 2.5 Conclusion

Of the extracellular enzymes tested, none stood out as having particularly good activity with the exception of α- or β-glucosidase. As a result, DHFR was the enzyme chosen for thermal characterisation. The reason for this choice was that DHFR is present in all bacterial cells, it could be purified relatively easily using a methotrexate affinity column and that results could be compared with those obtained from the characterisation of DHFRs from other sources.

# Chapter 3 Temperature Profiling of Antarctic Sampling Sites

## 3.1 Introduction

To gain an understanding of the range of temperatures Antarctic soil bacteria are exposed to within a day, a temperature profile of surface soils was measured for the sampling sites used.

## 3.2 Materials and Methods

### 3.2.1 Soil collection and ground temperature measurements

Antarctic soil samples were collected between 23 January and 7 February, 2002. Sampling sites were: the Upper Wright Valley, Miers Valley, Bratina Island and Cape Crozier, all located in the Ross Sea region of Antarctica.

Sampling was carried out aseptically by scooping between 20 to 80g of the top layer of soil into a sterile 50mL Falcoln tube. Sites were chosen where there were no obvious signs of human contamination, for example, footprints, huts and paths (see Figure 3.1).

Soil samples were stored at approximately -10°C while in the field and then transferred to a 4°C refrigerator at Scott Base and in the Thermophile Research Unit, University of Waikato, Hamilton.

A HOBO® H8 Pro Temperature/External temperature probe (internal sensor range: -30°C to +50°C, external sensor range: -40°C to +100°C, Part no. H08-031-08, Onset Computer Corporation) or StowAway TidbiT® temperature probe (sensor range:

-20°C to +50°C, Part no TBI32-20+50, Onset Computer Corporation) was placed in the top two centimetres of soil in close proximity to the sampling area. The three temperature probes were set to record temperature simultaneously at three different locations. Measurements were taken at one second intervals over approximately 24 hours. If extra soil samples were collected during this time, single ground temperature measurements were made at the sampling site using a hand held probe.

Temperature data was downloaded from the temperature probes into the Boxcar computer software (Part no. BC3.7-ON, Onset Computer Corporation), then transferred to MS Excel for analysis.

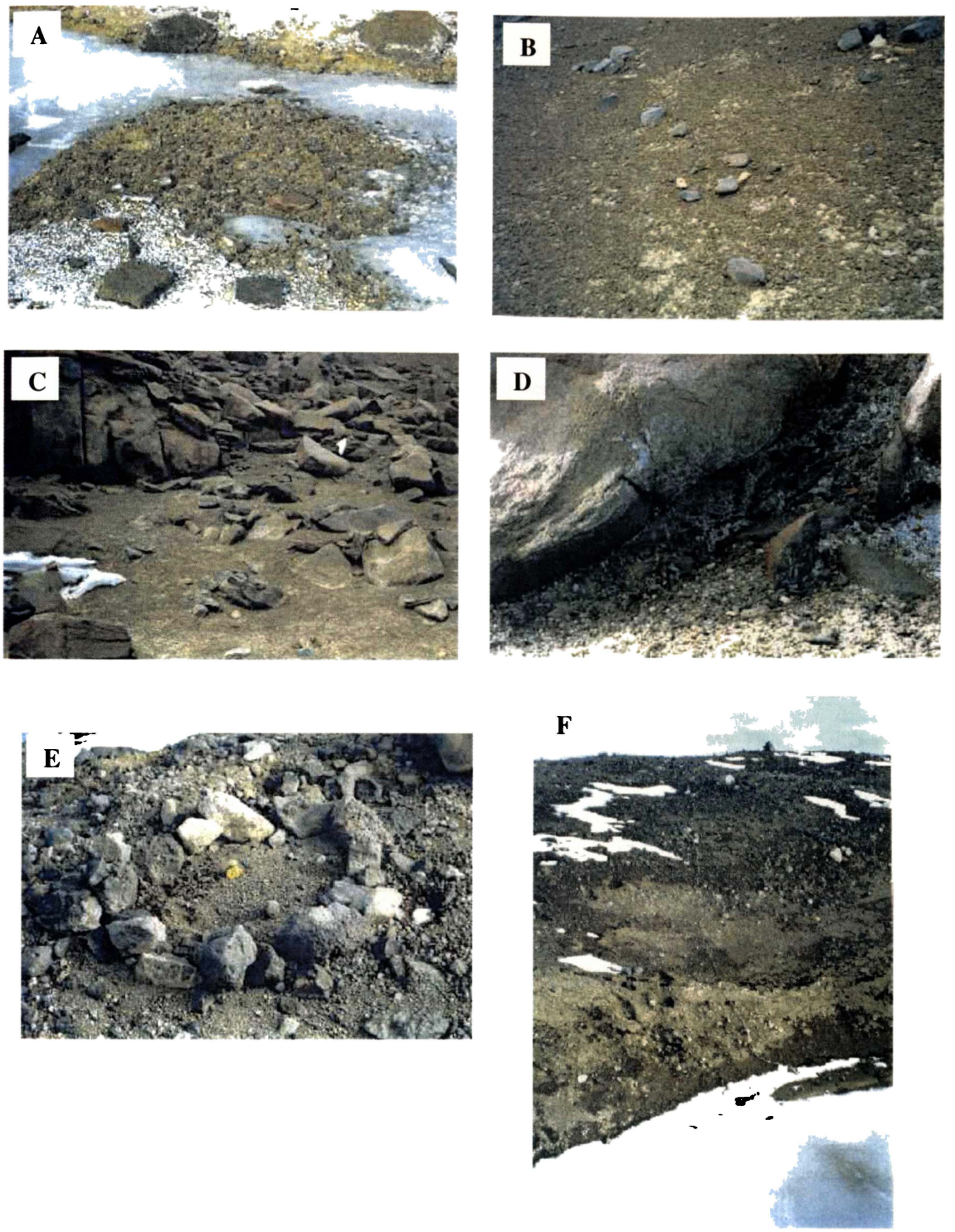



**Figure 3.1. Antarctic soil sampling sites. A, Upper Wright Valley melt-water stream (UWV-00-2); B, Upper Wright Valley exposed soil (UWV-TB-1); C, Upper Wright Valley rocky slope (UWV-HB-1A/1B); D, Upper Wright Valley salty soil (UWV-00-1); E, Bratina Island exposed ridge (BI-TB-1); F, Bratina Island pond (BI-HB-1A/1B)**

### 3.2.2 Soil collection and ground temperature measurements

Details of sampling sites and samples taken are summarised in Table 3.1.

**Table 3.1. Soil sampling locations and temperature details**

| Sample site | Latitude/ Longitude | Description | Sample name | Measured temperature |
|---|---|---|---|---|
| Upper Wright Valley | 77°31.432'S 160°44.463'E | Position 1. Soil surrounded by rocks, halfway up slope | UWV-HB-1A | See Fig 3.3A |
| | As above | Position 2. Soil under clump of ice protected by rocks, halfway up slope | UWV-HB-1B | See Fig 3.3B |
| | As above | Position 3. Exposed area at top of ridge | UWV-TB-1 | See Fig 3.2 |
| | 77°31.380'S 160°44.498'E | Salty soil under rock | UWV-00-1 | -4.0°C |
| | 77°31.373'S 160°44.320'E | Salty soil next to frozen meltwater stream | UWV-00-2 | -0.6°C |
| Miers Valley | 78°06.143'S 163°48.664'E | Position 1. Stony gravel on low slope | MV-HB-1A | See Fig 3.4B |
| | As above | Position 2. Stony gravel protected by rock | MV-HB-1B | See Fig 3.5 |
| | As above | Position 3. Edge of soil polygon, protected by rocks | MV-TB-1 | See Fig 3.4A |
| | 78°05.694'S 163°44.834'E | Mossy soil at base of glacier | MV-G | N/A |
| Bratina Island | 78°00.713'S 165°33.016'E | Position 1. Exposed upper slope of ridge leading down to pond | BI-HB-1A | See Fig 3.7 |
| | As above | Position 2. Snow covered soil at base of slope, near algal mat beside pond | BI-HB-1B | N/A |
| | As above | Position 3. Exposed dark, fine soil at top of ridge | BI-TB-1 | See Fig 3.6 |
| | 78°00.630'S 165°32.540'E | Lakeside algal mat | BI-1 | -0.2 °C |
| | 78°00.613'S 165°32.648'E | Hillslope with snow | BI-2 | -0.6 °C |
| Cape Crozier | N/A | Penguin rookery on coast | PR | -2.3 °C |

N/A, not available.

Other samples used (collected by C. Cary, 2002):

| | |
|---|---|
| MVT | Miers Valley Transect |
| MVS 4 | Soil samples collected around mummified seal carcass in Miers Valley |

### 3.2.3 Temperature profiles of Antarctic sampling sites

#### 3.2.3.1 Upper Wright Valley

Upper Wright Valley temperature profile was recorded over the 28-29 January, 2002 (Figures 3.2 and 3.3). From the start of the sampling period in the morning to the early evening, the sky was relatively bright, but it became overcast in the evening and during the night.

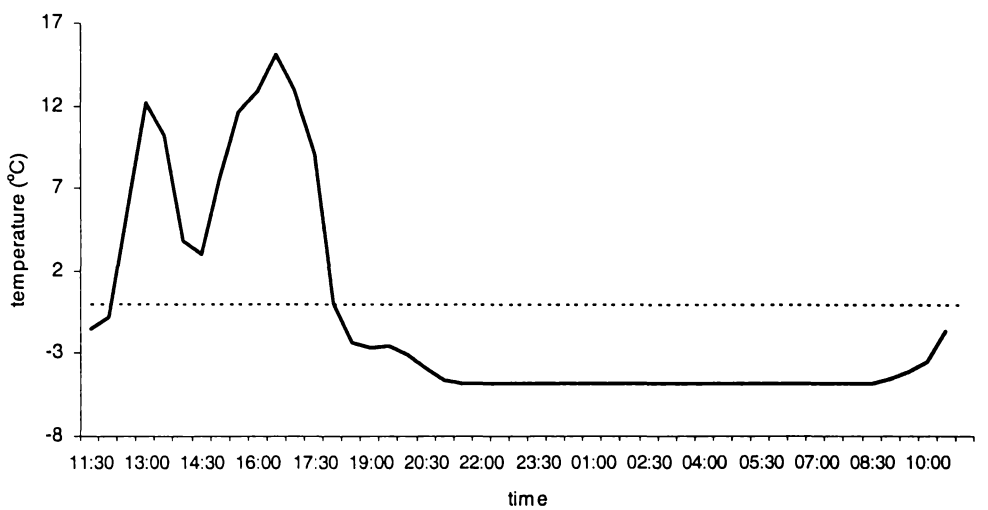



**Figure 3.2. Upper Wright Valley Position 3 temperature profile**

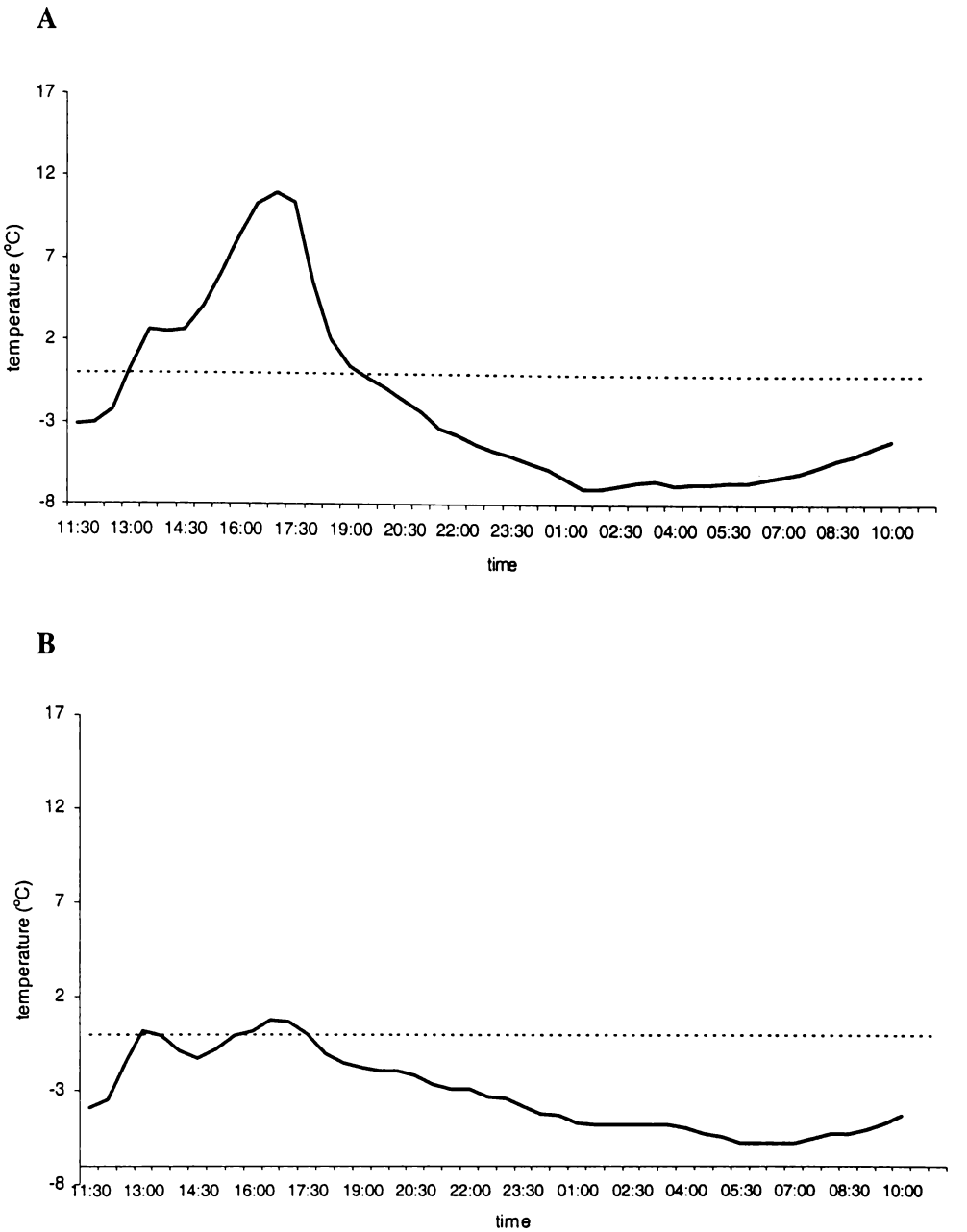
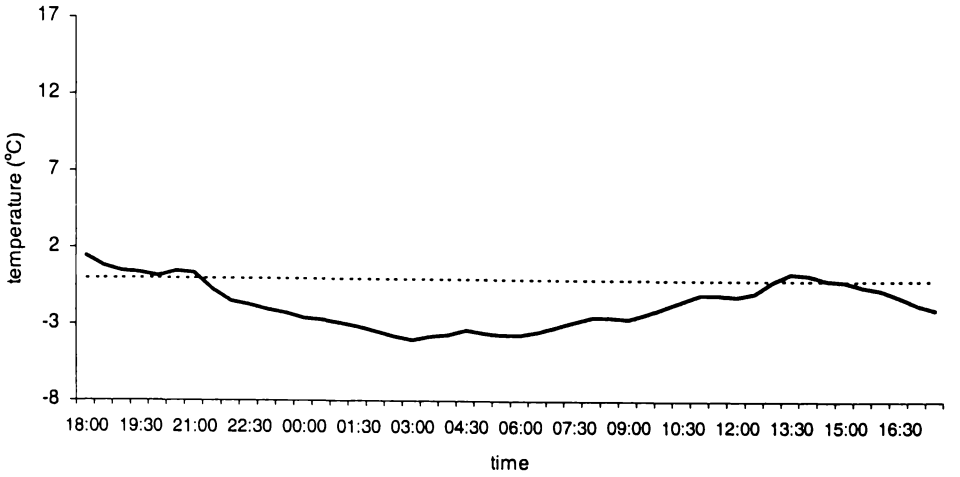



**Figure 3.3. Upper Wright Valley (A) Position 1 and (B) Position 2 temperature profile**

### 3.2.3.2 Miers Valley

Miers Valley temperature profiles were recorded over the 2-3 February, 2002 (Figures 3.4 and 3.5). This measurement period began in the evening when it was cloudy and snowing, in the morning the skies cleared and there was some sunlight.

A




B

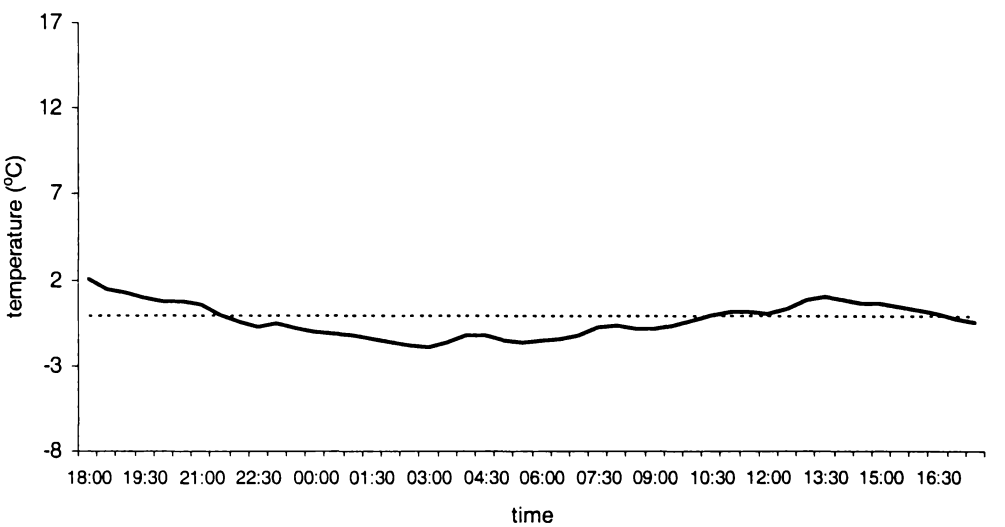



**Figure 3.4. Miers Valley (A) Position 3 and (B) Position 1 temperature profile**

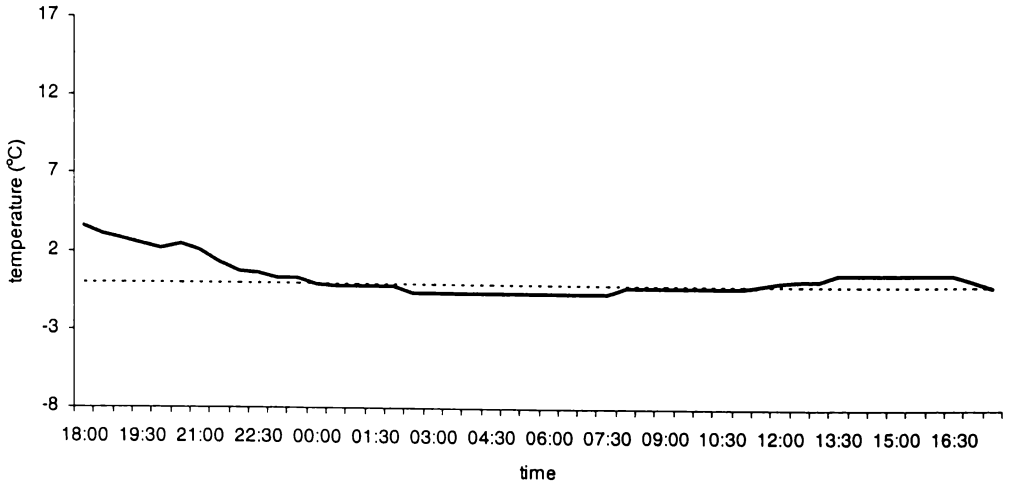



**Figure 3.5. Miers Valley Position 2 temperature profile**

### 3.2.3.3 Bratina Island

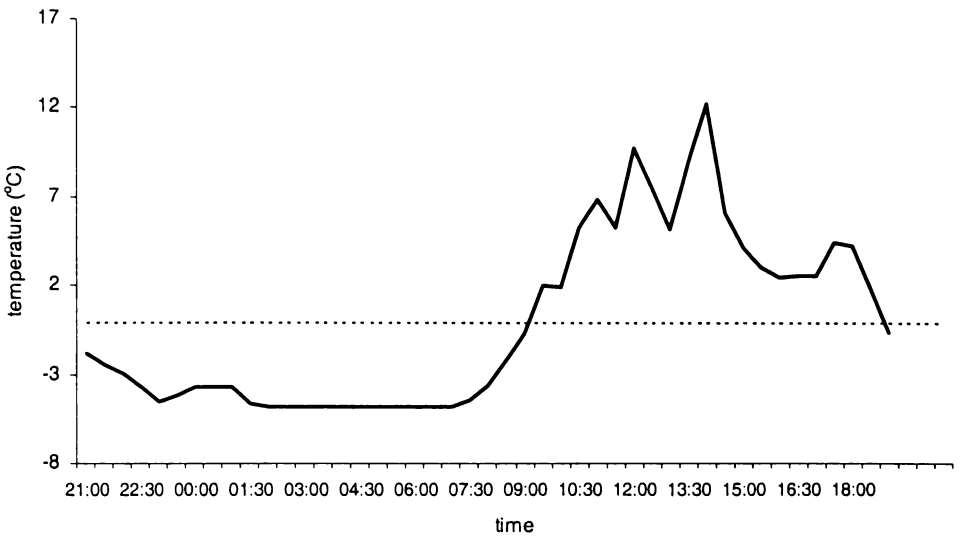



**Figure 3.6. Bratina Island Position 3 temperature profile**

The temperature profiles for Bratina Island sampling sites were recorded over the 4-5 February, 2002 (Figures 3.6 and 3.7). This measurement period began later in the evening than the previous two sites. Overnight it was overcast with strong winds, the next day was brighter.

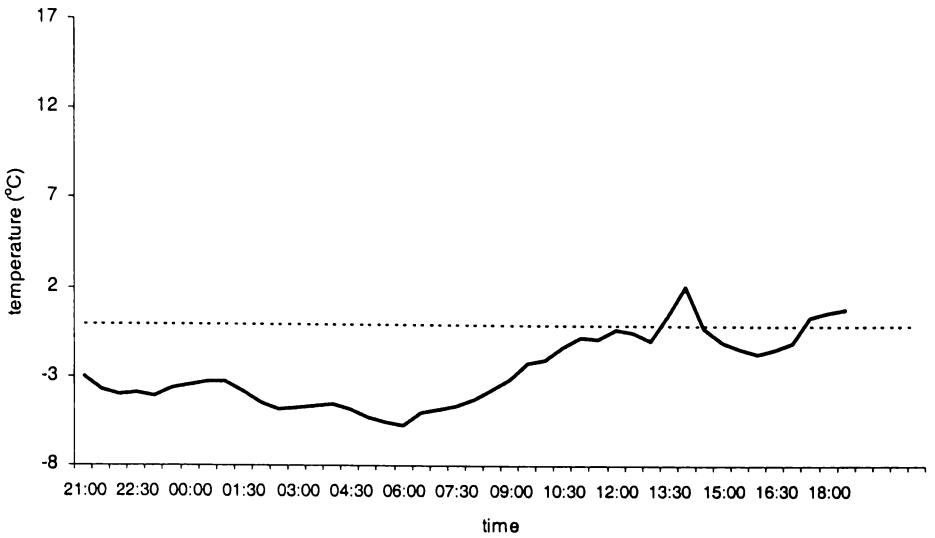



**Figure 3.7. Bratina Island Position 1 temperature profile**

The external HOBO sensor placed at Position 2 appeared to produce a fault at the beginning of the time period, resulting in a loss of information for this site.

A summary of the information collected about each site is presented in Table 3.2.

**Table 3.2. A summary of temperature data collected for Antarctic sampling sites**

| Location | Position | Maximum temp | Minimum temp | Temp range | Hours above 0°C |
|---|---|---|---|---|---|
| Upper Wright Valley | 1 | 11.01 | -7.02 | 18.03 | 6.0 |
| | 2 | 0.70 | -5.81 | 6.51 | 2.0 |
| | 3 | 15.10 | -4.82 | 19.92 | 5.5 |
| Miers Valley | 1 | 1.46 | -3.95 | 5.41 | 3.5 |
| | 2 | 3.62 | -0.61 | 4.23 | 12.0 |
| | 3 | 2.03 | -1.88 | 3.91 | 9.0 |
| Bratina Island | 1 | 2.16 | -5.72 | 7.88 | 1.5 |
| | 3 | 12.36 | -4.82 | 17.18 | 9.0 |

## 3.3 Discussion

The speed and magnitude of temperature changes in the top layer of soil is the result of solar radiation, temperature and windspeed of the overlying air and soil properties such as albedo. Maximum temperature is mainly due to solar radiation, while minimum temperature is effected by air temperature (Campbell *et al.*, 1998).

All temperature profiles showed the effect of sunlight on surface soil temperature, with highest temperatures recorded during sunny periods and lower temperatures during overcast conditions. This effect was observed by Thompson *et al.* (1971) at Vanda Station in the Lower Wright Valley, who were able to correspond temperature fluctuations at a soil depth of 8cm with solar radiation input.

Several sites were at a temperature above freezing for up to 12 hours (Table 3.2). This is important as water becomes freely available in these periods, allowing biological and chemical reactions to take place (Campbell and Claridge, 1987).

### 3.3.1 Upper Wright Valley

Position 3 had the most extreme temperature fluctuations over the time period, which was probably due to the sample sites exposed location at the top of a ridge. Rocks halfway up the slope surrounded position 1, while position 2 was further down the slope under a clump of ice closed in by rocks, which presumably accounted for increased temperature stability at these sites. The lowest maximum temperature recorded at position 2 was probably due to both ice and rocks shading most direct sunlight from this site during the sampling period.

The measurements recorded in the Upper Wright Valley over 24 hours fit in well with long-term temperatures previously recorded in the Lower Wright Valley. Thompson *et al.* (1971) found the maximum surface soil temperature at Vanda Station was

11.6°C, which is nearly the same as my values. Unpublished data collected by Patezold, Balks and Aislabie over three years at Bulls Pass showed that the top layer of soil can reach temperatures of nearly 28°C in January. December and January are the warmest months of the year, while the coldest is usually August (Thompson *et al.* 1971; Patezold *et al.*, unpublished data). The minimum temperature I recorded was –7.02°C, which corresponds with the data of Patezold *et al.* who found the minimum January temperature to be –7.9°C. The lowest surface soil temperature recorded by Patezold *et al.* was –56.4°C. This means that over one year, surface soils are exposed to a temperature range of up to 84°C in the Wright Valley.

### 3.3.2 Miers Valley

The large temperature variations recorded between sites in the Upper Wright Valley were not observed at Miers Valley sample sites. Position 2, situated at the base of a rock, measured temperatures slightly higher than the other two positions. Temperatures recorded at positions 1 and 3 were similar, however position 1 had significantly less time above zero degrees compared with the other two probes. Position 1 was the most exposed of all the sites, being on a shallow slope of stony gravel, both the other sites were close by on the same slope but located next to larger rocks. Reasons for the small variation in temperature between these sites is unclear. Perhaps the soil possessed a certain property that prevented it from retaining heat as well as nearby soils, or rocks surrounding the other sites provided better protection from the wind allowing the overlying air to warm up.

It could be assumed that generally the exposed soils in Miers Valley are subjected to the same temperature variations as the Wright Valley, receiving the same levels of sunlight and dropping to around the same minimum temperature in winter. Therefore the soil microorganisms present would need to be equally well adapted to cold and large temperature changes over short time periods.

### 3.3.3 Bratina Island

All three sampling sites at Bratina Island were situated in exposed areas amongst the ice ponds, although only position 3 was located on fine, dark soil, which may have contributed to the large fluctuations and high maximum temperature observed. Higher temperatures are usually recorded in darker soils because more solar radiation can be absorbed compared with lighter soils, such as found at positions 1 and 2, and snow (Campbell *et al.*, 1997).

The results for Bratina Island can be compared with temperature measurements previously collected at Scott Base as both areas are coastal, of similar altitudes and are located in the Ross Ice Shelf. Data collected by Campbell *et al.* (1997) showed that soil surface temperature varied between –2.3°C to 11.0°C over 24 hours during cloudy conditions in December. This is within the range of temperature measurements collected for Bratina Island in the current study.

Patezold *et al.* (unpublished data) carried out measurements of surface soil temperatures at Scott Base over three years and found that the average maximum temperature reached was 16.5°C in February, while the minimum was –22.1°C. Over the entire year, maximum temperatures reached an average of 22.6°C, while minimum temperatures got to –48°C.

The results of Campbell *et al.* (1997) and Patezold *et al.* show that coastal and dry valley soils are exposed to a high range of temperatures, both within a single day and also over an entire year. Therefore, populations of microorganisms present in these soils are expected to be psychrotolerant rather than psychrophilic, as temperatures above 20°C are lethal to truly psychrophilic microorganisms, as defined by Morita (1975).

Psychrotolerant bacteria have a reduced rate of metabolism when living in an environment that is often cooler than their optimum temperature for growth, which

may be an advantage if nutrient availability is poor (Baross and Morita, 1978). However, temperature profiles recorded for in the current study show that temperatures drop well below zero degrees. Baross and Morita (1978) suggest that survival below the minimum temperature for growth of psychrotolerant bacteria may occur due to secondary metabolism, such as spore formation in *Bacillus* species, which would occur just before freezing. Therefore, it is possible to isolate species of bacteria that cannot grow in such extremely cold conditions, but are adapted for survival at temperatures below their minimum for growth.

# Chapter 4 Culture and Identification of Bacterial Isolates from Antarctic Soils

## 4.1 Introduction

This section of work aimed to (1) isolate pure bacterial cultures from Antarctic soils, (2) characterise and identify the isolated cultures, and (3) further analyse any potential *Bacillus* isolates for future enzyme work. Only species of *Bacillus* were selected for enzyme characterisation because they share identical evolutionary histories. Therefore, any differences found between enzymes are more likely to be due to temperature adaptation only. Previous studies (Georlette *et al.*, 2000; Svingor *et al.*, 2001; Galkin *et al.*, 1999) have often compared temperature adaptations between bacteria from different, although sometimes related, genera which may decrease the validity of their results.

This research is also part of a larger project analysing microbial diversity in Antarctic soils using molecular biological methods (Cary *et al.*, in preparation). The temperature history of sampling locations, plus factors such as nutrient enrichment from desiccated seal carcasses, moisture content of soils, and the introduction of non-indigenous microorganisms as a result of human activities are all important contributing factors to microbial diversity and work is still being carried out by other research groups. Results obtained from the current study using classical methodology will be compared and incorporated with those obtained by others using 16S molecular phylogenetic methods to characterise the microbial communities present in these soils.

## 4.2 Materials and Methods

### 4.2.1 Growth of bacterial isolates

At Scott Base, a sterile cotton-tipped applicator was wetted with nutrient broth (Gibco BRL, Life Technologies Ltd, UK, 8g/L), dipped into the soil sample and inoculated into a McCartney bottle containing 10mL of nutrient broth. The cultures were incubated at temperatures ranging from approximately -10°C in the field to +20°C in New Zealand for approximately two weeks while they were couriered back to the laboratory from Scott Base.

In the laboratory, an inoculating loop was used to take a sample of each culture, which was streaked onto nutrient agar (Scharlau Microbiology, Barcelona) and incubated at 15°C or 35°C until visible growth had occurred (2-3 days). Single colonies of differing appearance (colour, size and morphology) were picked off each plate and streaked out again to obtain pure cultures.

Sample names summarised the location, e.g. MV - Miers Valley, temperature profile (if done), e.g. HB for HOBO, and soil sample e.g. A, B or 1, 2. If more than one colony was isolated from a particular soil sample they were given further annotation of (a), (b) etc.

### 4.2.2 Gram staining

Methods of preparation for solutions used are given in Appendix 2.

Newly grown bacterial cells were transferred from agar plates to microscope slides and fixed by passing quickly over a Bunsen flame. Slides were flooded with ammonium crystal violet for one minute, then this was tipped off and replaced with Grams iodine solution for 30 seconds. The iodine was drained off and the slides washed with gently running tap water followed by 95% ethanol for no longer than 30

seconds, until a pale violet colour was obtained. The ethanol was washed off with water, and slides counter-stained with carbol fuchsin for one minute. After the fuchsin was drained off, the slides were washed with water, air-dried and examined under the microscope. (C. Harfoot, personal communication).

### 4.2.3 DNA extraction

Genomic DNA was extracted from the bacterial isolates observed to be Gram positive rods. To do this, an IsoQuick kit (Catalogue no. MXT-020-100, ORCA Research Inc., USA) was used, following the protocol for total nucleic acid (DNA and RNA) extraction.

Isolates were grown in nutrient broth (8g/L) to maximal optical density (between 0.2-1.0 absorbance units) at 15°C. A volume of 10mL of culture was centrifuged (8 minutes, 13 200 rpm) and resuspended in 1mL of 15% glycerol in nutrient broth. 900$\mu$L was removed for storage at -70°C while the remaining 100$\mu$L was centrifuged again and used for genomic DNA extraction.

The chaotropic nature of the guanidine thiocynate (GuSCN) contained in the kit caused cell lysis and nuclease inhibition, resulting in DNA release and stabilisation. The cell lysate was mixed with extraction reagent and centrifuged, separating the organic phase from the aqueous phase, in which the nucleic acid was dissolved. Ethanol was added to precipitate the nucleic acid that was then dissolved in RNAse-free water.

Successful extraction was checked by running 2$\mu$L of extract plus 1$\mu$L of loading buffer (0.04% bromophenol blue, 30% glycerol) on a 1% agarose gel made with Tris-borate EDTA buffer (TBE buffer, 5X stock solution contains 54g Tris-HCl, 27.5g boric acid, 3.7g EDTA, pH 8.0, made up to 1L with distilled water) for 35 minutes at 100mV. A Hi-Lo DNA marker/mass ladder (BN2050, Bionexus Inc./Applied Bio Products) was loaded to allow estimation of molecular weight. The gel was stained 15

minutes in 100mL water containing 0.5mg/L ethidium bromide, then destained 10 minutes in running water. DNA bands were visualised using the Eagle Eye system (Stratagene, USA).

Based on the relative intensities of the genomic DNA extracts determined on the gel, these were diluted with molecular biology grade water up to $1/50^{th}$ the original concentration.

At this stage, the isolates were given abbreviated names to avoid confusion and will be referred to by these from now on.

### 4.2.4 Primer sequences

Sequences of primers used in research project are presented in Table 4.1. For sources of PCR reaction components see Appendix 2.

**Table 4.1. Sequences of primers (from 5′ to 3′ terminus) used in the current study. All primers obtained from Invitrogen, Life Technologies, Gaithersburg, USA.**

| Primer | Sequence |
|---|---|
| OPR 13 | GGACGACAAG |
| 338f | CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCTCCTACGGG AGGCAGCAG (GC clamp is underlined) |
| 519r | ATTACCGCGGCTGCTGG |
| 27f | AGAGTTTGATCCTGGCTCAG |
| 1522r | AAGGAGGTGATCCARCCGCA |
| 907r | CCGTCAATTCCTTTGAGTTT |
| 357f | CTCCTACGGGAGGCAGCAG |

### 4.2.5 Denaturing gradient gel electrophoresis

Analysis by denaturing gradient gel electrophoresis (DGGE) involves the amplification and electrophoresis of a small region of the highly conserved 16S

rRNA. By electrophoresing 16S rDNA fragments from different bacterial isolates on a DGGE gel, those found to produce the same banding pattern have the same, or highly similar, identities.

As the 16S rDNA fragments move through the polyacrylamide gel, they encounter a linearly increasing gradient of denaturant. Separation occurs due to the differing mobilities of the partially-melted DNA fragments, which is decreased compared with that of the completely helical form of the molecule (Muyzer *et al.*, 1993). Base pair composition produces discrete regions of stability within the DNA fragment so different sequences of DNA will vary in the way they denature and the amount of denaturant required to completely separate the two strands (Muyzer, 1999). A GC clamp at the 5′ terminus prevents the fully denatured fragments from migrating further through the gel.

DGGE is often used to analyse the members of a particular microbial community simultaneously, with each band representative of an individual member (Muyzer, 1999). However, it can be limited by the occurrence of multiple 16S rRNA genes and in detection of rare community members.

#### 4.2.5.1 DGGE-PCR and Electrophoresis

To amplify a small (approximately 220bp) region of the 16S rRNA gene, a PCR reaction was performed using universal bacterial 338f and 519r primers (see Table 4.1). All the PCR reagents, apart from the *Taq* polymerase, were prepared as a mastermix, and added to PCR tubes containing the template DNA. The tubes were loaded into the Eppendorf Mastercycler Gradient machine (Eppendorf AG, Hamburg, Germany) and heated to 94°C before the *Taq* polymerase was added. Using a "hot start" method ensured that extension of non-specifically annealed primers did not take place at lower temperatures. A touchdown program, which decreased the primer annealing temperature for each successive cycle, was employed to further guarantee the specificity of primer binding.

Each 25μL reaction mixture contained the following:

| | |
|---|---|
| DNA template (dil. extract) | 1μL |
| Milli-Q water | 15μL |
| 10X PCR buffer (without $MgCl_2$) | 2.5μL |
| $MgCl_2$ (25mM) | 1.5μL |
| dNTPs (2mM) | 2.5μL |
| Forward primer, 338f (0.25mM) | 1μL |
| Reverse primer, 519r (0.25mM) | 1μL |
| *Taq* polymerase (1U/μL) | 0.5μL |

For samples that did not amplify well the first time, the reaction mixture was adjusted to 4μL template and 12μL water.

The DGGE programme run on the thermocycler was 95°C for 2 minutes, 80°C for 1 minute, then a cycle of 94°C for 1 minute (hot start), 65°C for 1 minute (reducing by 0.5°C each cycle), 72°C for 1 minute for 21 cycles, followed by a cycle of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, which was repeated 8 times. The final step was 72°C for 5 minutes, then the tubes were held at 4°C.

To verify that the DNA had been amplified successfully, a volume of 3μL of PCR reaction and 1μL loading buffer were electrophoresed on a 1% agarose gel as described previously (Section 4.2.3) and checked for the presence of an approximately 220bp fragment.

#### 4.2.5.2 Denaturing gradient gel electrophoresis (DGGE)

The Gradient Delivery System (Model 475, Bio-Rad Laboratories, New York, USA) was used to pour parallel denaturant gradient gels with an increasing concentration of urea/formamide (25-60%), as described in the denaturing gel electrophoresis system instruction manual and applications guide (Bio-Rad, New York, USA). 12μL of

sample plus an equal volume of DGGE loading buffer solution (0.5% bromophenol blue, 0.05% xylene cyanol and 70% glycerol diluted in 1X TAE buffer) was loaded onto the gel. This was electrophoresed using a D GENE™ Denaturing Gel Electrophoresis System containing 7L of pre-heated 1X TAE buffer (50X TAE contains 242g Tris, 57.1mL acetic acid, 100mL 0.5M EDTA, pH 8.0, made up to 1L with Milli-Q water)

The gel was run at 60°C for 4.2 hours at 130V, then stained in ethidium bromide (Section 4.2.3) for 30 minutes, destained in tapwater for 15 minutes and visualised.

A second DGGE gel with isolates representing groups revealed in the first DGGE analysis was run. This had a 30-70% denaturant gradient and was prepared as described above.

### 4.2.6 RAPD analysis

RAPDs allow the separation of different bacteria by comparison of a characteristic gel pattern, produced by carrying out PCR using a primer of arbitrary sequence. The sequences of DNA that are amplified in this reaction are unknown, but whole genome comparisons can be obtained, allowing identification of different isolates. Sources of polymorphisms include base changes in genomic DNA, deletions of priming sites, insertions that separate priming sites by too large a distance to support amplification, or insertions that change the size of a DNA fragment (Williams *et al.*, 1990). The main advantage of the RAPD technique is that no prior knowledge of DNA sequence is required, and it allows detection of polymorphisms in closely related organisms at a higher resolution than 16S rRNA sequence comparisons (Zhang *et al.*, 2002).

#### 4.2.6.1 RAPD-PCR

A PCR mastermix was made using all reagents except the DNA template. *Taq* polymerase was added last, before the 23μL volumes were dispensed into the PCR tubes containing template DNA and loaded onto the thermocycler.

Each 25μL RAPD-PCR reaction mixture contained:

| | |
|---|---|
| Milli-Q water | 9.25μL |
| $MgCl_2$ (25mM) | 2.50μL |
| 10X PCR buffer (without $MgCl_2$) | 2.50μL |
| dNTPs (0.1mM) | 2.50μL |
| Primer OPR13 (2μM) | 5.00μL |
| *Taq* polymerase (1.25U) | 1.25μL |
| DNA template (dil. extract) | 2.00μL |

The following RAPD-PCR programme was used: the initial denaturation step was 94°C for 3 minutes 45 seconds, followed by 35 cycles of 94°C for 15 seconds, 36°C for 15 seconds and 72°C for 2 minutes. The final extension step was 72°C for 4 minutes, then the tubes were held at 4°C.

#### 4.2.6.2 Agarose gel electrophoresis

At the completion of the PCR, 7μL loading buffer (Section 4.2.3) was added to each reaction mixture. The total volume was then loaded onto a 1.5% agarose gel along with 14μL of DNA ladder (1 kb Plus DNA ladder, Life Technologies, Gaithersburg, USA) for comparison between gels.

### 4.2.6.3 Running conditions

The gel was run first at 20V until the samples left the wells (about 30 minutes) then power was increased to 70V for approximately two hours. The gel was stained in ethidium bromide (Section 4.2.3) for 40 minutes then destained under cold running water for 30 minutes and visualised.

### 4.2.7 Full 16S rDNA amplification

Amplification of the full 16S rRNA gene (approximately 1500bp) was carried out using universal bacterial primers 27f and 1522r in preparation for DNA sequencing.

The PCR reaction mix was made as described previously (Section 4.2.6.1), except the volumes were doubled to produce 50$\mu$L reactions. The same DGGE-PCR thermocycler programme was used as described in the previous section (Section 4.2.5.1). As poor results were obtained in the first attempt at full gene amplification, a new method using AmpliTaq Gold DNA polymerase was carried out instead. PCR reagents, except for the *Taq* polymerase, were also exposed to an UV light source for 7-8 minutes to remove contaminating DNA.

Each 50$\mu$L Amplitaq Gold reaction mixture contained:

| | |
|---|---|
| 10X PCR buffer (with $MgCl_2$) | 5$\mu$L |
| dNTPs (2mM) | 5$\mu$L |
| 27f primer (0.25mM) | 1$\mu$L |
| 1522r primer (0.25mM) | 1$\mu$L |
| Bovine serum albumin (10mg/mL) | 3$\mu$L |
| AmpliTaq Gold DNA polymerase* (1U/$\mu$L) | 0.25$\mu$L |
| Molecular biology grade water | 32.75$\mu$L |
| DNA template (dil. extract) | 2$\mu$L |

*AmpliTaq Gold DNA polymerase supplied by Applied Biosystems, USA .

### 4.2.8 PCR clean-up for DNA sequencing

#### 4.2.8.1 GenElute PCR cleanup kit method

To remove unwanted reaction components such as excess primers, nucleotides, and salts before sequencing, the full gene PCR products were purified using a GenElute™ PCR Clean-Up kit (Product code NA1020, Sigma, Missouri, USA).

The PCR product was bound to a silica membrane within a microcentrifuge binding column, while contaminants passed through into an attached collection tube. The bound DNA was washed then eluted in a purified form into a buffer solution.

To check that the purification was successful and no DNA was lost, a sample of purified DNA was electrophoresed in a 1% agarose gel and the presence of a DNA band observed.

#### 4.2.8.2 Freeze and squeeze method

PCR product clean up using the freeze and squeeze method required a much larger amount of product to start with, compared to the method used above. Six 50μL reactions were pooled together to give a total volume of 300μL PCR product for each sample. This volume, plus 85μL loading buffer (Section 4.2.3), was loaded onto a 1% agarose gel made with TAE buffer (50X TAE contains 242g Tris, 57.1mL acetic acid, 100mL 0.5M EDTA, pH 8.0, made up to 1L with Milli-Q water).

A large sample well was made by wrapping all but one of the teeth of the gel comb with sellotape before pouring the gel. A 1 kb Plus DNA molecular weight ladder (Section 4.2.6.2) was loaded into the extra well to check the position of the band. A large gel used 400mL 1% agarose in TAE, with 3L TAE in the electrophoresis chamber and was run overnight at 30V. A small gel used 100mL 1% agarose in TAE, plus 1L TAE in the electrophoresis chamber and was run for 4 hours at 40V. The gel

was stained as previously described (Section 4.2.3), then the band of DNA was visualised using a UV light box, cut out using a clean coverslip, wrapped in parafilm and stored at –20°C overnight.

The next day, DNA was removed from the gel by squeezing the wrapped frozen segment gently by hand, causing the liquid containing the DNA to drip out into an Eppendorf tube. The following steps were then carried out:

1. The tube was spun for 1 minute at 16100 r.c.f. and 600μL supernatant transferred to a new tube. An equal volume (600μL) of phenol:chloroform:isoamyl alcohol (25:25:1) was added, the tube mixed and spun for 4 minutes at 9240 r.c.f.
2. The top layer (550μL) was transferred to a new tube, an equal volume of chloroform:isoamyl alcohol (24:1) was added, mixed and the tube spun for 2 minutes at 9240 r.c.f.
3. The supernatant (430μL) was transferred to a new tube, and ammonium acetate was added to provide a final concentration of 2.5M. Isopropyl-alcohol was added in a volume equal to that of the supernatant (430μL), then the mixture was incubated at room temperature for 15 minutes.
4. The tube was spun at 16100 r.c.f for 15 minutes, after which a pellet was visible at the bottom of the tube. The supernatant was carefully removed and the pellet washed with 70% ethanol. Most of the ethanol was pipetted off; the pellet left to dry for 1 hour at 37°C then resuspended in 20μL Milli-Q water.

### 4.2.9 DNA Quantification

DNA quantification was carried out using an ethidium bromide fluorescence method. A set of 20μL standards of increasing DNA concentration was prepared (Table 4.2).

**Table 4.2. Volume of stock DNA and Milli-Q water required to generate DNA standards for quantification**

| Stock DNA (x10ng/μL) | Milli-Q water |
|---|---|
| 1μL | 19μL |
| 2 | 18 |
| 3 | 17 |
| 5 | 15 |
| 7.5 | 12.5 |
| 10 | 10 |
| 20 | 0 |
| 0 | 20 |

Droplets were placed on a perspex gel plate then 20μL droplets of the DNA solutions to be measured were positioned around them. Ethidium bromide fluorescence was detected using the Eagle Eye visualisation system, a picture taken, then the intensities of the samples was compared to the standards and DNA concentration estimated.

### 4.2.10 DNA sequencing reaction

Nine isolates were sequenced using the DYEnamicET protocol and DYEnamic mastermix provided by the Waikato DNA Sequencing Facility.

Each 10μL reaction contained:

| | |
|---|---|
| DYEnamic mastermix | 4μL |
| 357f primer (5μM) | 1μL |
| DNA template | 1μL |
| Milli-Q water | 4μL |

Each reaction was cycled at 95°C for 20 seconds, 50°C for 30 seconds and 60°C for 2 minutes over 30 cycles.

The reactions were then cleaned using cleaning buffer (1.5M sodium acetate with 250mM EDTA) and ethanol and then centrifuged to produce a DNA pellet for sequencing.

Reverse sequencing, using a bacterial 907r primer, was carried out on isolates identified as being *Bacillus* species, or closely related *Planococcus* species from the results of the forward sequencing.

### 4.2.11 Analysis and BLAST search of DNA sequences

The returned nucleotide sequences were aligned using the BioEdit program (Version 5.0.9, © Tom Hall, Dept of Microbiology, North Carolina State University). Unassigned nucleotides had their identities predicted by comparison between aligned conserved sequences or by referral to the electropherogram. Sequences were truncated at the ends to remove less reliable data. Edited sequences were submitted to the Basic Local Alignment Search Tool (BLAST) search engine hosted on the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov) to obtain a list of bacterial species with varying levels of similarity.

An identity was assigned to each isolate based on the most significant alignments (producing a high bit score and low E score). A tentative description to species level was allocated to isolates that generated multiple high-scoring matches to a particular species.

### 4.2.12 Phylogenetic analysis

Nucleotide sequences of members of the *Bacillus* and *Planococcus* groups were downloaded from the NCBI and Deutsche Sammlung von Mikroorganisem und Zellkulturen (DSMZ, www.dsmz.de) websites. These were then aligned using the

BioEdit program and submitted to the Clustalw program (Version 1.7) to create a phylogenetic tree.

## 4.3 Results

### 4.3.1 Growth of bacterial isolates

At the end of the incubation period (approximately two weeks), nearly all (21 out of 24) culture bottles were turbid except for the Upper Wright Valley samples, indicating microbial growth had occurred. Bacterial colonies took 2 - 3 days to grow on nutrient agar plates. On average, 2.1 isolates of differing appearance were obtained per soil sample at 15°C (total number = 52), while 0.7 isolates were obtained per soil sample at 35°C (total number = 17).

### 4.3.2 Initial characterisation

Colony and cell morphology for the 15°C isolates is described in Table 4.3.

Out of 52 isolates, 26 were observed to be Gram positive rods, therefore potential species of *Bacillus*. Two of the 26 isolates were not analysed further due to being mixed cultures. These were stored for future purification if necessary.

The remainder of the Antarctic isolates consisted of:

- 4 Gram positive cocci
- 13 Gram negative rods
- 7 Gram negative cocci
- 2 mixed cultures - Gram negative rods and cocci

**Table 4.3. Preliminary characterisation of Antarctic soil bacterial isolates**

| Soil sample | Isolate | Description of colonies | Gram stain | Morphology | Possible *Bacillus*? |
|---|---|---|---|---|---|
| UWV-00-1 | UWV-00-1(a) | Yellow | + | cocci | N |
| | UWV-00-1(b) | Yellow | + | cocci | N |
| UWV-00-2 | - | No growth | N/A | N/A | N/A |
| UWV-HB-1A | - | No growth | N/A | N/A | N/A |
| UWV-HB-1B | - | No growth | N/A | N/A | N/A |
| UWV-TB-1 | - | No growth | N/A | N/A | N/A |
| MV-Glacier | MV-G(a) | Beige, small colonies | - | rod/comma | N |
| | MV-G(b) | Beige, small colonies | - | rod | N |
| | MV-G(c) | Orange | - | rod | N |
| MV-HB-1A | MV-HB-1A(a) | Peach, medium colonies, turn agar orange | + (some -) | large rod | Y |
| | MV-HB-1A(b) | Orange/brown, rough surface | + | rod | Y |
| | MV-HB-1A(c) | Beige | + | rod | Y |
| MV-HB-1B | MV-HB-1B | Beige | + | rod | Y |
| MVS 4.1 | MVS 4.1(a) | Beige, small colonies | - | rod + cocci mix | N |
| | MVS 4.1(b) | Light yellow, medium colonies | - | short rod | N |
| MVS 4.2 | MVS 4.2(a) | Beige | - | cocci | N |
| | MVS 4.2(b) | Beige, very small colonies | + | short rod | Y |
| | MVS 4.2(c) | Orange | + | short rod | Y |
| | MVS 4.2(d) | Beige, medium colonies | + | short rod | Y |
| MVS 4.3 | MVS 4.3(a) | Beige, very small colonies | + | short rod | Y |
| | MVS 4.3(b) | Pink/beige, medium colonies | - | rod | N |
| MVS 4.4 | MVS 4.4(a) | Beige, medium colonies | - | rod | N |
| | MVS 4.4(b) | Light yellow, small colonies | + | small rod | Y |
| | MVS4.4(c) | Peach, medium colonies | + | rod | Y |
| MVT 1 | MVT 1(a) | Beige, large colonies | - | cocci/short rod | N |
| | MVT 1(b) | Beige, large colonies | + | rod | Y |
| MVT 3 | MVT 3(a) | Pink, small colonies | +/- | rod | Y |
| | MVT 3(b) | Orange | + | short rod | Y |
| | MVT 3(c) | Beige | - | cocci | N |
| MVT 5 | MVT 5(a) | Beige, small colonies | + | rod | Y |
| | MVT 5(b) | Beige, small | +/- | rod + cocci | Y* |

| | | | | | |
|---|---|---|---|---|---|
| | | colonies | | mix? | |
| MVT 7 | MVT 7 | Orange, medium colonies | - | rod | N |
| MVT 9 | MVT 9 | Beige, small colonies | + | rod | Y |
| MVT 11 | MVT 11(a) | Beige, small colonies | + | rod | Y |
| | MVT 11(b) | Bright pink, small colonies | + | cocci | N |
| | MVT 11(c) | Beige, small colonies | - | cocci | N |
| MV-TB-1 | MV-TB-1 | Beige | + | small rod | Y |
| BI-1 | BI-1(a) | Beige, medium colonies | - | rod | N |
| | BI-1(b) | White/beige, medium colonies | - | rod | N |
| | BI-1(c) | Beige, large colonies | - | rod | N |
| | BI-1(d) | Beige, small colonies | - | rod | N |
| BI-2 | BI-2A(a) | BI-2A = yellow, medium colonies, later found | + | long rod | Y |
| | BI-2A(b) | to be a mix of three different bacteria | + | rod | Y |
| | BI-2A(c) | | +/- | rod mix? | Y* |
| | BI-2B | Beige, rough surface, medium colonies | - | cocci | N |
| BI-HB-1A | BI-HB-1A(a) | Beige, diffuse colonies | - | rod + cocci mix? | N |
| | BI-HB-1A(b) | Beige, small colonies | - | cocci | N |
| | BI-HB-1A(c) | Beige, small colonies | + | short rod | Y |
| BI-HB-1B | BI-HB-1B(a) | Beige | - | small rod | N |
| | BI-HB-1B(b) | Beige, diffuse colonies | - | rod | N |
| | BI-HB-1B(c) | Beige | + | short rod | Y |
| BI-TB-1 | BI-TB-1(a) | Beige, small colonies | - | cocci | N |
| | BI-TB-1(b) | Beige | + | rod | Y |
| Penguin rookery | PR(a) | Yellow, small colonies | + | cocci | N |
| | PR(b) | Yellow, small colonies | + | rod | Y |
| | PR(c) | Beige | + | rod | Y |

*Not analysed further

### 4.3.3 DNA Extraction

The relative intensities of genomic DNA extracts are shown in Figure 4.1 and the resulting dilutions, with abbreviations for each isolate, summarised in Table 4.4. Isolates MVT 11 (a) and MVT 9 were extracted at an earlier time to confirm that the technique was working, therefore they are not shown in this figure.

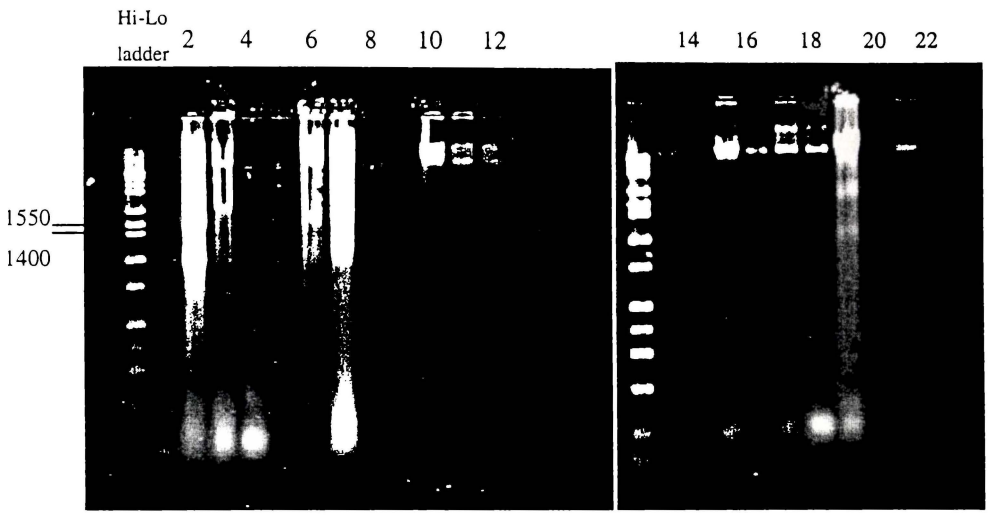



**Figure 4.1. Electrophoresed genomic DNA extracts from Antarctic isolates, for sample lanes, refer to Table 4.4.**

**Table 4.4. Dilution factors for genomic DNA extracts (with abbreviated isolate names).**

| Lane | Isolate | Intensity | Dilution | Abbreviated Name |
|---|---|---|---|---|
| 1 | PR (c) | None | 1 | PR1 |
| 2 | MVS 4.2 (c) | High | 1/50 | MVS1 |
| 3 | MVS 4.2 (d) | Medium | 1/20 | MVS2 |
| 4 | MVT 3 (a) | None | 1 | MVT1 |
| 5 | PR (b) | None | 1 | PR2 |
| 6 | BI 2A (a) | Medium | 1/20 | BI1 |
| 7 | BI 2A (b) | High | 1/50 | BI2 |
| 8 | MVS 4.4 (b) | None | 1 | MVS3 |
| 9 | MV-HB-1A (a) | None | 1 | MV1 |
| 10 | MV-HB-1A (c) | Light | 1/10 | MV2 |
| 11 | MV-TB-1 | Light | 1/10 | MV3 |
| 12 | MV-HB-1B | Light | 1/10 | MV4 |
| 13 | MVS 4.4 (c) | None | 1 | MVS4 |
| 14 | MV-HB-1A (b) | None | 1 | MV5 |
| 15 | MVT 5 (a) | Light | 1/10 | MVT2 |
| 16 | BI-HB-1B (c) | Light | 1/10 | BI3 |
| 17 | MVS 4.2 (b) | Light | 1/10 | MVS5 |
| 18 | MVS 4.3 (a) | Light | 1/10 | MVS6 |
| 19 | MVT 3 (b) | Medium | 1/20 | MVT3 |
| 20 | MVT 1 (b) | None | 1 | MVT4 |
| 21 | BI-HB-1A (c) | Light | 1/10 | BI4 |
| 22 | BI-TB-1 (b) | None | 1 | BI5 |
| N/A | MVT 11 (a) | N/A | N/A | MVT5 |
| N/A | MVT 9 | N/A | N/A | MVT6 |

Several lanes produced a bright smear indicating a high amount of genomic DNA was extracted, while others produced a very faint band or nothing at all. In these cases the genomic DNA concentration was assumed to be below the detection limit of the visualisation system

### 4.3.4 Denaturing Gradient Gel Electrophoresis

#### 4.3.4.1 DGGE analysis of Antarctic isolates

A DGGE gel of all potential *Bacillus* isolates is presented in Figure 4.2.

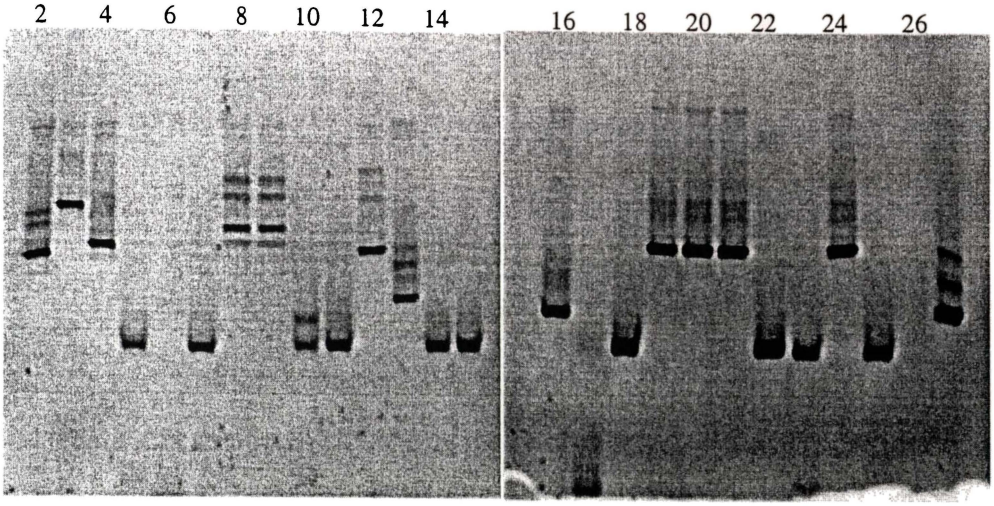



**Figure 4.2. DGGE profile of Antarctic isolates. Lane numbers and corresponding isolates are: (1) PR1; (2) MVS1; (3) MVS2; (4) MVT5; (5) MVT1; (6) PR2; (7) BI1; (8) BI2; (9) MVT6; (10) MVS3; (11) MV1; (12) MV2; (13) MV3; (14) MV4; (15) MVS4; (16) MV5; (17) MVT2; (18) BI3; (19) MVS5; (20) MVS6; (21) MVT3; (22) MVT4; (23) BI4; (24) BI5; (25) Negative control; (26) Positive control, *E. coli***

Certain isolates produced the same banding patterns (Table 4.5). These groups are likely to represent the same species, or closely related species.

**Table 4.5. Groups of Antarctic isolates producing matching DGGE profiles.**

| Group | Isolates |
|---|---|
| A | PR1, BI1, BI2 |
| B | BI3, BI4, MVS5, MVS6 |
| C | PR2, MVT5, MVT6, MVS3, MV3, MV4 |
| D | MVT2, MVT3, MVT4, BI5 |
| E | MVS2, MV1 |

Isolates MVS1, MVS4, MV5 and MV2 produced unique banding patterns, while MVT1 did not generate a pattern.

### 4.3.4.2 DGGE summary gel

Isolates representative of each group identified in the first DGGE gel were electrophoresed on a summary gel (Figure 4.3).

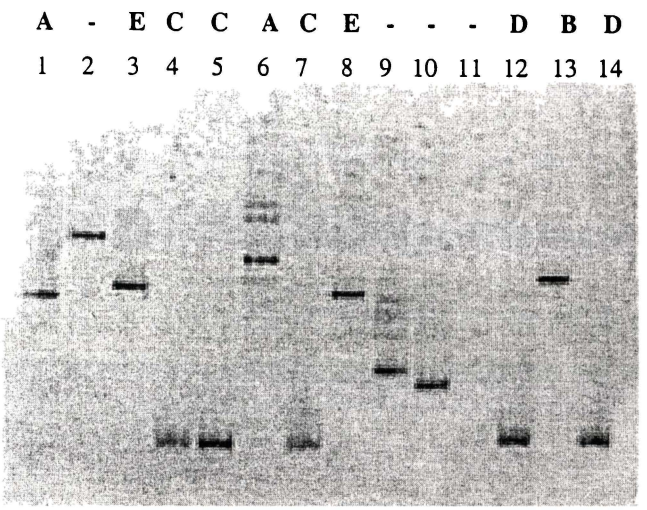



**Figure 4.3. DGGE profile of Antarctic isolates representing groups A – E. Lane numbers and corresponding isolates are: (1) PR1; (2) MVS1; (3) MVS2; (4) MVT5; (5) PR2; (6) BI1; (7) MVT6; (8) MV1; (9) MV2; (10) MVS4; (11) MV5; (12) MVT2; (13) BI3; (14) MVT3**

### 4.3.5 RAPD analysis

A RAPD gel showing the genome-wide differences in bacterial isolates shown to be similar by DGGE analysis is presented in Figure 4.4. Isolates with unique DGGE banding patterns were not included.

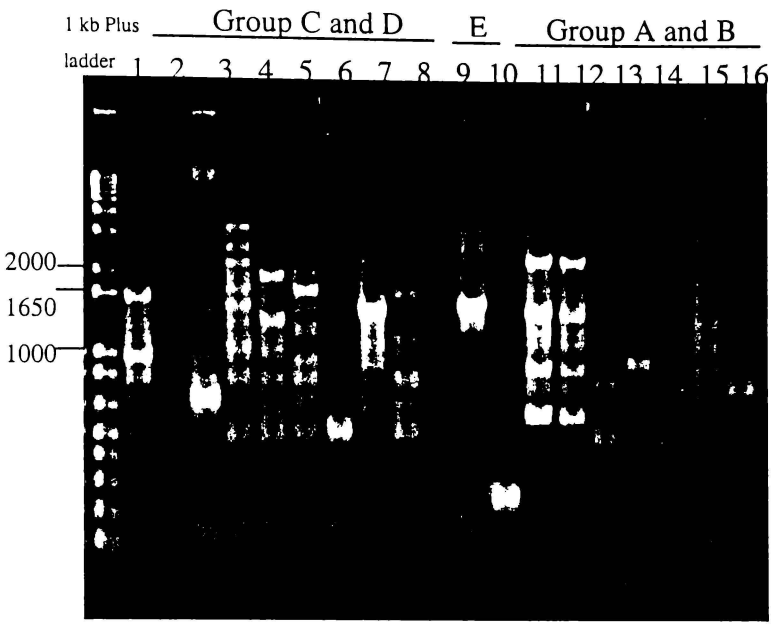



**Figure 4.4. RAPD profile of Antarctic isolates. Lane numbers and corresponding isolates are: (1) MVT5; (2) PR2; (3) MVS3; (4) MV3; (5) MV4; (6) MVT2; (7) MVT3; (8) MVT4; (9) BI5; (10) MV1; (11) MVS2; (12) PR1; (13) BI1; (14) BI2; (15) BI3; (16) MVS5; (17) MVS6; (18) BI4; (19) Negative control**

Except for lanes 13 and 14, each sample electrophoresed on this gel produced a unique banding pattern. The negative control produced a positive result but this did not have a significant effect on the results, as the purpose of this analysis was to check for differences between isolates, which is easily apparent.

### 4.3.6 Full 16S rDNA amplification

Figure 4.5 shows the DNA products of the full 16S rRNA gene amplification (1500bp approximately), required for sequencing.

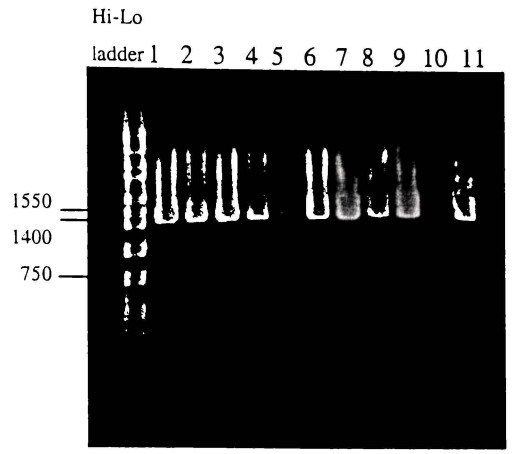



**Figure 4.5. Full 16S rDNA amplification product (approximately 1500bp length). Lane numbers and corresponding isolates are: (1) PR1; (2) MVS1; (3) MVS2; (4) MVT5; (5) MVT1; (6) BI1; (7) MV2; (8) MVS4; (9) BI3; (10) Negative control; (11) Positive control, *E. coli***

All isolates produced a good full gene amplification except for MVT1, which had previously given poor results for DNA extraction. Bands are smeared due to the buffer solution being accidentally omitted from the agarose gel preparation.

### 4.3.7 PCR cleanup

#### 4.3.7.1 GenElute™ method

The good signal of the bands (Figure 4.6) indicated that the PCR cleanup was successful and no DNA was lost. An obvious band for sample MVT1 was visible; confirming that some DNA was present.

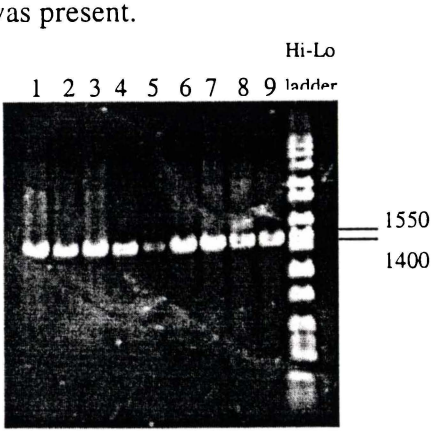



**Figure 4.6. PCR products purified using GenElute™ kit. Lanes are as for Figure 4.5.**

### 4.3.7.2 Freeze and squeeze method

A successful purification of the PCR product was confirmed for all three isolates (Figure 4.7)

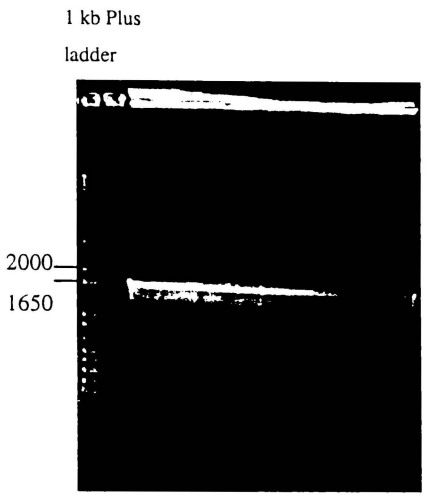



**Figure 4.7. Freeze and squeeze gel for isolate MV2.**

### 4.3.8 DNA Quantification

By comparison of sample spot intensity to standards, DNA concentration was estimated as presented in Table 4.6.

**Table 4.6. Quantification of full 16S rDNA amplified product. Underlined numbers represent the most likely DNA concentration within the range of values given.**

| Isolate | DNA conc. (ng/μL) |
|---|---|
| PR1 | 20 |
| MVS1 | 0 - 10 |
| MVT5 | 0 - 10 |
| PR2 | 0 - 10 |
| BI1 | 10 - 20 |
| BI2 | 10 - 20 |
| MVS3 | 20 – 30 |
| MV1 | 0 - 10 |
| MV3 | 0 - 10 |
| MV4 | 0 - 10 |
| MVT2 | 0 - 10 |
| BI3 | 0 - 10 |
| MVS5 | 0 - 10 |
| MVS6 | 0 - 10 |
| MVT3 | 0 - 10 |
| MVT4 | 0 - 10 |
| BI4 | 0 - 10 |
| BI5 | 0 - 10 |

### 4.3.9 DNA sequencing

Using the 357f primer (Section 4.2.4), partial 16S rRNA sequences of good quality between 370–620 nucleotides were produced for isolates PR1, MVS1, MVS2, MVT5, BI1, MV2, MVS4 and BI3 in the forward direction. Isolate MVT1 failed to produce a 16S rRNA sequence.

Reverse sequences of 457-695 nucleotides were produced using the 907r primer (Section 4.2.4) for isolates PR1, MV2 and MVS4.

### 4.3.10 BLAST search

BLAST search results with highest scoring matches are displayed in Table 4.7. Allocation of isolates at genus level was supported by a large number of high-scoring alignment matches. Only isolates PR1 and MV2 were assigned an identity to species level, due to their significance in identifying with the targeted genus and by meeting the criteria described in Section 4.2.11.

**Table 4.7. Significant alignments to Antarctic isolates produced by BLAST search.**

| Isolate | Matched identity | Accession number | Percentage similarity |
|---|---|---|---|
| PR1 | *Bacillus psychrophilus* | BP16SRRB | 98% |
| MVS1 | *Pseudomonas sp.* | AF501361.1 | 99% |
| MVS2 | *Psychrobacter sp.* | AJ297439 | 98% |
| MVT5 | *Arthrobacter sp.* | AF235091 | 98% |
| BI1 | *Pseudomonas sp.* | AF411854 | 98% |
| MV2 | *Bacillus subtilis* | AY030331.1 | 99% |
| MVS4 | *Planococcus sp.* | AF041791 | 97% |
| BI3 | *Carnobacterium sp.* | AF450136 | 98% |

Isolates PR1, MV2 and MVS4 were added to the TRUCC as freeze-dried cultures TG863, TG864 and TG862, respectively.

### 4.3.11 Phylogenetic analysis

A phylogenetic tree (Figure 4.8) showed the relationships between the three most closely related isolates, PRI (isolated from a penguin rookery), MV2 (isolated from Miers Valley) and MVS4 (isolated from soil near a desiccated seal in Miers Valley).

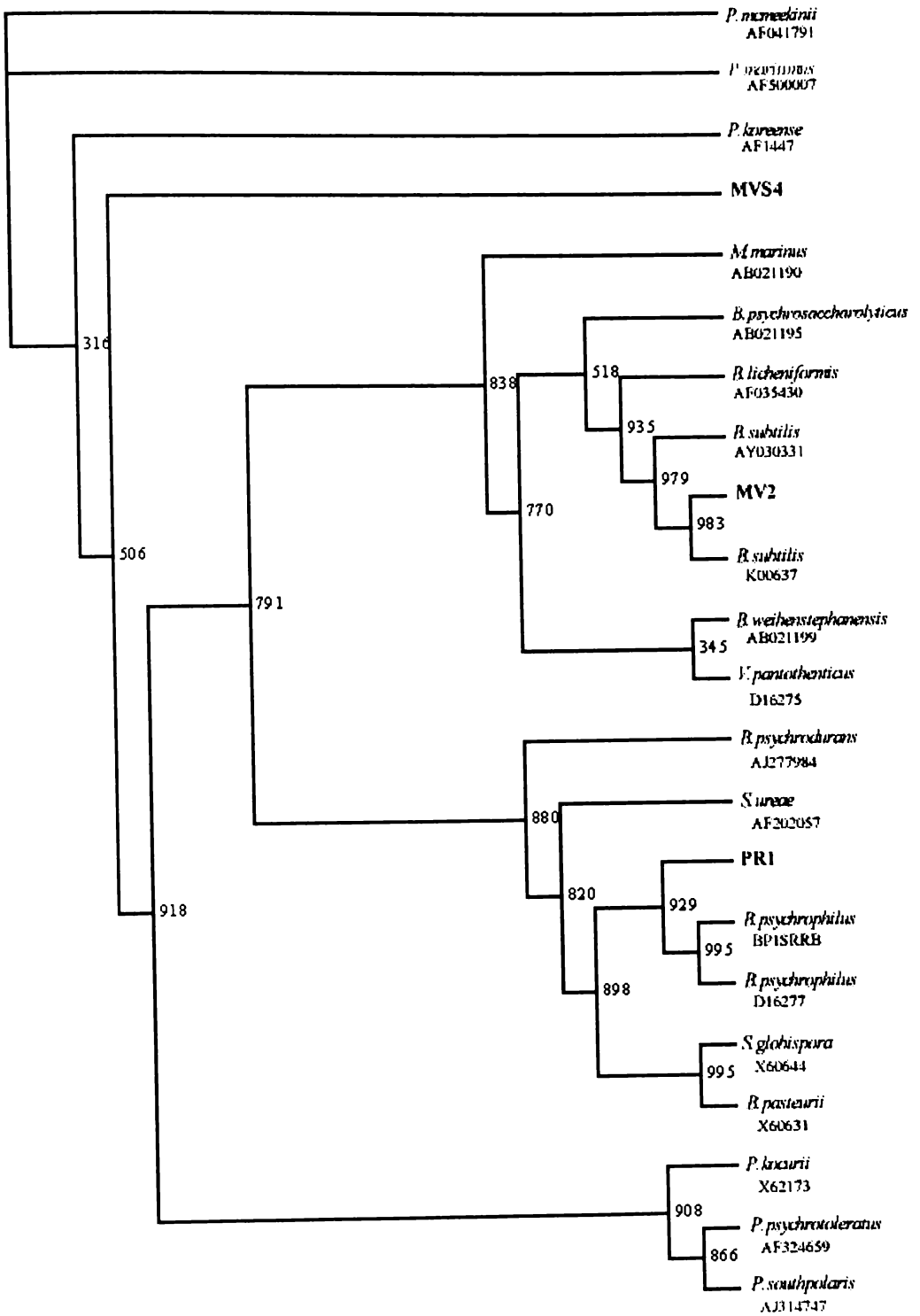



**Figure 4.8. Phylogenetic relationships between Antarctic isolates PR1, MV2 and MVS4 and other species from the *Bacillus* and *Planococcus* genera**

## 4.4 Discussion

### 4.4.1 Growth of bacterial isolates

Bacteria were isolated from all soil samples apart from those taken in the Upper Wright Valley. Only one of the Upper Wright Valley samples produced any isolates, interestingly, this was a sample consisting of salty soil next to a rock. Soils with high concentrations of salt are normally unfavourable for the growth of microorganisms, as it causes osmotic stress (Cameron *et al.*, 1968).

Previous studies carried out in the lower Wright Valley (Boyd *et al.*, 1966) found that around half (22/40) of the soil samples collected across four sites to be sterile. These sites had a relatively low moisture content, with an average of 0.8%. However, the authors discovered that other sites within the area in close proximity to Lake Vanda or glacial meltwater supported high numbers of bacteria. The average moisture content of these soils was 7.6%, and most of them were covered with large masses of the blue-green algae *Nostoc commune*, which has been shown to fix nitrogen under laboratory conditions (Boyd *et al.*, 1966).

A recent paper (Reddy *et al.*, 2000) describes the isolation of *Arthrobacter flavus*, a psychrotolerant bacterium from a pond in the Labyrinth, Upper Wright Valley, close to where my samples were collected. This confirms findings by Boyd *et al.* (1966) and suggests that bacteria are likely to be found in moist areas in the Upper Wright Valley. In the current study, however, soil collected next to a meltwater stream failed to produce any isolates.

From the soil samples that produced a high number of bacterial isolates in the study by Boyd *et al.* (1966), twice as many isolates grew at 22°C compared to 2°C. One third of the Antarctic soil samples analysed by Straka and Stokes (1960) had much higher bacterial counts at 30°C compared to 0°C, indicating that in these samples most of the bacteria are psychrotolerant rather than psychrophilic.

In the current study, a comparison of the number of isolates grown at 15°C to 35°C shows a much higher number grew at the lower temperature. This probably means that many of the isolates were psychrotolerant bacteria, with a maximum growth temperature of less than 35°C. They are also less likely to be human contaminants, as these should grow at 37°C.

### 4.4.2 Initial characterisation

Half of the isolates cultured in this study were found to be Gram positive rods, one quarter were Gram negative rods, while the remainder were cocci or mixed cultures of rods and cocci. This contrasts with Johnson *et al.* (1978) who did not find Gram negative bacteria in any soils, including those from the Dry Valleys and several coastal locations around the Ross Dependency, apart from those taken at McMurdo Station. However, the majority (28/30) of the isolates sampled by Straka and Stokes (1960) were Gram negative rods and cocci. Unfortunately, the location of the sampling sites was not reported, it is assumed to be in the Ross Dependency region as samples included soil, animal faeces and debris and were collected by the American Navy. Madden *et al.* (1979) found Gram positive, rod-shaped *Corynebacteria* to be the dominant bacteria in the Dry Valleys. Their conclusion is in accordance with the results of my preliminary characterisation.

Non-pigmented bacteria have been found to be more abundant than pigmented in Dry Valley soils (Cameron *et al.*, 1968). In the current research, non-pigmented bacteria were also more abundant (19/33 isolates) than pigmented for Dry Valley soil samples. The incidence of pigmented bacteria may reflect adaptation towards high light intensity that is often found in extreme environments. Pigments help to shield cellular components from light and reduce the generation of damaging oxidants (Whitelam and Codd, 1986).

Three isolates were grown from a sample of soil in the Adelie penguin rookery at Cape Crozier. Soils from penguin rookeries contain guano and its breakdown

products, providing nitrogen and phosphorus to nutrient deficient areas (Baross and Morita, 1978). Bacterial populations within these soils are not limited to microorganisms originating from the penguin gastrointestinal tract, but can contain normal soil bacteria due to succession over time (Boyd *et al.*,1966). Therefore, it is possible that a psychrotolerant soil bacterium could be isolated from this soil, as opposed to a faecal contaminant that would be unlikely to be adapted to cold temperatures.

Other possible sources of nutrients in Dry Valley soils are mummified seal carcasses, such as those found in Miers Valley. Sampling of surface and 5-10cm depth soils in a grid formation around the seal carcass was carried out by C. Cary, and will be analysed by his laboratory to assess the impact of local C and N sources on microbial diversity and biomass. The current study found a similar number of isolates grew from soil samples taken around the seal carcasses compared to other soil samples. One of these isolates was identified (according to the criteria in Section 4.2.11) as a *Planococcus* species, being highly similar to *Planococcus mcmeekinii*, which was first isolated from Antarctic sea ice. A previous study in the Wright Valley (Boyd *et al.*, 1966) showed that although many soil samples were sterile, those taken next to carcasses had relatively high levels of bacteria present. The authors suggested that these bacteria originated from the seal, although it is also possible that the increased levels of carbon, nitrogen and phosphorus in the soil around the seal allowed an indigenous population of Antarctic bacteria to exist at that site.

### 4.4.3 Denaturing gradient gel electrophoresis

An assessment of the DGGE gel in Section 4.3.4.1 indicated that several isolates were identical, or highly similar, and could therefore be joined to form two larger groups. To confirm this, a summary gel was run with representatives of each group (Section 4.3.4.2).

All the isolates that did not fit within a group in the first DGGE gel still had unique patterns in the summary gel (apart from MV5, which did not produce a banding

pattern). Members of groups C and D appeared to be the same, as their DNA banding patterns were the same on the gel. The isolates chosen from group A produced different banding patterns on this gel, indicating they should not have been grouped together. Neither of the isolates from group A matched the patterns of isolates from group B. The representatives from group E also appeared to be slightly different in this gel. However, one matched up with a group A isolate (PR1 and MV1), while the other matched group B (MVS2 and BI3).

In the first DGGE analysis (Section 4.3.4.1), isolate MV2, loaded into lane 16, appeared to have run right to the end of the gel. If the gel had been run for longer, this DNA fragment might have moved off the gel, indicating that the level of denaturant used was not sufficient to completely separate the DNA strands, i.e. this isolate had a higher GC content than the other isolates. Using a steeper gradient of denaturant concentration would cause the fragment to denature and stop before it got too close to the end of the gel.

Isolate MVT1 did not produce a banding pattern. Unlike the other samples, this isolate was observed to form a diffuse pellet of cells during genomic DNA extraction that was hard to break up. Possibly the cells did not lyse, causing only a very small amount of DNA to be extracted or the quality of the DNA was bad resulting in inhibition of the PCR.

An important feature of the DGGE banding patterns in Figures 4.2 and 4.3 is the presence of multiple 16S rRNA bands for some isolates, for example PR1, BI1 and MV2. rRNA sequences are generally believed to show low variability within species, which is one of the reasons they are used as molecular chronometers (Woese, 1987). The phenomena of intraspecific polymorphisms in 16S rRNA genes has been observed across many bacterial taxa, with some being more variable than others (Clayton *et al.*, 1995). For example, *Paenibacillus turicensis* has seven different 16S rRNA genes, while other bacteria can have up to 15 (Bosshard *et al.*, 2002).

The presence of multiple 16S rRNA bands in this DGGE gel caused no problems, as the purpose of this analysis was to detect similarities and differences between the isolates. However, the presence of heteroduplex 16S rRNA molecules in a DGGE gel assessing community diversity, which is the main use of DGGE analyses, would cause problems. This is because the DGGE banding pattern provides a profile of the microbial community and in theory, each band is representative of a single member (Muyzer *et al.*, 1993). The generation of multiple rRNA bands from a single member would give a profile misrepresentative of the whole community.

The implications of multiple 16S rRNA genes with regards to phylogenetic analysis is also important, and will be discussed in a later section.

### 4.4.4 Random amplified polymorphic DNA analysis

RAPD analysis showed that members of groups closely related, as determined by 16S rRNA similarities, still had genome-wide differences. Previous studies (Ronimus *et al.*, 1997, Zhang *et al.*, 2002) have used RAPD analysis to differentiate between thermophilic and mesophilic strains of *Bacilli*. The technique screens between closely related microorganisms with a higher resolution than 16S rRNA gene comparisons as random, genome-wide differences are tested (Zhang *et al.*, 2002). Although the RAPD analysis performed in this study was not optimised, it showed that genome-wide differences existed between isolates that were shown to be the same, or related, by DGGE analysis.

The RAPD profile obtained could possibly be improved by decreasing the concentration of DNA template, changing the primer sequence to get a greater number of bands for each isolate (as the primer used was optimised for use with *Bacillus* strains, and the isolates analysed were unlikely to all come from this genus). Furthermore, changing the primer concentration has been shown to alter the banding profiles (Ronimus *et al.*, 1997). To get a clearly negative control the PCR reagents could be UV treated to remove any contaminating DNA, or filtered pipette tips could be used.

### 4.4.5 BLAST search

Of the eight isolates sequenced, two were identified as *Bacillus* species using the criteria defined in Section 4.2.11, while another was identified as being a closely related *Planococcus* species.

- Isolate PR1 from the Adelie penguin colony, Cape Crozier, was identified as most likely being *Bacillus psychrophilus*.
- Isolate MV2 from a gravel slope in Miers Valley was identified as most likely being *Bacillus subtilis* with a high level of confidence, as 20 of the top 25 matches were for *B. subtilis*.
- Isolate MVS4 from soil surrounding a desiccated seal carcass in Miers Valley was identified as most likely being a *Planococcus* species, most closely resembling *Planococcus mcmeekinii*, which was originally isolated from Antarctic sea ice.

Of the remaining isolates:

- Two were found to be *Pseudomonas* species, which are Gram negative rods. The characterisation of these isolates as Gram positive may have been due to the age of the colony analysed, as some bacteria are known to be Gram variable at certain stages of growth. Isolate BI1, was most similar to *Pseudomonas stutzeri*, an animal pathogen often isolated from environmental and human sources (Brock and Madigan, 1991).
- An *Arthrobacter* species was isolated from Miers Valley. *Arthrobacter* species are among the most common of the soil bacteria, being one of the main genera of Coryneform bacteria, and are particularly resistant to desiccation and starvation (Brock and Madigan, 1991).
- A *Carnobacterium* species (related to the *Lactobacillus* genus) was isolated from Bratina Island

- A *Psychrobacter*, most species of which are psychrotolerant with the ability to grow at 5°C (Holt *et al.*, 1994) was cultured from soil surrounding a desiccated seal in Miers Valley

Overall, only two *Bacillus* species were isolated out of 52 cultures. This matches the results of Johnson *et al.* (1978), who found that there were relatively few *Bacilli* in soil but a surprisingly large concentration of *Bacilli* in air samples taken in the Dry Valleys. Low numbers of *Bacillus* species in Dry Valley soils has been suggested to be due to an inability to germinate from spores (Baross and Morita 1978). In contrast, Cameron *et al.* (1968) listed *Bacillus* species as some of the dominant bacterial species isolated from the Antarctic Dry Valleys. These inconsistencies are probably due to the use of different culturing techniques and sampling sites, and are likely to be clarified with the information gained using a molecular approach. Studies of microbial communities using 16S rRNA sequencing will remove the biases associated with classical culturing techniques i.e. a lack of knowledge of nutritional requirements resulting in most bacteria being unculturable.

Cameron *et al.* (1972) found that many of the *Bacillus* species they isolated had optimum growth temperatures of 37-45°C, suggesting they could be human contaminants. The optimum growth temperature of the *Bacillus* isolates from the current study will be examined in the next chapter.

### 4.4.6 Phylogenetic analysis

The purpose of creating a phylogenetic tree was to determine the relationship between the three isolates that were going to be most useful, i.e. the two *Bacilli* and the *Planococcus* species. From the phylogenetic tree the identification of PR1 as *B. psychrophilus* was confirmed, as well as the identification of MV2 as *B. subtilis*. It can be seen that MVS4, identified as a *Planococcus* species, is more distantly related to the other two isolates. Its placement in the phylogenetic tree, along with many of the other *Planococcus* species, is not so well defined.

As discussed previously (Section 4.4.3), PR1 and MV2 were both observed to have multiple 16S rRNA bands on a DGGE gel. Clayton *et al.* (1995) found that the presence of multiple 16S rRNA genes can have a significant effect on phylogenetic analyses of some bacterial taxa, while others are less affected depending on the extent of the variability. They suggest that to perform an accurate phylogenetic analysis, the sequencing of each of the multiple 16S rRNA genes is necessary. In the current study, however, the primary aim of the 16S rRNA sequencing of these isolates was to confirm their genus, identification to species level was of lesser importance therefore the presence of multiple genes was not considered to be a limitation in this study.

## 4.5 Conclusion

Two *Bacillus* isolates, identified as most likely being *B. psychrophilus* and *B. subtilis*, and a closely related *Planococcus* isolate were cultured from soils exposed to large thermal fluctuations over their history. Data (from three laboratories) relating to the general ecology of the soils sampled has not been fully processed and integrated at this time.

# Chapter 5 Characterisation of Dihydrofolate Reductase

## 5.1 Introduction

The two *Bacilli* and one *Planococcus* were further characterised with regards to their optimum growth temperature. Isolate PR1, identified as *Bacillus psychrophilus* in the previous chapter, was chosen for enzyme characterisation due to its good growth compared with isolate MV2 (identified as *Bacillus subtilis*) and because isolate MVS4 (*Planococcus* sp.) was more distantly related to the *Bacillus* genus. The choice to purify and characterise dihydrofolate reductase (DHFR) was due to several factors including (1) that work on this enzyme had already been carried out using thermophilic and mesophilic enzymes, (2) it is an enzyme common to all bacteria and (3) the enzyme is easy to purify with a methotrexate agarose affinity column.
The enzyme was extracted and an initial characterisation of $K_m$ values and temperature optimum was performed. To gain a comparison between psychrotolerant PR1 DHFR and a thermophilic DHFR, a previously purified enzyme from *Bacillus stearothermophilus* was also characterised.

DHFR, E.C. 1.5.1.3, is an enzyme essential for folate metabolism in eukaryotes and prokaryotes (Rajagopalan and Benkovic, 2002). It regulates levels of tetrahydrofolate in cells, necessary for the synthesis of purines, pyrimidines and amino acids. Due to its role in actively dividing cells, much research has been carried out on DHFR inhibitors as a treatment for cancer (Schweitzer *et al.*, 1990). One such inhibitor is methotrexate, an analogue of dihydrofolic acid that can be also be used as an affinity ligand for the purification of DHFR. DHFR catalyses the reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate using nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. Thus an assay for DHFR activity measures the decrease in NADPH concentration.

## 5.2 Materials and Methods

### 5.2.1 Growth in two media

The growth of isolates PR1, MVS4 and MV2 had been poor in nutrient broth, so two other media were tested to try to increase cell mass, Bacillus EA1 Medium and Oshima Neutral Medium (Appendix 1).

Approximately 100mL of media was inoculated with a loopful of culture, either isolate PR1 or MVS4, scraped from an agar plate. This was placed at room temperature in a shaking incubator until visible growth appeared. Culture density was measured by absorbance at 650nm.

### 5.2.2 Optimum growth temperature determination

Bellco tubes containing 10mL of ONM were equilibrated in a temperature gradient incubator, set to generate a temperature range of 5-30°C, for approximately 36 hours. Tubes were inoculated using a sterile syringe with 400μL of cultures PR1 in early log phase, and MV2 and MVS4 in late log phase.

Growth was monitored by reading culture absorbance at 650nm using a PYE Unicam SP6-450 UV/Vis spectrophotometer, which the Bellco tubes could slot into. Measurements were initially taken at 2-4 hour intervals depending on rate of growth, this was increased to 5-8 hour intervals after 48 hours. Ten temperature points were monitored for each isolate and at each temperature cultures were grown in triplicate.

The change in absorbance per hour during log phase was calculated from the slope of the growth curves. This was plotted against temperature to determine the optimum temperature of growth.

### 5.2.3 Growth of isolate PR1 for enzyme

Cultures of isolate PR1 were grown in ONM overnight, until an absorbance of around 2.0 was reached. A 5mL volume of culture was inoculated into 22 x 2L flasks, each containing 700mL ONM. Flasks were incubated at 21°C rotating 110rpm in a shaking incubator, until an absorbance at 650nm of 1.6 was reached (16h).

The cultures from each flask were pooled into one volume and concentrated using an Amicon Hollow Fibre cartridge (0.1μm cut-off, Millipore Corporation, USA). The bacterial cell suspension was centrifuged at 7695 r.c.f. for 20 minutes using an SLA 3000 rotor. The supernatant was decanted and the cell pellet frozen at –20°C.

### 5.2.4 Assay for enzyme production

All assays were performed using a Pharmacia Biotech Ultrospec 3000 UV/Visible spectrophotometer.

#### 5.2.4.1 DHFR assay

Lysed cultures of PR1 and MVS4 were assayed for DHFR activity. The culture supernatant of PR1 was also assayed.

| | | |
|---|---|---|
| Assay contained: | 0.1M Na phosphate buffer, pH 6.0 | 600μL |
| | 50mM DTT | 18μL |
| | 4mM DHFA | 9μL |
| | 8mM NADPH | 12μL |
| | Enzyme solution | 10-200μL |

Continuous assays were run for around 1 minute. Absorbance was measured at 340nm.

### 5.2.4.2 *p*-NP assay

Enzyme activity against the following *p*-NP substrates was also measured for PR1:

*p*-NP α-L-arabinopyranoside
*p*-NP β-D-xylopyranoside
*p*-NP β-D-fucopyranoside
*p*-NP β-D-maltoside
*p*-NP β-D-galactopyranoside
*p*-NP α-D-glucopyranoside
*p*-NP β-D-glucopyranoside

| | | |
|---|---|---|
| Assay contained: | 1mg/mL substrate in 100mM MOPS, pH 7.0 | 100μL |
| | Enzyme solution (whole culture) | 50μL |

Assay was incubated for 20 minutes at room temperature or 37°C, placed on ice and 100μL 1M $Na_2CO_3$ added to terminate the reaction. Cells were centrifuged at 16100 r.c.f. for 5 minutes, and absorbance read at 420nm. Enzyme was not added to the negative controls until after addition of the $Na_2CO_3$.

### 5.2.4.3 α-amylase and phosphatase assay

Assays were carried out for α-amylase and alkaline or acid phosphatase at 37°C using whole and lysed cultures.

| | | |
|---|---|---|
| Assay contained: | 1mg/mL *p*-NP phosphate in 100mM MOPS, | |
| | or ½ dilution of α-amylase reagent* | 100μL |
| | Enzyme solution | 50μL |

*(Diagnostic Chemicals Ltd, Canada, Cat. No. 321-15)

Reactions were incubated at 37°C for 20 minutes, then placed on ice. Assays using *p*-NP phosphate were terminated with 100μL 1M $Na_2CO_3$. Reactions containing whole cultures were spun for 2 minutes at 16100 r.c.f. then held on ice. Absorbance was read at 405nm for α-amylase activity or 420nm for phosphatase activity.

#### 5.2.4.4 Maltase assay

Assays were carried out for *p*-NP α-D-maltoside using cultures of PR1 that had been grown in the presence of 0.2% maltose.

| | | |
|---|---|---|
| Assay: | 1mg/mL *p*-NP α-D-maltoside in 100mM MOPS, pH 6.3 | 100μL |
| | Enzyme solution | 10μL |

Reactions were incubated at room temperature and 40°C for 10 minutes. Assay was stopped by the addition of 100μL 1M $Na_2CO_3$. The cells were spun down for 2 minutes at 16100 r.c.f. then absorbance read at 420nm. Enzyme extract from E34.A1 was used as a positive control.

### 5.2.5 Preparation of affinity column

Chicken liver extract was prepared by blending 123g chicken livers with 250mL lysis buffer (50mM Tris-HCl pH 8.0, 7.5mM β-mercaptoethanol, 7.5mM EDTA, 200mM PMSF) in a Waring blender until most of the tissue was broken up. The resulting mixture was spun for 20 minutes at 13873 r.c.f. using the SLA 1500 rotor. The supernatant was drawn off the pellet using a 5mL pipette, then filtered through a coarse and fine glass membrane to remove the fat. A small sample was removed and assayed for DHFR activity, using the following assay:

| | |
|---|---|
| 0.1M Na phosphate buffer, pH 6.0 | 100μL |
| 50mM DTT | 18μL |
| 4mM DHFA | 9μL |
| 8mM NADPH | 12μL |
| Enzyme solution | 10-20mL |

Approximately 200mL chicken liver extract was diluted with 800mL lysis buffer and 1 L distilled water to make a volume of 2L which was loaded onto the methotrexate column (Product no. M0269, Sigma) overnight at around 1mL/minute. A total volume of 800mL chicken liver extract was loaded onto the column, this was then washed with equilibration buffer (10mM $KP_i$, pH 8.0, 0.1mM EDTA, 1mM β-mercaptoethanol) until the red colour had disappeared. The column was further washed with 20mL equilibration buffer-KCl (10mM $KP_i$, pH 8.0, 0.1mM EDTA, 1mM β-mercaptoethanol, 0.5M KCl), 10mL elution buffer (10mM $KP_i$, pH 9.0, 0.1mM EDTA, 1 M KCl, 3mM folate, 1mM β-mercaptoethanol) then 10mL equilibration buffer again.

To check that the column was properly conditioned, *Escherichia coli* DHFR that had been previously purified was reconstituted from a freeze-dried powder to a concentration of 1mg/mL in distilled water. This was assayed for activity then 5mL of the enzyme solution was loaded onto the methotrexate column. Washes with 10mL equilibration buffer, followed by 10mL equilibration buffer-KCl were performed, then the enzyme was eluted in 10mL fractions using elution buffer. These fractions were assayed for DHFR activity.

For short term storage, equilibration buffer-KCl was pumped through the column to prevent microbial contamination. This was washed out with equilibration buffer immediately before an enzyme solution was loaded onto the column.

### 5.2.6 Extraction of DHFR from isolate PR1 cells

Bacterial cells were resuspended in an approximately equal volume of lysis buffer (50mM Tris-HCl pH 8.0, 7.5mM β-mercaptoethanol, 7.5mM EDTA), ie 45g cells = 45mL lysis buffer. Lysozyme, enough to make a 0.05% in resuspended cells, was dissolved in a small amount of buffer, added to cells and left to act for 60 minutes at room temperature. At the end of the incubation period, 0.02% DNase and 30mM $MgCl_2$ were added to the lysed suspension.

The cell suspension was sonicated on ice for four cycles of five minutes sonication, followed by five minutes cooling. Phenylmethane-sulphonyl fluoride (PMSF) from a 50mM stock was added after the first and third cycle of sonication to a final concentration of 200μM in the cell suspension. The progress of sonication was determined by looking at a small drop of cell suspension diluted with water under the microscope. Lysed cells had a patchy internal appearance or had lost their inner contents with only a transparent membrane remaining.

The lysed solution was centifuged for 30min at 11235 r.c.f. using the SS-34 rotor. The supernatant was carefully removed to be loaded onto the methotrexate affinity column, and the pellet frozen at –20°C.

### 5.2.7 Purification of DHFR using affinity column

A small volume (300μL) of supernatant was stored on ice for assaying later, while the remainder was diluted with an equal volume of equilibration buffer (to give 40mL), then loaded onto the methotrexate affinity column, which had previously been equilibrated with the same buffer.

The column was washed with 10mL equilibration buffer, followed by 10mL equilibration buffer-KCl. Elution buffer was then used to elute fractions of

approximately 10mL. Ten fractions were collected altogether and assayed for DHFR activity (Section 5.2.5).

As activity was difficult to detect, all fractions were pooled and ultrafiltrated using an Amicon Ultrafiltration cell with a YM3 membrane. As the volume of enzyme solution reduced, it was topped up with Milli-Q water to dilute the folate. This was repeated a couple of times, then the enzyme solution was reduced to a final volume of 4mL, giving a concentration of 19 times the original value. DHFR activity was assayed again, varying the buffer solution (100mM Na-phosphate, pH 6.0; 100mM K-phosphate, pH 6.0 or 6.5) to optimise measured activity.

### 5.2.8 Substrate requirements for isolate PR1 DHFR

In preparation for the temperature optima assays, determinations were performed to confirm that the concentration of substrate and cofactor used would not be rate-limiting. Assays using DHFA were performed at a low (14°C) and higher (30°C) temperature, to check that $K_m$ for the substrate would not vary greatly over the temperature range of the temperature optima assays, as described by Daniel and Danson (2001).

Approximate $K_m$ values were derived from a direct linear plot (Appendix 4) due to the reasons described by Henderson (1993). Results were also graphed as Michaelis-Menten and Lineweaver-Burke plots to confirm that experimental conditions were satisfactory. Assays were performed using a Thermospectronic™ Helios γ-spectrophotometer equipped with a Thermospectronic™ single cell peltier-effect cuvette holder.

Due to the limited amount of PR1 enzyme available, $K_m$ determinations could only be approximated.

### 5.2.8.1 Approximate $K_m$ for DHFA

(a) A 1mL assay was carried out at 20°C, with volume of buffer and 0.1mM DHFA altered to give the correct final concentration of DHFA:

| | | |
|---|---|---|
| Assay: | 0.1M Na-phosphate buffer, 25mM DTT, pH 6.0 | $967 - x$ µL |
| | 8mM NADPH | 18µL |
| | 0.1mM DHFA | $x$ µL |
| | Enzyme solution | 15µL |

The following concentrations of DHFA were assayed: 0.0008, 0.0009, 0.001, 0.002, 0.004, 0.005, 0.01, 0.02, 0.03 and 0.05mM.

(b) A 1mL assay was carried out at 14°C and 30°C, with volume of buffer and 0.1mM DHFA adjusted as previously:

| | | |
|---|---|---|
| Assay: | 0.1M Na-phosphate buffer, 25mM DTT, pH 6.0 | $972 - x$ µL |
| | 8mM NADPH | 18µL |
| | 0.1mM DHFA | $x$ µL |
| | Enzyme solution | 10µL |

The following concentrations of DHFA were assayed: 0.0008, 0.001, 0.002, 0.005, 0.02mM.

Note: There are too few values to obtain an accurate $K_m$, but sufficient to check for substrate limitation.

### 5.2.8.2 Approximate $K_m$ for NADPH

A 1mL assay was performed at 20°C, with volume of buffer and 8mM NADPH altered to provide the correct final concentration of NADPH:

| | | |
|---|---|---|
| Assay: | 0.1mM Na-phosphate buffer, 25mM DTT, pH 6.0 | 977- x μL |
| | 4mM DHFA | 13μL |
| | 8mM NADPH | x μL |
| | enzyme solution | 10μL |

The concentrations of NADPH used were: 0.025, 0.05, 0.15, 0.2, 0.4mM.
Note: There are too few values to obtain an accurate $K_m$, but sufficient to check for substrate limitation.

### 5.2.9 Temperature optimum of PR1 DHFR activity

#### 5.2.9.1 Temperature optima assay method

Enzyme activity was measured using continuous assays on a Thermospectronic™ Helios γ-spectrophotometer equipped with a Thermospectronic™ single cell peltier-effect cuvette holder. This system was networked to a computer installed with Vision32™ (Version 1.25, Unicam Ltd) software. The concentration of NADPH was maintained at 50 times $K_m$ to minimise the effects of any possible increases in $K_m$ with temperature, while the concentration of DHFA was approximately 40 times $K_m$.

To give a total reaction absorbance of around 1 at 340nm, the assay mixture was composed of:

| | |
|---|---|
| 100mM Na phosphate buffer, pH 7.0 | 943μL |
| 2mM DHFA (0.04mM final conc.) | 20μL |
| 8mM NADPH (0.056mM final conc.) | 7μL |
| Enzyme solution | 30μL |

The reaction was carried out every 3°C between a temperature range of 15°C to 59°C. At temperatures above 27°C, a denaturation curve for NADPH was also measured in

duplicate by monitoring change in absorbance of the reaction mixture without the addition of the enzyme.

### 5.2.9.2 Data collection

Three assay progress curves were collected at each temperature using Vision32™ (Version 1.25, Unicam Ltd) software. Absorbance was measured every second over a three minute period, and reactions were repeated if they deviated significantly from the other replicate curves obtained. Temperature was measured inside the cuvette immediately before and after the reaction, using a Cole-Parmer Digi-Sense® thermocouple thermometer accurate to ± 0.1% of the reading and calibrated using a Cole-Parmer NIST-traceable high-resolution glass thermometer. The reaction was repeated if the temperature was found to change by more than 0.2°C over the assay period (at higher temperatures, this parameter was increased to 0.9°C).

### 5.2.9.3 Data analysis

At each temperature, catalytic rate ($\mu M.s^{-1}$) was calculated at 1s time intervals for the three progress curves. The averages of each time-point value were used to generate 3D plots of rate (v) versus temperature (T in degrees Celcius) versus time (t in seconds) [SigmaPlot® 2001 for Windows, Version 7.101, SPSS Inc.]. Data were smoothed using a Loess transformation. As the first data point was recorded after a delay of approximately 2 seconds, 'zero time' data were gained from the smoothing process, which extrapolates back to zero using the trend derived from the data.

### 5.2.9.4 Other thermal parameters of isolate PR1 DHFR

The values of $\Delta G^{\ddagger}_{cat}$ (the activation energy of the catalytic reaction), $\Delta H_{eq}$ (the enthalpy change for the transition between active and inactive forms of the enzyme) and $T_{eq}$ (the temperature for the mid-point of this transition) were calculated from the data. Firstly, for the data at t = 0 (where there is no thermal inactivation), Eyring

plots of ln(v/T) versus 1/T gave values of $\Delta H^{\ddagger}_{cat}$ and $\Delta S^{\ddagger}_{cat}$ (from the gradient and intercept, respectively), from which $\Delta G^{\ddagger}_{cat}$ could be calculated at any temperature of assay. Values of $K_{eq}$ were calculated from an Arrhenius plot [ln(v) versus 1/T] for the data at time zero by comparison of the observed values with those from the extrapolated linear portion. Finally, using the equation below, values of $\Delta H_{eq}$ and $T_{eq}$ were determined from a plot of $\ln(K_{eq})$ versus 1/T.

$$\ln (K_{eq}) = \Delta H_{eq} / R * [1/ T_{eq} - 1/ T ]$$

### 5.2.10 Substrate requirement for *B. stearothermophilus* DHFR

Approximate $K_m$ values were determined using the methods described in Section 5.2.8.

#### 5.2.10.1 $K_m$ for DHFA at 30°C

At a temperature of 30°C, the $K_m$ value for DHFA was determined using a 2mL assay volume, with the volume of 1mM DHFA stock solution altered to provide the correct concentration:

| | | |
|---|---|---|
| Assay: | 100mM Na phosphate buffer, pH 6.5 | 1916µL |
| | 1 mM DHFA | 40µL |
| | 8mM NADPH | 14µL |
| | 0.1mg/mL freeze dried *B. stearothermophilus* DHFR | 20µL |

Concentrations used were: 0.001, 0.002, 0.003, 0.004, 0.005, 0.006, 0.008, 0.01, 0.02 and 0.04mM DHFA.

### 5.2.10.2 $K_m$ for DHFA at 68°C

At the higher temperature, a 1mL assay volume was used as this took a shorter time to equilibrate to the correct temperature. Buffer pH was also increased to 7.0 to reduce the amount of degradation of the substrates. A stock solution of 1mM DHFA was used.

| | | |
|---|---|---|
| Assay: | 100mM Na phosphate, pH 7.0 | 953µL |
| | 1mM DHFA | 20µL |
| | 8mM NADPH | 7µL |
| | 0.5mg/mL freeze dried *B. stearothermophilus* DHFR | 20µL |

Activity was measured at the following concentrations: 0.002, 0.003, 0.004, 0.005, 0.006, 0.008, 0.01 and 0.02mM DHFA.

### 5.2.10.3 $K_m$ for NADPH at 30°C

A 2mL assay was used, with 1mM and 10mM stock solutions of NADPH:

| | | |
|---|---|---|
| Assay: | 100mM Na phosphate buffer, pH 7.0 | 1916µL |
| | 1mM DHFA | 40µL |
| | 1mM NADPH | 14µL |
| | 0.5mg/mL freeze-dried *B. stearothermophilus* DHFR | 20µL |

Concentrations of NADPH assayed were: 0.001, 0.002, 0.005, 0.008, 0.01, 0.05, 0.1, 0.2 and 0.3mM.

### 5.2.10.4 $K_m$ for NADPH at 68°C

A 1mL assay was used, with 1mM and 10mM stock solutions of NADPH:

| | | |
|---|---|---|
| Assay: | 100mM Na phosphate buffer, pH 7.0 | 946µL |
| | 2mM DHFA | 20µL |
| | 8mM NADPH | 14µL |
| | 0.5mg/mL freeze-dried *B. stearothermophilus* DHFR | 20µL |

The concentrations of NADPH assayed were the same as those used at 30°C.

## 5.2.11 Temperature optimum for *B. stearothermophilus* DHFR

### 5.2.11.1 Temperature optima assay method

Activity was measured by monitoring decrease in absorbance at 340nm using the following assay:

| | | |
|---|---|---|
| Assay: | 100mM Na phosphate buffer, pH 7.0 | 930µL |
| | 2mM DHFA (0.08mM final conc.) | 40µL |
| | 8mM NADPH (0.08mM final conc.) | 10µL |
| | 0.02mg/mL *B. stearothermophilus* DHFR | 20µL |

The reaction was carried out in triplicate every 5°C between the temperatures of 25°C and 80°C. At temperatures of 35°C and above, a denaturation curve for NADPH was also carried out as in the previous section.

Data was collected and analysed as described in Sections 4.2.9.1 - 4.2.9.3. The concentration of NADPH was maintained at approximately six times $K_m$ to minimise the effects of any possible increases in $K_m$ with temperature, while the concentration of DHFA was between 4 and 30 times $K_m$ (depending on the $K_m$ value used).

### 5.2.11.2 Other thermal parameters of *B. stearothermophilus* DHFR

These were calculated as described in Section 4.2.9.4. Arrhenius activation energy ($E_A$) was also derived from the slope of ln(v) plotted against 1/T.

## 5.3 Results

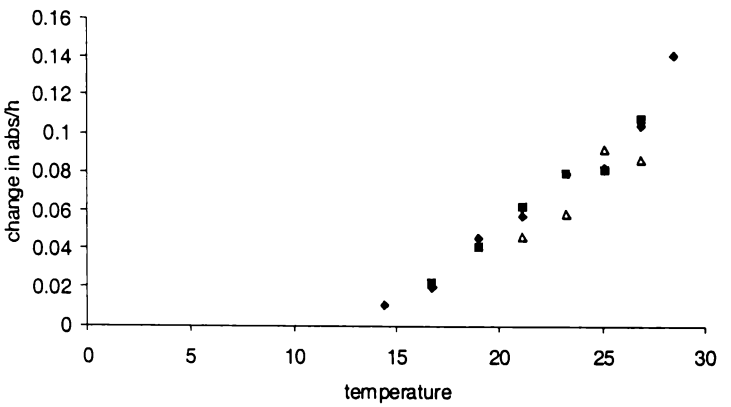
### 5.3.1 Growth in two media

Isolate PR1 had not grown after six days in the *Bacillus* medium, however, it had reached an optical density of 1.70 after 3 days growth in ONM.
In comparison, isolate MVS4 grew to an optical density of 1.0 after 6 days in *Bacillus* medium, and had grown to 0.35 absorbance units after 3 days in ONM.

### 5.3.2 Optimum growth temperature determination

PR1 grew between the temperature of 13°C to the highest temperature, 28°C, while MV2 grew between 14°C and 29°C. Growth of MVS4 was poor, limited growth occurred between 17°C and 27°C. Because of the inadequate data obtained for MVS4, growth results were not analysed further. A plot of log phase growth rate versus temperature for PR1 and MV2 (Figure 4.1) showed that MV2 had probably not reached its optimum growth temperature, while the results for PR1 were less conclusive.

A




B

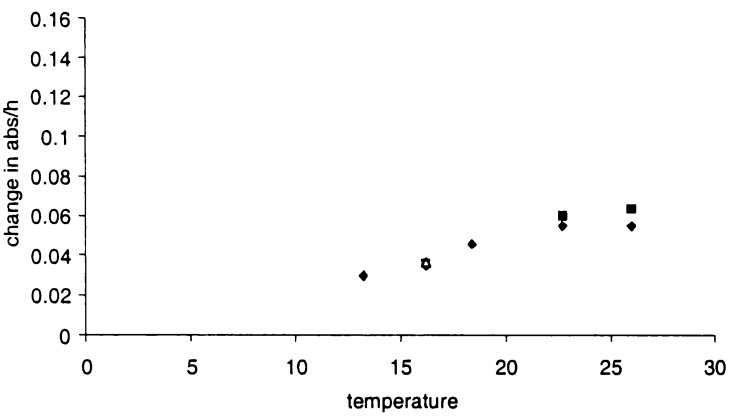



**Figure 5.1. Log phase growth rate versus temperature. (A) MV2 did not reach maximal growth rate within this temperature range; (B) PR1 appears to reach maximal growth rate at 27°C**

### 5.3.3 Growth of isolate PR1 for enzyme

A final mass of 42g PR1 cells was produced in preparation for enzyme extraction. These were frozen at –20°C until required.

### 5.3.4 Assay for enzyme production

Activity was found against certain substrates, as summarised in Table 5.1. No enzyme activity was detected towards the rest of the substrates tested.

**Table 5.1. Enzyme activity produced by PR1 and MV2 cultures; L, lysed culture; S, culture supernatant; W, whole culture**

| Substrate | Enzyme solution | Activity present (μmol/mL/min) |
|---|---|---|
| Dihydrofolic acid | PR1 - L | 0 |
| | PR1 - S | 0.0680 |
| | MVS4 - W | 0 |
| *p*-NP β-D-galactopyranoside | PR1 - W | 0.0022 |
| *p*-NP α-D-glucopyranoside | PR1 - W | 0.0004 |
| α-amylase commercial | PR1 - W | 0 |
| | PR1 - L | 0.0004 |
| *p*-NP-phosphate | PR1 - W | 0.0008 |
| | PR1 - L | 0 |

### 5.3.5 Preparation of affinity column

A 0.2mg/mL solution of *E. coli* DHFR gave an activity of 1.17 μmol/mL/min. Six fractions of the *E. coli* enzyme were collected from the methotrexate column and assayed for activity (Table 5.2).

**Table 5.2. DHFR activity in eluted fractions**

| Fraction | Activity μmol/mL/min |
|---|---|
| 1 | 0.029 |
| 2 | 0.196 |
| 3 | 0.113 |
| 4 | 0.078 |
| 5 | 0.038 |
| 6 | 0.038 |

It appeared that most of the enzyme was eluted from the column, mainly in fractions 2-4.

### 5.3.6 Purification of DHFR using affinity column

DHFR activity in the sample before loading onto the column was measured as around 0.098μmol/mL/min. In the eluted fractions, most activity was found in fraction 4 (see Table 5.3).

**Table 5.3. PR1 DHFR activity in eluted column fractions**

| Fraction | Activity μmol/mL/min |
|---|---|
| 1 | 0.064 |
| 2 | 0.021 |
| 3 | 0 |
| 4 | 0.289 |
| 5 | 0 |
| 6 | 0 |

An assay for DHFR activity in the enzyme solution (after ultrafiltration) using different buffers gave the following results:

| | |
|---|---|
| 100mM Na phosphate, pH 6.0 | 0.095μmol/mL/min |
| 100mM K phosphate, pH 6.0 | 0.050μmol/mL/min |
| 100mM K phosphate, pH 6.5 | 0.062μmol/mL/min |

### 5.3.7 Substrate requirements for isolate PR1 DHFR

Approximate $K_m$ values for PR1 DHFR are presented in Table 5.4. Due to the variability of some results Michaelis-Menten and Lineweaver-Burke plots are included in Appendix 4.

**Table 5.4. Approximate $K_m$ values for PR1 DHFR**

| Substrate | Temperature (°C) | Approximate $K_m$ (mM) |
|---|---|---|
| DHFA | 14 | 0.0004 |
| DHFA | 30 | 0.0011 |
| DHFA | 20 | 0.001 |
| NADPH | 20 | 0.0011 |

Concentration of DHFA and NADPH used in the temperature optima assays were at least ten times the concentrations given in Table 5.4.

### 5.3.8 Temperature optimum of isolate PR1 DHFR

The three-dimensional curves generated for PR1 DHFR are displayed in Figure 5.2. A clear optimum temperature of activity at 44°C was revealed at zero time.

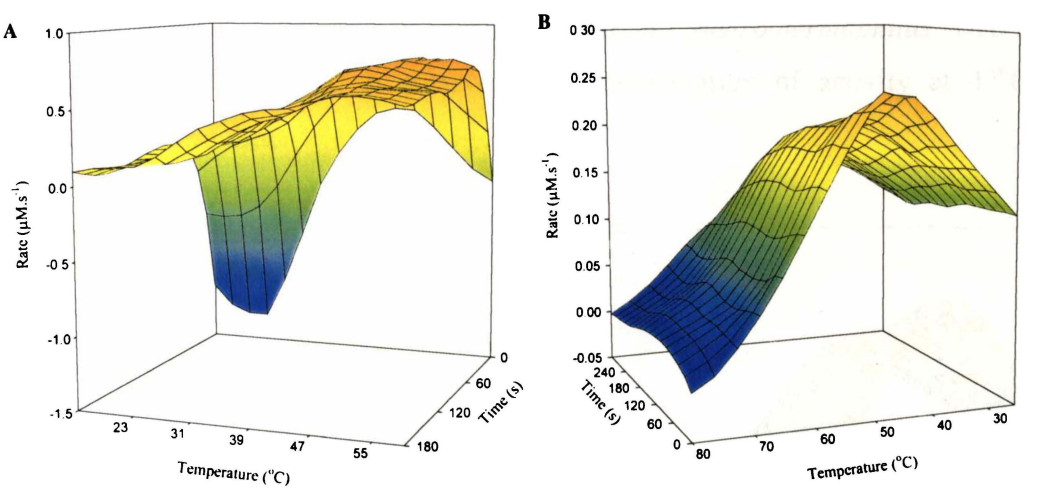



**Figure 5.2. Plots of PR1 DHFR activity versus temperature versus time. (A) 3D plot presented from the standard viewing angle; (B) 3D plot presented to display temperature optimum of activity at zero time**

### 5.3.9 Substrate requirements for *B. stearothermophilus* DHFR

Approximate $K_m$ values for *B. stearothermophilus* DHFR are presented in Table 5.5.

**Table 5.5. Approximate $K_m$ values for *B. stearothermophilus* DHFR**

| Substrate | Temperature (°C) | $K_m$ (mM) |
|---|---|---|
| DHFA | 30 | 0.0026 |
| DHFA | 70 | 0.019 |
| NADPH | 30 | 0.013 |
| NADPH | 70 | 0.015 |

Concentration of DHFA and NADPH used in temperature optima assays for *B. stearothermophilus* DHFR were 5-10 times higher than those given in Table 5.5.

### 5.3.10 Temperature optimum for *B. stearothermophilus* DHFR

The three-dimensional curves generated for *B. stearothermophilus* DHFR are displayed in Figure 5.3. A clear optimum temperature of activity at 47°C was revealed at zero time.

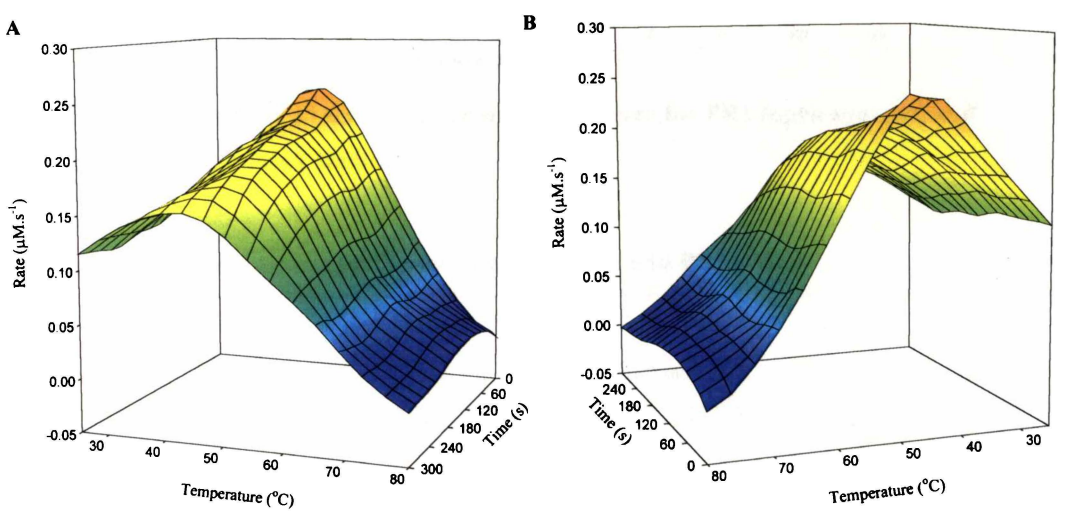



**Figure 5.3. Plots of *B. stearothermophilus* DHFR activity versus temperature versus time. (A) 3D plot presented in the standard viewing angle; (B) 3D plot presented to display temperature optimum of activity at zero time**

### 5.3.11 Other thermal parameters of PR1 and *B. stearothermophilus* DHFRs

DHFR activity versus temperature at zero time was plotted for both enzymes (Figure 5.4). This showed a difference in optimal temperature peak widths between the psychrotolerant and thermophilic enzymes and a slightly higher optimum temperature for the thermophilic enzyme.

Thermal parameters $T_{eq}$, $\Delta G^{\ddagger}_{cat}$, $\Delta H_{eq}$ and $E_A$ are presented for both enzymes in Table 5.6.

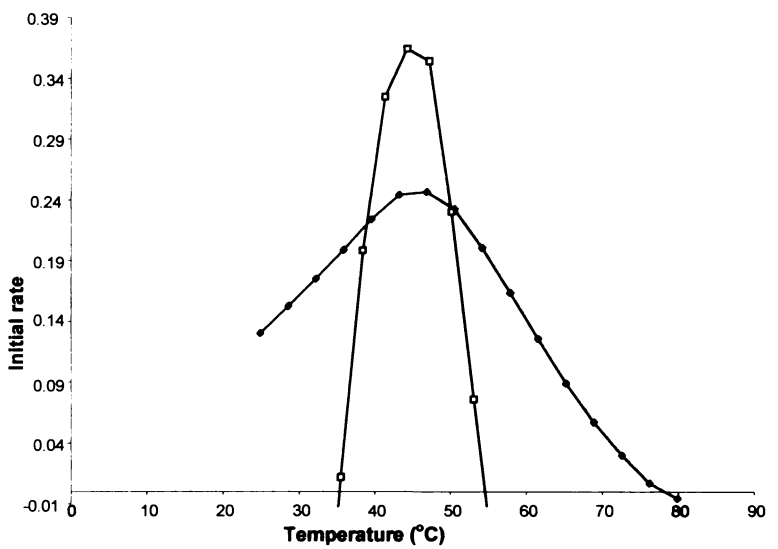



**Figure 5.4. Enzyme activity at zero time versus temperature for PR1 (open squares) and *B. stearothermophilus* (closed diamonds).**

**Table 5.6. Thermal parameters for *B. stearothermophilus* and PR1 DHFR.**

| Organism | Opt. growth temp | $T_{opt}$ (°C) | $T_{eq}$ (°C) | $\Delta G^{\ddagger}_{cat}$ (KJ.mol$^{-1}$.K$^{-1}$) | $\Delta H_{eq}$ (KJ.mol$^{-1}$.K$^{-1}$) | $E_A$ (KJ.mol$^{-1}$.K$^{-1}$) |
|---|---|---|---|---|---|---|
| *B. stearothermophilus* | 65°C | 47 | 59 | 31 | 185 | 72 |
| PR1 | 27°C | 44 | 49 | 83 | 470 | N/D* |

*N/D, not determined.

## 5.4 Discussion

The Antarctic bacteria isolated were slow growing, especially in liquid media. Rate of growth needed to be increased so the cultures could be used for enzyme isolation later on. ONM was determined to be the best for growth of these isolates, due to the increased rate of culture growth and final cell density compared to the *Bacillus* Medium.

Characterisation of the optimum temperature of growth for isolates PR1 and MV2 confirmed that neither isolate was psychrophilic, as both had optimum temperatures higher than 20°C. The temperature range tested did not appear to encompass the optimum growth temperature of isolate MV2, as the growth rate was still increasing at the highest temperature measured (30°C). For isolate PR1, the trend of the curve (Figure 4.1B) suggested that the optimum growth temperature would be not much higher than 25°C. The highest temperature point was not included for analysis as the result was equivocal; two replicates failed to grow while the other was contaminated.

As pointed out by Glansdorff and Xu (2002), the temperature at which maximal growth is observed may not necessarily be the optimal temperature for growth of a particular isolate. For cultures PR1 and MV2, little to no growth was seen at the colder temperature range, however, these authors suggested that slow growth at cold temperatures is likely to be advantageous in nutrient poor environments, such as Antarctic soils, as rapid use of the limited resources would result in starvation. It is also possible that MV2 originated from human sources, especially since its sampling site in the Miers Valley would be easy to reach by field parties.

Feller *et al.* (1994) discovered that although exposure to temperatures higher than normally experienced caused their Antarctic isolates to grow faster, cell development and enzyme secretion was negatively affected in the majority of isolates studied. This was not investigated here.

These studies suggest that assigning the temperature at which maximal growth occurs to be the optimum temperature of growth may not be wise, as other environmental factors should be considered. Therefore, although these results reveal that both Antarctic isolates have a faster rate of growth at higher temperatures, this may not necessarily mean that they are not adapted to growing in the Antarctic environment.

The culture PR1 was tested for production of a range of enzymes, however, only a few showed detectable activity. A small level of activity was found for DHFR in the culture supernatant, suggesting cell lysis had occurred. Many other enzymes use NADPH as a cofactor, and it is also possible that the decrease in absorbance measured was due to the activity of a different enzyme. A relatively good activity was seen against *p*-NP β-D-galactopyranoside, suggesting the secretion of an extracellular β-glucosidase into the culture medium. This is another enzyme that could be worked on in future research.

After conditioning with chicken liver extract, the affinity column appeared to release most *E. coli* DHFR activity. It was important to check that activity was recoverable and that the column was working correctly due to the low activities of PR1 DHFR previously observed. Optimisation of column conditions could have improved the elution of purified enzyme activity, however, recovery of a sufficient amount of enzyme activity, and time constraints imposed, meant that an optimisation step was not carried out.

$K_m$ values for PR1 and *B. stearothermophilus* enzymes (see Tables 5.4 and 5.5) were found to be in the same general range as those determined for the *E.coli* DHFR at 23°C (0.0004mM DHFA, 0.0065mM NADPH ) by Poe *et al.* (1972) and trimethoprim-resistant *E. coli* at 30°C (0.0032mM DHFA, 0.0068mM NADPH) by Baccanari *et al.* (1975).

$K_m$ did not appear to change significantly over the temperature range assayed for both enzymes. In the case of the *B. stearothermophilus* enzyme, assays performed at 68°C

produced less reliable data due to the problems of temperature equilibration and substrate degradation.

Although temperature optima analysis of these two enzymes needs further work to improve the data obtained, the current study shows that both the psychrotolerant and thermophilic DHFRs characterised fit the equilibrium model proposed by Daniel *et al.* (2001), as opposed to the classical model. Enzymes clearly display an optimum temperature of activity at zero time, when no thermal denaturation could have occurred.

The optimum temperature of activity for *B. stearothermophilus* DHFR was lower than might have been expected from its optimum growth temperature. A major problem discovered with carrying out these assays at high temperature was the degradation of the substrate and cofactor during the equilibration of the cuvette (which was slower at higher temperatures), and throughout the assay. As discussed by Daniel and Danson (2001), there are few ways to overcome substrate instability. Degradation of the reaction components was measured separately to the assay to gain an estimation of the effect of this on resulting enzyme activity. Equilibration of the enzyme in buffer with addition of pre-mixed DHFA and NADPH as a small concentrated volume was another technique employed, however, it was later found that the thermophilic enzyme had also lost activity over this time period.

The result for PR1 is likely to be closer to its true value than that of *B.stearothermophilus*, there being less problems with denaturation at the lower temperatures at which assays were performed. A problem with the temperature optimum determination of PR1 was that only a low concentration of enzyme was used. As no more than 20μL of enzyme extract could be added to the cuvette at one time without the temperature of the equilibrated assay mixture dropping
(M. Peterson, personal communication), activity was too low to get an ideal assay result. Earlier attempts to freeze-dry the enzyme solution and re-dissolve the enzyme in a more concentrated form did not result in an increased level of activity. In the future, the best way to improve the activity obtained may be to clone the gene

encoding the DHFR enzyme from the native organism into an expression vector, allowing a much greater amount of enzyme to be purified and facilitating measurement of enzyme activity at each temperature.

Thermal stability was not measured for either enzyme; *B. stearothermophilus*, because denaturation of the enzyme occurred during the equilibration step and PR1, because enzyme activity measured over the three minutes of the assay did not follow a smooth trend (enzyme activity was delayed at lower temperatures).

Previous studies have found the $T_{opt}$ to be 20-40°C higher than the optimum growth temperature of the source organism (Thomas and Scopes, 1998, M. Peterson, unpublished data). In the case of PR1 this appears to be so, however, the results for *B.stearothermophilus* do not support this finding, due to the reasons stated above.

A plot of enzyme activity at zero time versus temperature (Figure 4.4) shows that PR1 DHFR has a narrow temperature of optimal activity compared to *B.stearothermophilus*. This was unexpected, as the environmental temperature range that isolate PR1 was exposed to in the Antarctic soil was very large, approximately 70°C over the year, and it was thought that adaptation may have occurred to allow good activity over this wider temperature range. It is possible that when the temperature optimum characterisation is repeated, a wider peak width is observed for PR1, however, assuming that the current analysis is correct, this suggests that enzyme activity is adapted to work optimally over a small temperature range. As DHFR is expressed in actively growing cells (Stryer, 1988), and Antarctic soil bacteria would normally grow only during warm periods when soil temperature may reach 25°C or higher, this result may accurately represent enzyme properties of psychrotolerant bacteria, as higher soil temperatures are often achieved during the summer months (see Chapter 2).

# Chapter 6 Concluding Discussion

True psychrophiles are rarely found in Antarctic soils due to their exposure to (relatively) high temperatures in the summer months. In this study, all Antarctic bacteria isolated were found to be psychrotolerant. These are better adapted to survival in thermally fluctuating environments. Even over a single day, a large variation in temperature was recorded in the Upper Wright Valley and other sampling sites.

Genomic characterisation using DGGE and RAPD methods showed that some of the bacteria isolated were from the same genus and species, although there were many different strains. Future work should compare enzyme thermal properties of these very similar bacteria from different sampling locations, as differences in their properties would most likely be due to their varied habitat. A more detailed characterisation of all the isolates obtained using culture-based methods would give an insight into the microbial diversity of these soils.

The data obtained here will also be integrated into a larger ecological study into the microbial diversity of Antarctic soils. So far, Antarctic microbes studied have come from a restricted group of genera and most of the isolates from the current study fall within these. Moisture content, localised C and N enrichments (from desiccated seal carcasses and penguin guano) and temperature regime are all environmental factors affecting microbial diversity. The use of different methods to assess microbial abundance and diversity has a major impact on the results, i.e. culture-based versus molecular biological. In the absence of the supporting information from other laboratories involved in this project, it is difficult to draw conclusions about microbial diversity at this stage.

Initially, extracellular enzymes from thermophilic Antarctic bacteria were surveyed, as it was thought that these would be easy to purify and available at a relatively high concentration. In hindsight, the final choice of an intracellular enzyme, DHFR, for temperature characterisation was preferable. Unlike the enzymes initially tested, all bacteria express DHFR activity and by using this enzyme results could be compared to those obtained from work involving DHFRs from other sources.

The optimum temperature of enzyme activity is a new concept. It is supported by the results here from both the psychrotolerant and thermophilic enzyme characterisations, where a peak of activity is revealed at zero time. However, at this stage the data obtained are insufficient to confirm the environmental significance of this intrinsic property, although the idea that 'true' temperature optimum reflects the environmental temperature is validated, being lower for the psychrotolerant enzyme compared to the thermophilic DHFR.

The work here has laid the foundation for future studies. These should include the analysis of enzymes from several different temperature regimes, i.e., a range of constant temperature environments (psychrophilic, mesophilic, thermophilic), as well as environments of fluctuating temperature (eurythermal) as originally planned for this work. A comparison between a psychrotolerant and psychrophilic enzyme would be interesting to see how growth temperature affects the width of the $T_{opt}$ peak. It might be expected that a psychrophilic enzyme would generate a narrow peak compared to an enzyme from a psychrotolerant organism. Or, as the results presented in the current study indicate, the psychrotolerant enzyme may also have a narrow temperature range of optimal activity, but shifted towards a higher temperature than the psychrophilic enzyme.

# APPENDIX 1 Culture media

## Casein agar

| | |
|---|---|
| Hammersten casein | 10.0g |
| Yeast extract | 1.0g |
| Trypticase peptone | 1.0g |
| Agar | 17.5g |

Adjust pH to 5.6 and make up to 1L with distilled water. Boil to dissolve agar and autoclave.

## Casein broth

| | |
|---|---|
| $(NH_4)_2SO_4$ | 1.3g |
| $KH_2PO_4$ | 0.28g |
| $CaCl_2.2H_2O$ | 0.074g |
| $MgSO_4.7H_2O$ | 0.274g |
| Trypticase peptone | 1.0g |
| Yeast extract | 1.0g |
| Hammersten casein | 5.0g |

Dissolve by heating, adjust pH to 6.5 and make up to 1L with distilled water. Autoclave.

## Modified R medium

| | |
|---|---|
| $MgSO_4.7H_2O$ | 7.5g |
| $KH_2PO_4$ | 1.5g |
| $FeSO_4.7H_2O$ | 0.03g |
| Yeast extract | 30g |

| | |
|---|---|
| Trypticase peptone | 30g |
| $CaCl_2.2H_2O$ | 4.41g |

Adjust pH to 7.0 and make up to 1L with distilled water. After autoclaving, add D-glucose to produce a final concentration of 1g/L.

**Claudia medium**

Dissolve 3.25g nutrient broth (Gibco BRL, Life Technologies Ltd, UK) and 1g $CaCl_2$ in 980mL distilled water and adjust pH to 7.1. Mix 15g olive oil and 5g gum arabic with 20mL water, add to the medium in drops, then homogenise using a Waring blender. Dispense the medium into culture flasks and autoclave.

**Bacillus EA1 Medium**

| | |
|---|---|
| $MgSO_4.7H_2O$ | 0.25g |
| $KH_2PO_4$ | 0.05g |
| $FeSO_4.7H_2O$ | 0.001g |
| Yeast Extract | 1g |
| Trypticase Peptone | 1g |
| D-glucose | 1g |
| $CaCl_2.2H_2O$ | 0.147g |

Make up to 1L with distilled water and autoclave.

**Oshima Neutral Medium (ONM)**

| | |
|---|---|
| Trypticase Peptone | 8g |
| Yeast Extract | 4g |
| NaCl | 3g |

Make up to 1L with distilled water and autoclave.

# APPENDIX 2 Gram's Stain and PCR reaction components

## Ammonium crystal violet

| | | |
|---|---|---|
| Solution A | Crystal violet | 20g |
| | 95% ethanol | 200mL |
| Solution B | Ammonium oxalate | 8g |
| | Distilled water | 800mL |

Add solution A to solution B and filter.

## Gram's iodine

| | |
|---|---|
| Resublimed iodine | 20g |
| 1N NaOH | 100mL |
| Distilled water | 900mL |

Dissolve iodine in NaOH then add water

## Dilute carbol fuchsin

| | |
|---|---|
| Basic fuchsin | 1g |
| Phenol | 5g |
| 100% ethanol | 10mL |
| Distilled water | 990mL |

Dissolve fuchsin in phenol by placing in 1L flask and holding over boiling waterbath for 5 minutes, shaking occasionally. When dissolved, add alcohol, mix, then add distilled water. Filter before use.

**Table A2.1. PCR reaction components**

| PCR component | Source |
|---|---|
| PCR grade water | Eppendorf, Hamburg, Germany |
| PCR buffer 10X conc (without $MgCl_2$) | Roche Diagnostics, Mannheim, Germany |
| dNTPs (2mM conc., dil. in Milli-Q water) | Boehringer Manheim, Germany |
| $MgCl_2$ (25mM) | Roche Diagnostics, Mannheim, Germany |
| Taq DNA polymerase | Roche Diagnostics, Mannheim, Germany |

# APPENDIX 3 16S rDNA alignment

```
PR1             ------------------------------------AGGGAATCTTCCACAATGGACGAA
MVS4            TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGCA
MV2             ----------------------------------------------------GGACGAA
BI3             ------------------------------------------------------------
MVS1            ------------------------------------------------------------
BI1             ------------------------------------------------------------
MVS2            ----------------------------------------------------GGGGGAA
MVT5            ------------------------------------------------------------

PR1             AGTCTGATGGAGCAACGCCGCGTGAGCGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGT
MVS4            AGTCTGACGGAGCAATGCCGCGTGAGTGACGAAGGTTTTCGGATCGTAAAACTCTGTTGT
MV2             AGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGT
BI3             ------------------------------------------------------------
MVS1            ------------------------------------------------------------
BI1             ------------------------------------------------------------
MVS2            ACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTGTAAAGCACTTTAAG
MVT5            ------------------------------------------------------------

PR1             AAGGGAAGAACAAGTACGGGAGTAACTGT-CCGTACCATGACGGT-ACCTTATTAGAAAG
MVS4            AAGGGAAGAACAAGCCCCATT-TAACTGA-TGGGGCCCTGACGGT-ACCTTACCAGAAAG
MV2             TAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGT-ACCTAACCAGAAAG
BI3             ----------------------------------CCCCCGACGNAGATCTAACCAGAAAG
MVS1            --------------------------------GCTGTTTTGAC-GTTACCGACAGAATAAG
BI1             ----------------CTAATACGTCA--CGTGTCTGGACAGTNACCGANAAAAANNTA
MVS2            CAGTGAAGAAGACTCTTTNGTTTATACCC--GGAGACGATGACATTAGCTGCAGAATAAG
MVT5            ----------------------------------------CGGTACCTGCAGAAGAAG
                                                          * *     *

PR1             CCACG-GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGA
MVS4            CCACG-GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGA
MV2             CCACG-GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGA
BI3             CCACG-GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGA
MVS1            CACCG-GCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGA
BI1             CANCGAGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGA
MVS2            CACCG-GCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGA
MVT5            CGCCG-GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGA
                *  ** *******  ********************** ***  *********   ****

PR1             ATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAAGCCCACGGCT
MVS4            ATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAAGCCCACGGCT
MV2             ATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCT
BI3             TTTATTGGGCGTAAAGCGAGCGCAGGCGGTTCTTTAAGTCTGATGTGAAAGCCCCCGGCT
MVS1            ATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCT
```

```
BI1             ATTACTGGGCGTAAAGCGCGCGTANGTGGTTTGTTAAGTTGGATGTGAAAGCCCCGGGCT
MVS2            ATTACTGGGCGTAAAGGGAGCGTAGGTGGCTCGATAAGTCAGATGTGAAATCCCCGGGCT
MVT5            ATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCT
                *** *********** *  ** * * **        **  *  ******  **  ****

PR1             CAACCGTGGAGGGTCATTGGAAACTGGAGGACTTGAGTACAGAAGAGGAAAGCGGAATTC
MVS4            CAACCGTGGAGGGTCATTGGAAACTGGGGGACTTGAGTGCAGAAGAGGAAAGTGGAATTC
MV2             CAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTC
BI3             CAACCGGGGAAGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTC
MVS1            CAACCTGGGAACTGCATTCAAAACTGACAAGCTAGAGTATGGTAGAGGGTGGTGGAATTT
BI1             CAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTATGGCAGAGGGTGGTGGAATTT
MVS2            CAACCTGGGAACTGCATCTGATACTGTTGAGCTAGAGTATGTGAGAGGAAGGTAGAATTC
MVT5            CAACTCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTC
                ****   ***    *       ** *     ** ****     ** **      *****

PR1             CACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTC
MVS4            CATGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTTTC
MV2             CACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTC
BI3             CACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTC
MVS1            CCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACC
BI1             CCTGTGTANCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAANGCGACCACC
MVS2            CAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCTTC
MVT5            CTGGTGTACCGGNGAAATGCGCAGATATCANGAGGAACACCGATGGCGAANGCAGGTCTC
                *  ***** *** ******** *** **    ***** ***  ******* **      *

PR1             TGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
MVS4            TGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC-TG
MV2             TGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTG
BI3             TGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
MVS1            TGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATNANATACCCTG
BI1             TGGGCTAATACTGACACTGANGTGCGAAAGCGTGGNGAGCAAACAGGATNANATACCCTG
MVS2            TGGCATAATACTGACACTGAGGCTCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTG
MVT5            TGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGNGAGCGAACAGGATTANATACCCTG
                ***      ****** **** *  ******* ***  *** ******** * ***** **

PR1             GTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTC-CGCCCCTTAGTGCT
MVS4            GTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTC-CGCCCCTTAGTGCT
MV2             GTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTC-CGCCCCTTAGTGCT
BI3             GTAGTCCACGCCGTAAACGATGAGTGCTANGTGTTGGANGGTTTC-CGCCCTTCAGTGCT
MVS1            GTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTT--GAGCTCTTAGTGGC
BI1             GTAGNCCACGCCGTAAACGATGTCGACTAGCCGTNGGGATCCTT--GACATCTTANTGGC
MVS2            GTAGTCCACGCCGTAAACGATGTCTACTAGTCGTTGGGTCCCTT--GAGGACTTAGTGAC
MVT5            GTANNCCATGCCGTAAACGTTGGGCACTANGTGTGGGGGACATTCCANGTTTTCCGCGCC
                ***  *** ********** **    ***   **  *     **        *    *

PR1             GCAGCTAACGCATTAAGCACTCCGCC-TGGGGAGTACGGCCGCAAGGCTGAAACTCAAAG
MVS4            GCAGCTA-CGCAATAAG----------------------------------------
MV2             GCAGCTAACGCATTAAGCACTCCGCC-TGGGGAGTACGGTCGCAAGACTGAAACTCAAAG
```

```
BI3             GCAGCTAACGCATNAAGCACTCCGCCCTGGGGAGTACGACCGCAANGTTGAAACTCAAAG
MVS1            GCAGCTAACGCATTAAGTNGACCGCC-TGGGGAGTACGGCCGCAANGTTAAAACTCAAAT
BI1             GCAGCTAACGCATTAANTCGACCGCC-TGGNGAGTACGGCCGCAAGGTTAAAACTCAAAT
MVS2            GCAGCTAACGCAATAAGTAGACCGCC-TGGGGAGTACGGCCGCAAGGTTAAAACTCAAAT
MVT5            GTAGCTAACGCATTAAGTGCCCCGCC-TGGGGAGTACGGCCGCCANGCTAAAACTCAAAG
                * ***** ****  **

PR1             GAATTGACGGGGACCC-GCACAAGCGGTGGAGCATGTGGTTTAATT-CGAAGCAACGCGA
MVS4            ------------------------------------------------------------
MV2             GAATTGACGGGGGCCC-GCACAAGCGGTGGAGCATGTGGTTTAATT-CGAAGCAACGCGA
BI3             GAATTGACGGGGACCC-GCACAAGCGGTGGAGCATGTGGTTTNATTTCGAAGCAACGCGA
MVS1            GAATTGACGGGGGCCC-GCACAAGCNGTGGAGCATGTGGTTTAATT-CGAAGCAACGCGA
BI1             GAATNGACGNNGGCCCCGCACAAGCGGTGGA----------------------------
MVS2            GAATTGACGGGGGCCC-GCACAAGCGGTGGAGCATGTGGTTTAATT-CGATGCAACGCGA
MVT5            GAATNGACNGNGGCCCCGCACAANCGGCGCGA----------------------------

PR1             AGAACCTT-ACCAGGTCTTGACATCCCACTGACCGGTGTAGAGATACGCCCTTTCCCTTC
MVS4            ------------------------------------------------------------
MV2             AGAACCTT-ACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTTGG
BI3             AGAACCTTTACCAGGTCTTGACATCCCTTCGACAACCCTAANAGATAGGGCTTTCCCTTT
MVS1            AGAACCTTACCAGGCCTTGACATCCAATGAACTT--------------------------
BI1             ------------------------------------------------------------
MVS2            AGAACCTTACTGGTCTTGACATATCTAGAATCCTGCAGAGATGCGGGAGTGCCTTCGGGA
MVT5            ------------------------------------------------------------

PR1             GGGGACAGTGGTGACAAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGG
MVS4            ------------------------------------------------------------
MV2             GGGGCAGAGTGACAGGTGGTGCATGGTTGGTCGTCAGCTCCTGGTCGTGAA---------
BI3             CGGCGGACAA--------------------------------------------------
MVS1            ------------------------------------------------------------
BI1             ------------------------------------------------------------
MVS2            ATTAGAATACAGGTGCTGCATGGGCTGTCGTCAGCTCGTGGTCGTGAGATTGTTGGGGTT
MVT5            ------------------------------------------------------------

PR1             TTAAGTCCCCAACGAGCGCAACCCTTGATCCTT
MVS4            --------------------------------
MV2             --------------------------------
BI3             --------------------------------
MVS1            --------------------------------
BI1             --------------------------------
MVS2            AAGT----------------------------
MVT5            --------------------------------
```

# APPENDIX 4 Enzyme graphs

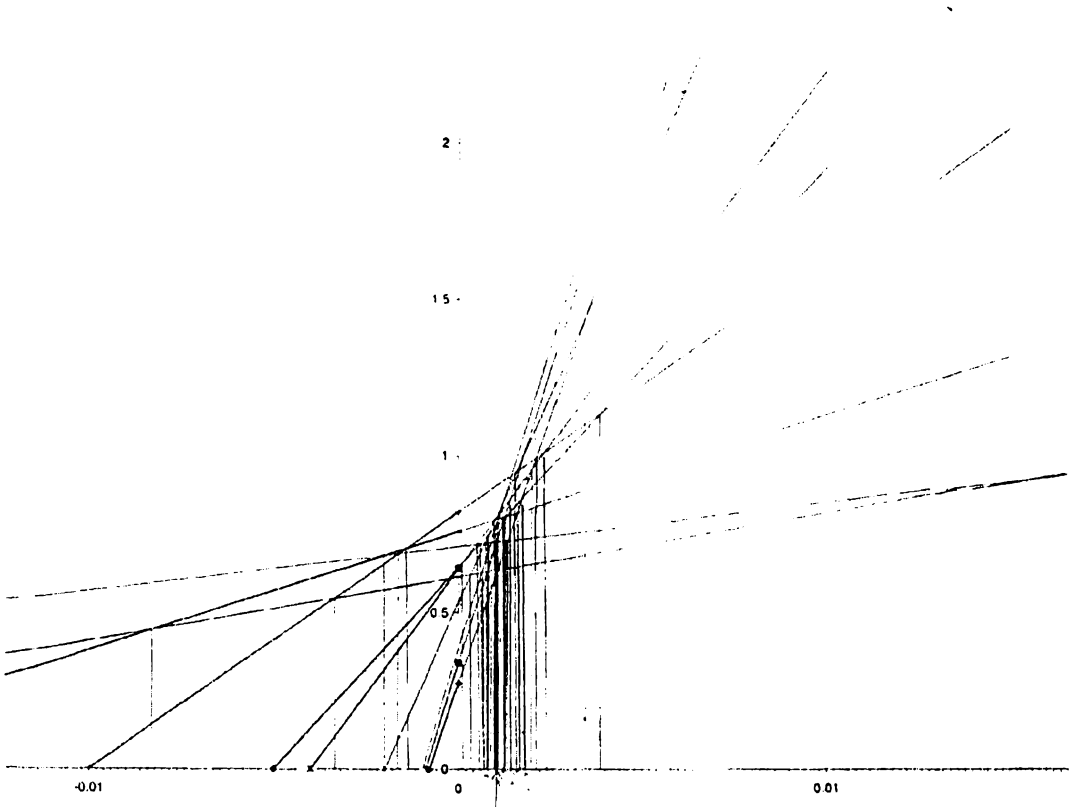



**Figure A4.1. Direct linear plot of DHFA at 20°C, PR1 DHFR, $K_m$ = 0.001mM**

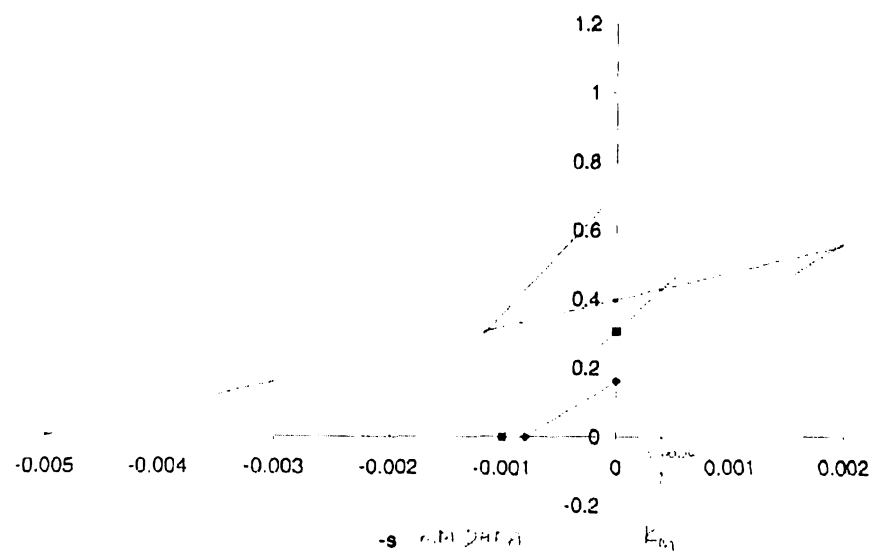
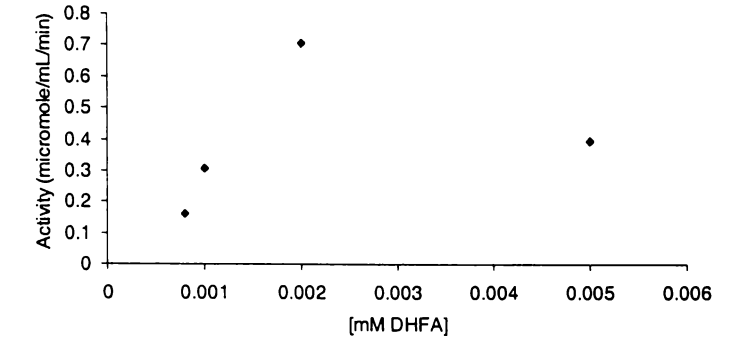



**Figure A4.2. Direct linear plot of DHFA at 14°C, PR1 DHFR, $K_m$ = 0.0004mM**

A




B

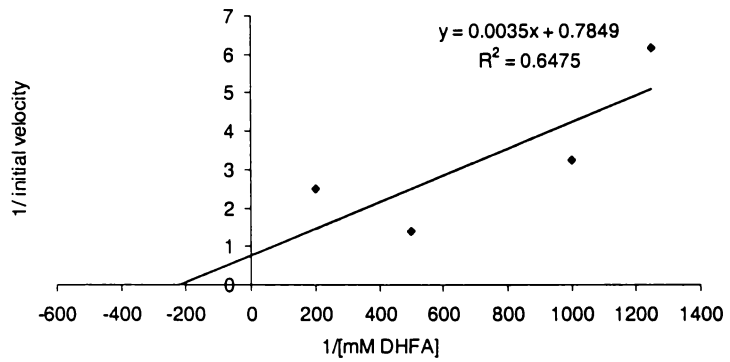



**Figure A4.3. (A) Michaelis-Menten plot and (B) Lineweaver-Burke plot of DHFA at 14°C, PR1 DHFR, $K_m$ = 0.0004mM**

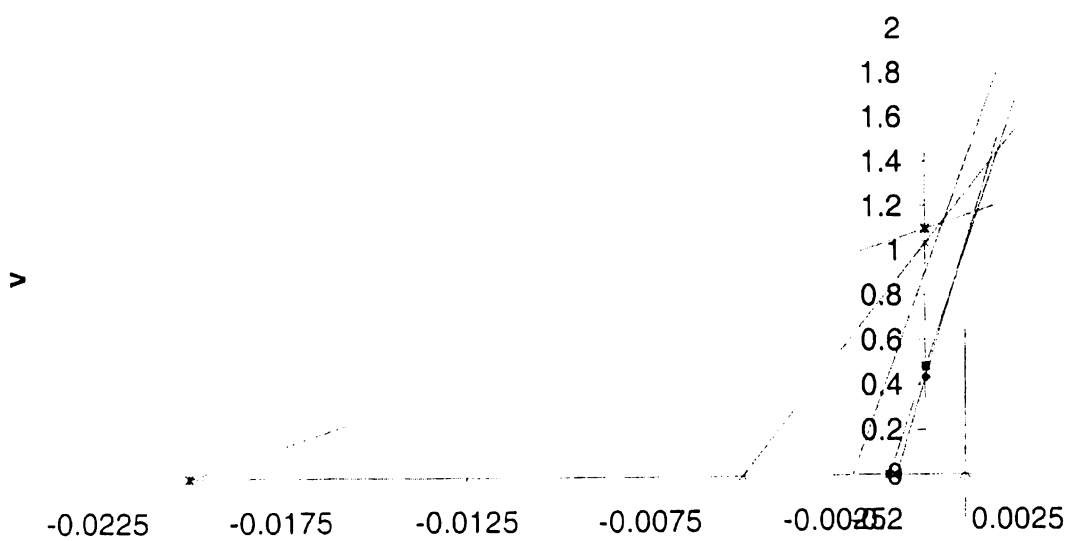
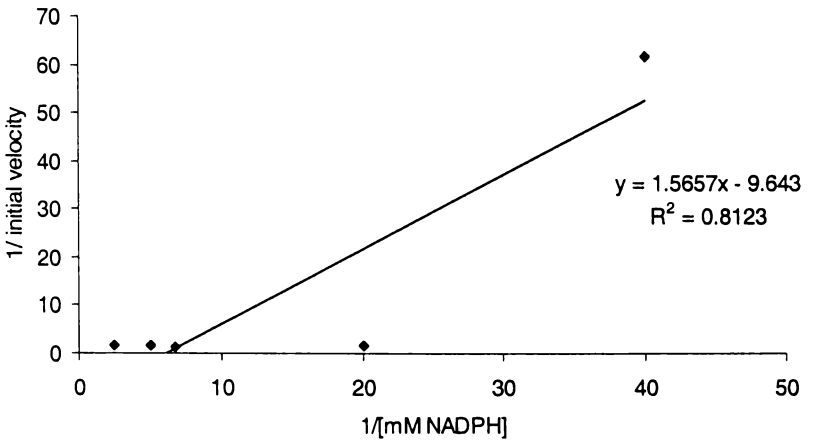



**Figure A4.4. Direct linear plot of DHFA at 30°C, PR1 DHFR, $K_m$ = 0.0011mM**

A




B

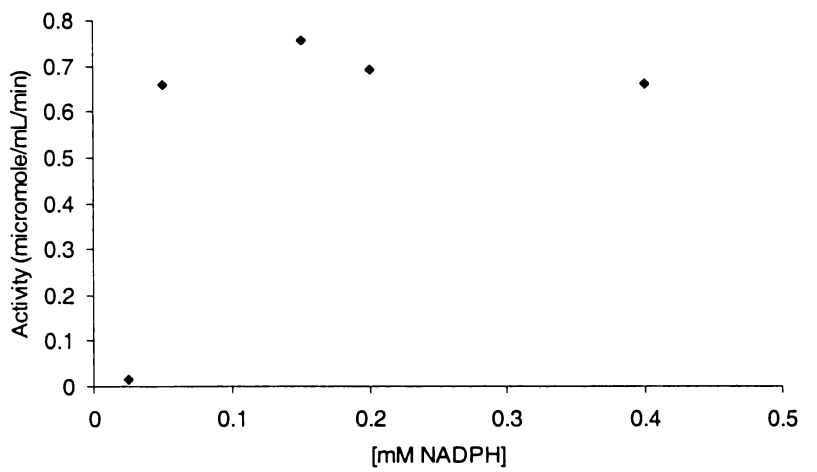



**Figure A4.5. (A) Michaelis-Menten plot and (B) Lineweaver-Burke plot of DHFA at 30°C, PR1 DHFR, $K_m$ = 0.0011mM**

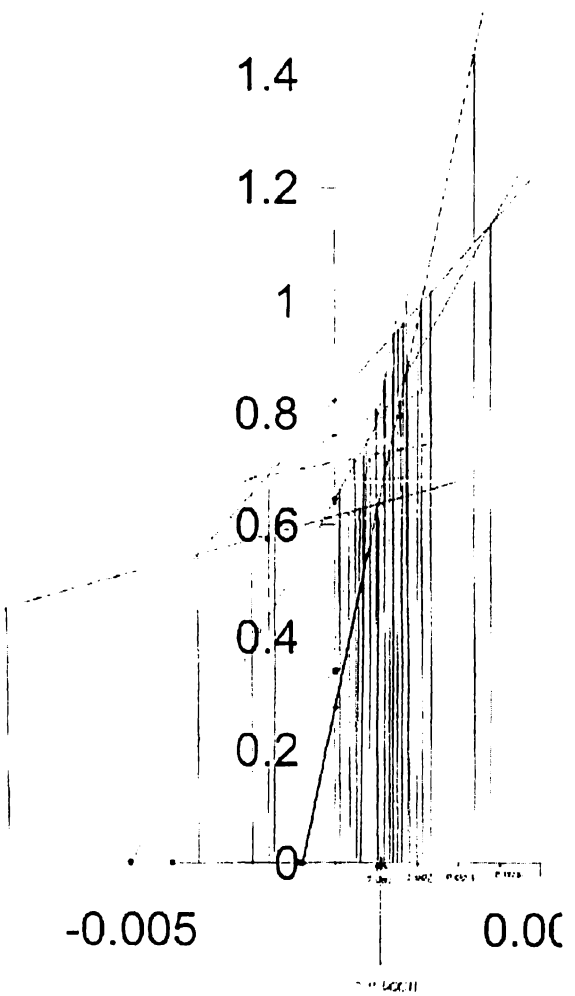



**Figure A4.6. Direct linear plot of NADPH at 20°C, PR1 DHFR, $K_m$ = 0.0011mM**

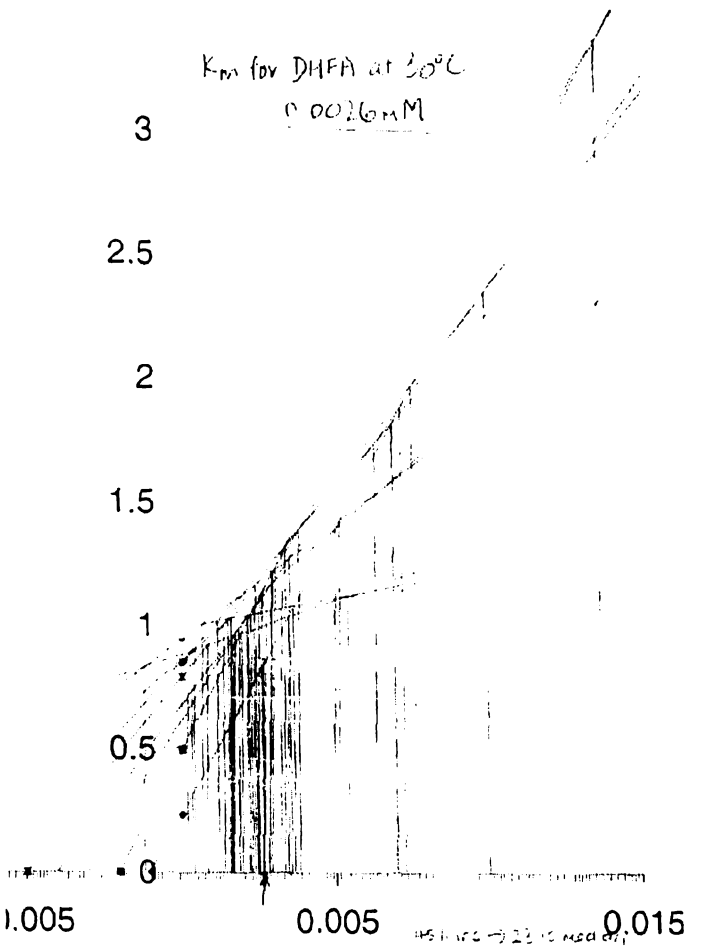



**Figure A4.7. Direct linear plot of DHFA at 30°C, *B. stearothermophilus* DHFR, $K_m$ = 0.0026mM**

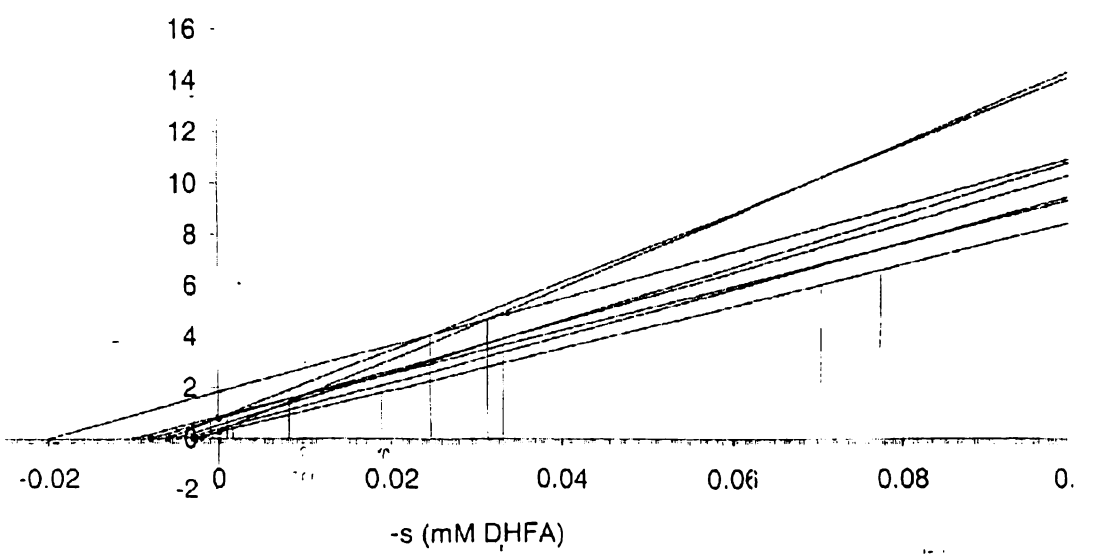



**Figure A4.7. Direct linear plot of DHFA at 68°C, *B. stearothermophilus* DHFR, $K_m$ = 0.019mM**

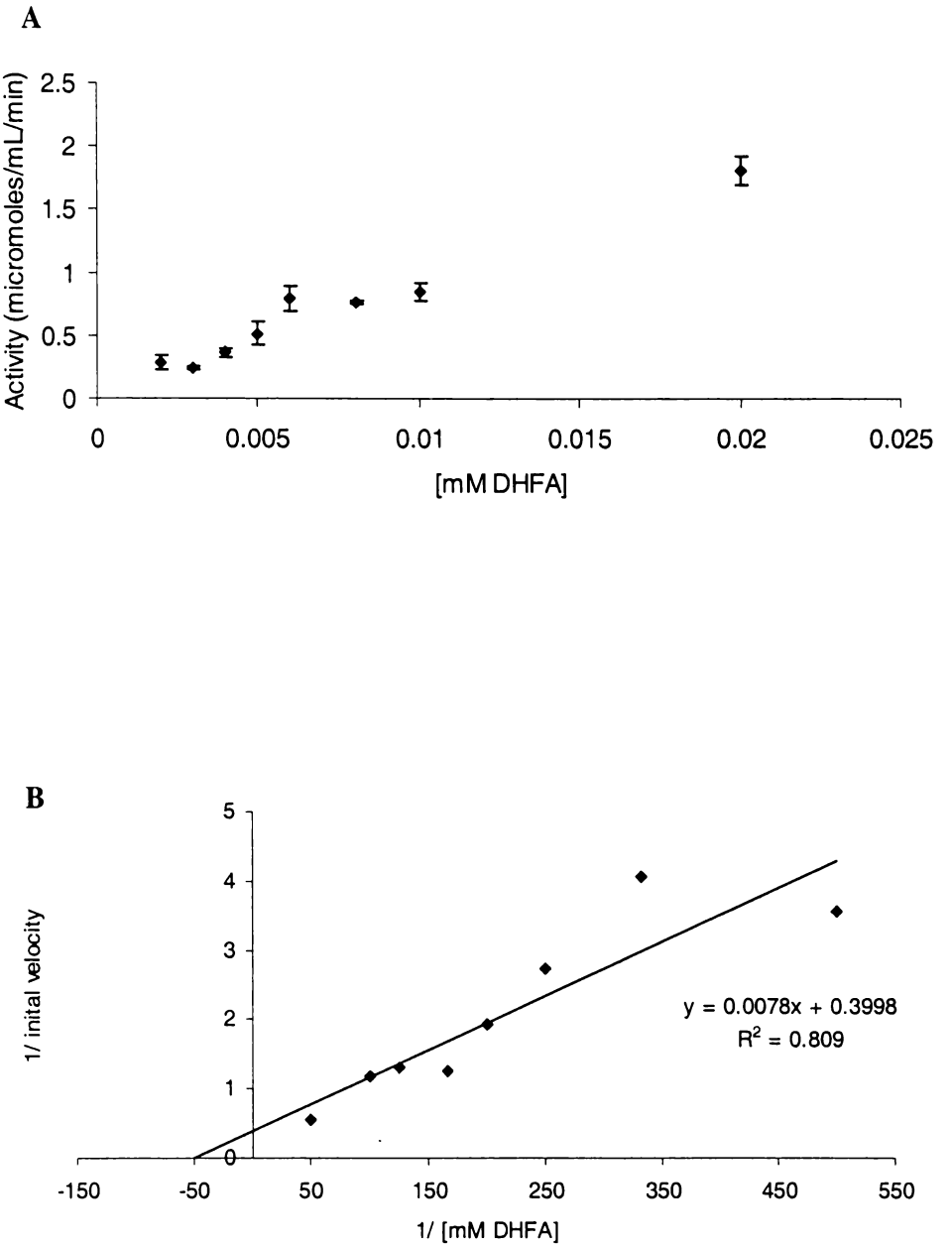



**Figure A4.8. (A) Michaelis-Menten plot and (B) Lineweaver-Burke plot of DHFA at 68°C, *B. stearothermophilus* DHFR, $K_m$ = 0.019mM**

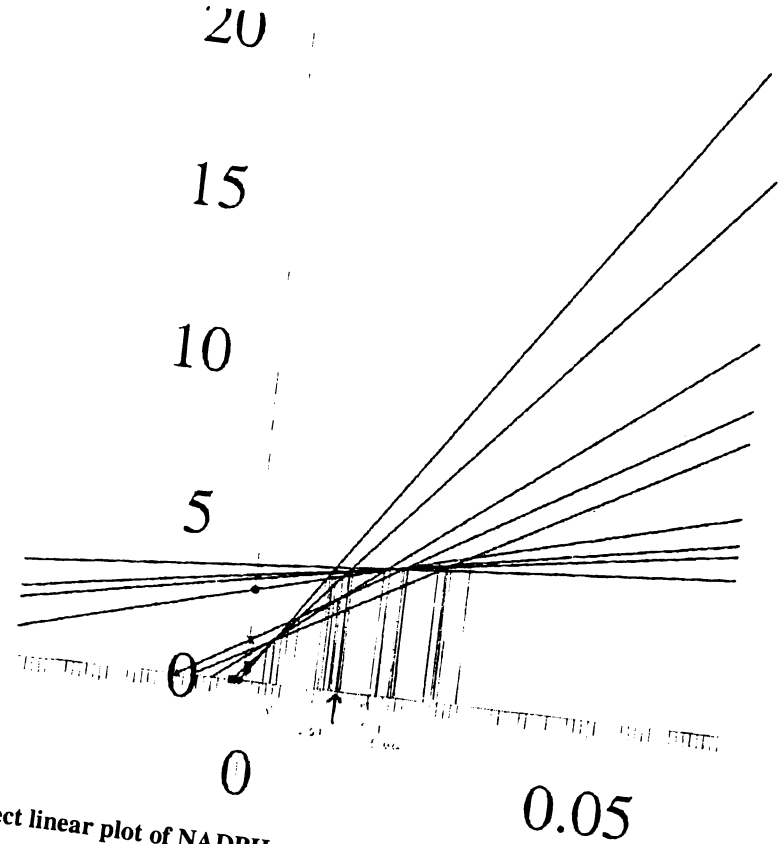



**Figure A4.9. Direct linear plot of NADPH at 30°C, *B. stearothermophilus* DHFR, $K_m$ = 0.013mM**

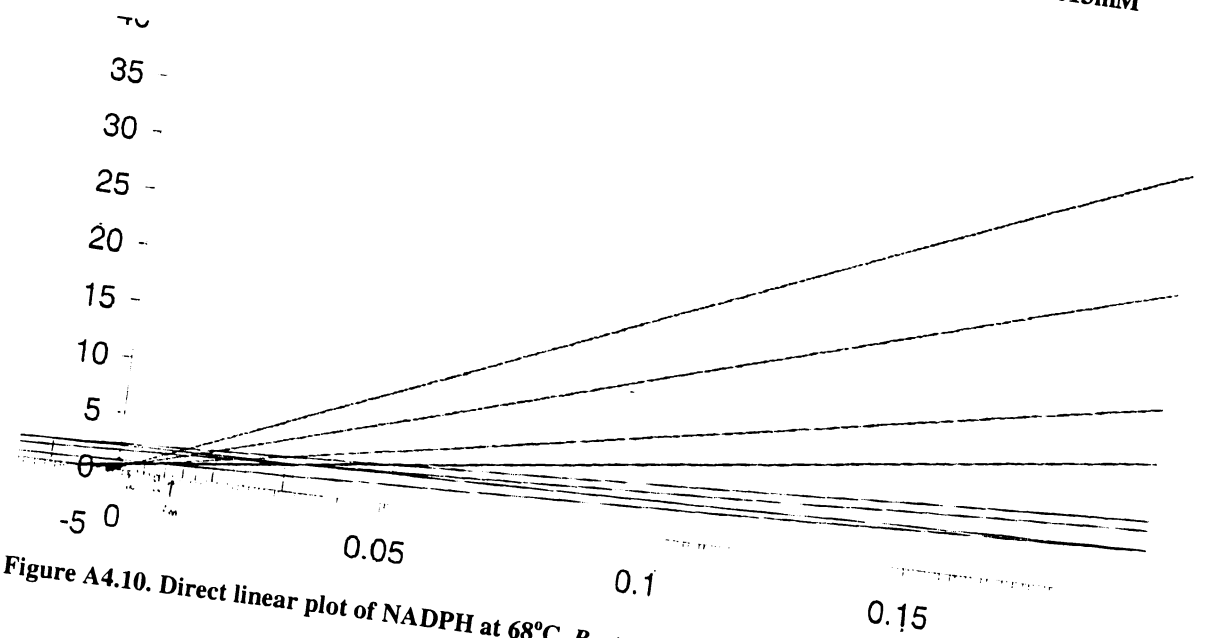



**Figure A4.10. Direct linear plot of NADPH at 68°C, *B. stearothermophilus* DHFR, $K_m$ = 0.015mM**

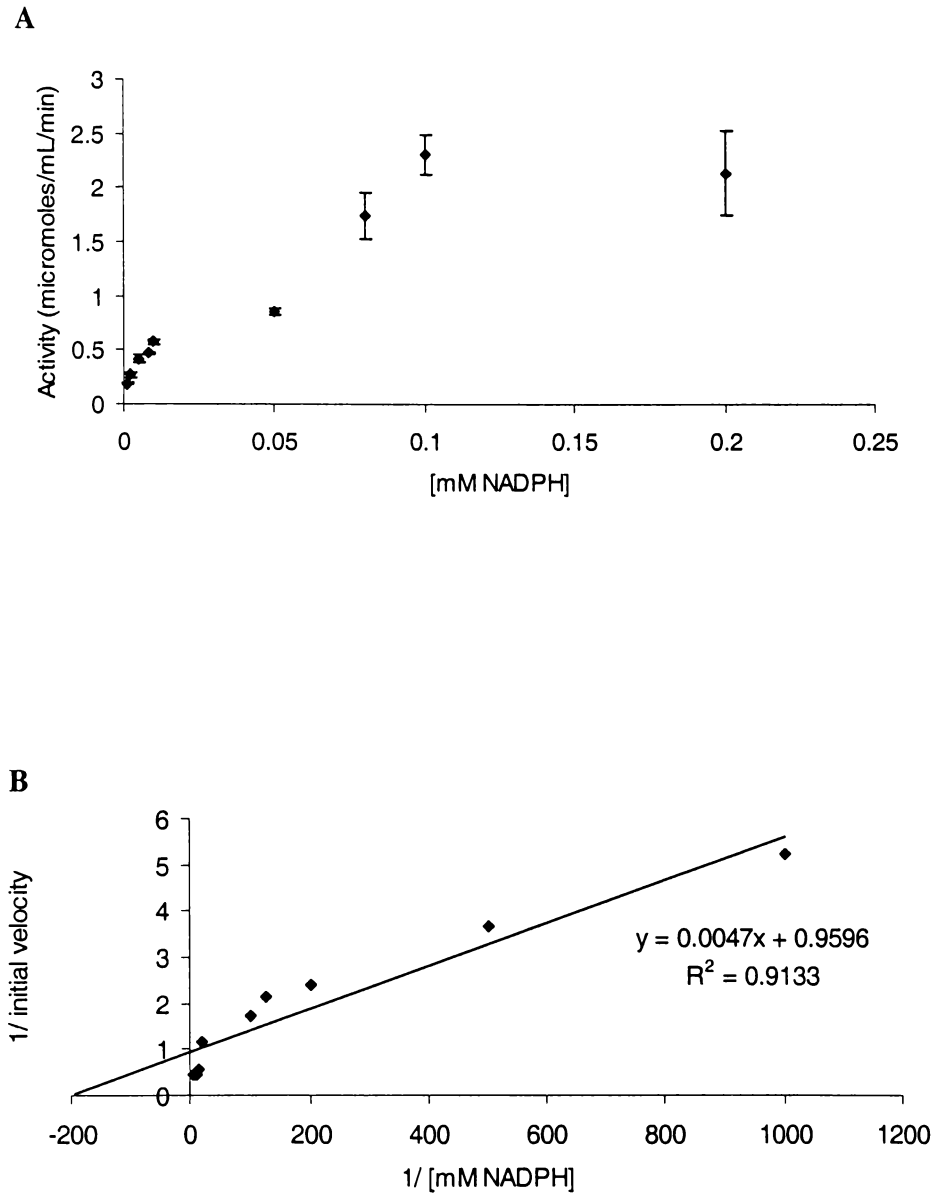



**Figure A4.11. (A) Michaelis-Menten plot and (B) Lineweaver-Burke plot of NADPH at 68°C, *B. stearothermophilus* DHFR, $K_m$ = 0.015mM**

# APPENDIX 5 Antarctic information

## Event K023 Terrestrial Microbiology, Antarctica New Zealand 2001/02

### Event personnel

| | |
|---|---|
| Professor DA Cowan | University of the Western Cape, SA |
| Professor C Cary | University of Delaware, USA |
| Miss S Whiting | University College London, UK |
| Miss S Hawkins | University of Waikato, NZ |

### Proposed programme

- Investigation of bacterial and archaeal diversity in dessicated terrestrial Dry Valley gravels
- Comparisons of Dry Valley mineral gravel microbial diversity with microbial diversity in nutrient rich ornithogenic soils
- In situ analysis of microbial biomass in Dry Valley mineral gravels using ATP quantitation
- Analysis of introduction of non-indigenous microorganisms into the Antarctic environments as a result of human activities
- Effects of localised C and N enrichments on microbial diversity in Dry Valley soils
- Microbial diversity associated with "fossil" Antarctic marine sponges
- Isolation of microbial strains from thermally fluctuating sites
- Field testing of mobile molecular biology laboratory (comprising of a Bead-beater, microcentrifuge, PCR thermocycler, electrophoresis unit, transilluminator and camera unit)

**Activities and achievements at field sites**

Upper Wright Valley (4 days)

1. Collection of mineral soil samples from regions in the Upper Wright Valley, the labyrinth, and from a vertical sampling transect from the valley floor to a circ on the eastern side of the valley. In situ ATP analysis indicated a significant variation in ATP content with increasing elevation, possibly reflecting soil moisture content. Samples will be used for studies of microbial diversity using 16s rRNA analysis.
2. Successful extraction and PCR amplification of microbial DNA from valley floor mineral soils. PCR using universal bacterial primers resulted in the successful electrophoretic separation and visualisation of DNA bands. The success of these trials indicated that more detailed molecular ecological studies could be carried out in the field.

Don Juan Pond

1. Volumes of hypersaline water and sediment were collected to be used for subsequent isolation of extreme halophilic bacteria and archaea, and for molecular phylogenetic analysis.

Miers Valley

1. Collection of mineral soil samples from regions in the Miers Valley and from a vertical sampling transect from the valley floor to a high saddle on the top of the Marshall Valley. Samples will be used for studies of microbial diversity using 16s rRNA analysis and DGGE, for the PCR based analysis of bacterial integron diversity in Antarctic soils, and for the isolation if psychrotolerant microbial strains.
2. Successful extraction and PCR amplification of microbial DNA from 20 valley floor mineral soil samples.
3. Transect sampling of surface and 5-10cm depth soils in the vicinity of seal carcasses. These samples will be used to assess the impact of local C and N sources on microbial diversity and biomass. As a working hypothesis, we propose

that microbial diversity in the dry valleys is influenced by the known C and N limitations of the soils.

Bratina Island

1. Collection of water and sediment samples from saline ponds in the vicinity of Bratina Island. Samples will be used for analysis of microbial diversity using 16s rRNA analysis and DGGE, for PCR based analysis of bacterial integron diversity in Antarctic soils and for the isolation of psychrotolerant microbial strains.
2. Collection of exposed "fossil" sponge materials. Marine sponges entrained into glacial ice at the glacial-marine sediment interface, and eventually exposed on the upper surface of the glacier through the processes of ablation. The transit time is estimated to be in the order of 500-5000 years. These samples may include signals from microbial endosymbionts, and may be used for comparitive analyses with homologous extant organisms. It may be possible to acquire evidence of microbial molecular evolution from these studies.

Cape Crozier

1. Sampling of ornithogenic soil samples from various sites in the Adelie penguin colony.

**Time-line for Antarctic Trip**

| | |
|---|---|
| 22 Jan | Leave Christchurch for Antarctica |
| 23 Jan | Field training (AFT) |
| 24 Jan | Field training |
| 25 Jan | Scott Base, McMurdo Station, trip to Discovery Hut |
| 26 Jan | Scott Base, climb up Observation Hill |
| 27 Jan | Helicopter to Upper Wright Valley, set up camp |
| 28 Jan | Set up UWV temperature probes, walk up valley slope |
| 29 Jan | Molecular biology lab work, walk along upper glacier |
| 30 Jan | Walk along moraine and through labyrinth |

| | |
|---|---|
| 31 Jan | Collect spot soil samples, picked up by helicopter, short stop at Don Juan pond. Arrive at Miers Valley |
| 1 Feb | Microbiological transect, locate mummified seals |
| 2 Feb | Set up MZ temperature probes, DNA extractions using mol bio lab |
| 3 Feb | Don and Craig sample up valley, PCR work and electrophoresis |
| 4 Feb | Helicopter from Miers Valley to Bratina Island, BI temperature probes |
| 5 Feb | Collect samples, sponge sampling, picked up and returned to Scott Base |
| 6 Feb | Cape Crozier sampling at Adelie penguin colony |
| 7 Feb | Return to New Zealand |

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