MICROBIAL DIVERSITY IN RELATION TO HUMAN ACTIVITY IN HISTORIC AREAS OF ROSS DEPENDENCY, ANTARCTICA

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE IN BIOLOGY

BY

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The goal of this study was to undertake a relatively broad microbiological investigation at historic areas of Cape Evans and Ross Island. The two phylogenetically diverse targets were *Bacillus anthracis*, the causative agent for anthrax disease, and filamentous micro-fungi associated food products. The human activities were presumed to have played a significant contribution to the introduction of non-indigenous *Bacillus anthracis* and many filamentous micro-fungi at the historic areas on Ross Island.

Bacillus anthracis was suspected to be present at Cape Evans based on a circumstantial clinical analysis of the death of the member in Captain Robert F Scott's Terra Nova Expedition in 1912. Detection methods based on polymerase chain reaction (PCR) targeting *B. anthracis* specific genes on chromosome and two plasmids were employed. DNA extraction was performed by a bead-beating technique from 74 environmental samples. PCR efficiency was compromised probably due to inhibitors in DNA extracts, but improved with higher concentrations of *Taq* polymerase. Initially a total of 74 environmental samples were screened with one set of primers before positively tested 19 samples were rigorously investigated with seven sets of primers. Nested PCR also increased the target specificity and detection levels. Sequence analyses of the several positive samples from PCR reactions were characteristic to *B. anthracis*.

A diverse range of filamentous micro-fungi were isolated on three different media at two different temperatures, 15 °C and 25 °C, and identified by classical morphological taxonomy from the foodstuffs and internal environmental samples of Captain Robert F Scott's historic hut at Hut Point built in 1901. In total, there were 22 taxa and 14 genera recorded including many cosmopolitan species isolated from the samples, in particular *Penicillium* species. An extensive literature review of the filamentous micro-fungi found in Antarctica identified that 7 taxa isolated in the study were not reported previously. Many isolates were obtained at 15 °C while some isolates grew in the presence of antibiotics.

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A	adenosine
A_{260}/A_{280}	absorbance at 260/280 nm
AHT	Antarctic Heritage Trust
ANZ	Antarctica New Zealand
bp	base pairs
C	cytosine
DNA	deoxyribose nucleic acid
dNTP	deoxy-nucleotide triphosphates (dATP, dCTP, dGTP and dTTP)
EDTA	ethylenediamine tetraacetic acid
FAM	5-carboxyfluorescein
fg	femto gram
FRET	fluorescence resonance energy transfer
G	guanosine
IGY	International Geophysical Years
kb	kilo-base
LLNL	Lawrence Livermore National Laboratory
M	molarity
Milli-Q	Millipore Corporation
mM	millimolar
μM	micromolar
N	any nucleotide (adenosine, thymine, guanosine, or cytosine)
NCBI	National Center for Biotechnology Information
NCDI	National Centre for Disease Investigation
ng	nanogram
nm	nanometre
PCR	polymerase chain reaction
pg	picogram
PSB	phosphate, SDS, and bead-beating
rDNA	ribosomal DNA
RNA	ribose nucleic acid

rRNA	ribosomal RNA
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
SDS	dodecyl sulphate sodium salt
T	thymine
TAE	tris acetic acid EDTA buffer
TE	tris EDTA buffer
TIGR	The Institute of Genomic Research
Tris	trihydroxymethyl amino methane
tRNA	transfer RNA
U	units
UV	ultraviolet
V	volts
VNTR	variable number tandem repeats
vrr	variable repetitive repeat
vol/vol	volume per volume
vol/wt	volume per weight
YM	yeast malt agar

CHAPTER ONE

1. General Introduction

1.1. Antarctica

1.1.1. Geographical

Antarctica, in a geographical sense, is defined as the Antarctic Continent, and 'the Antarctic' has a diversity of definitions that loosely indicate the area south of latitude 60 degrees south (McGonigal and Woodworth 2001). It also refers to the area south of the nautical boundary called the Antarctic Convergence which is between 50 and 60 degrees south where cold water from the continent meets warmer water from lower latitudes. The Antarctic Treaty (1959) defines Antarctica as "the area south of 60 degrees south latitude, including all ice shelves ..... to the high seas within that area". Figure 1.1 shows geographical locations of Ross Island, Antarctica and New Zealand.

Antarctica is the fifth largest continent; together with land and permanent ice, it contains approximately 14 million square kilometres (Campbell and Claridge 1987). It has seasonal variability in the total area, for instance, surface area at the height of winter is approximately double that of summer. If all of the polar ice cap should melt, the continent would be divided into two main geographical regions; the Trans-Antarctic mountains range at the western edge of East Antarctica and the archipelago of West Antarctica with its extending arm of Antarctic Peninsula to Patagonia.

The geographical uniqueness of Antarctica can be easily summarised as it is the highest, driest, windiest, and coldest continent on Earth (McGonigal and Woodworth 2001). The average elevation is 2,160 metres above sea level and at its thickest point the ice extends to a depth of 4,800 metres. In comparison, the

Asian continent is the second highest at about 1,000 metres and North America is, on average, 700 metres above sea level.

The extremely arid environment of Antarctica is due to the restricted availability of moisture under sub-zero temperatures (Campbell and Claridge 1987). The mean annual precipitation of snow and ice crystals is equivalent to less than 50 mm of water, slightly more than that of the Sahara Desert in Africa. However, the Antarctic Peninsula receives relatively high levels of precipitation owing to prevalent westerly wind and close proximity to coastal areas at low latitudes.

The Dry Valleys, on the eastern side of McMurdo Sound in the southern Victoria Land, cut through the Trans-Antarctic mountains which create a precipitation shadow. Sublimation and katabatic wind effectively removes all moisture even if there is any precipitation, and consequently the Dry Valleys have less moisture than the Sahara Desert. The area is the driest place on Earth and is known to have analogous features to Mars (Campbell and Claridge 1987).

The relative flatness of the polar ice cap enhances the overall speed due to the lack of any physical impedance (i.e., friction force). Extreme wind velocity, called katabatic wind, is often associated with characteristic topographical features where cold and dense air near the surface of the polar plateau rolls down under the influence of gravity to the coastal line. As a classic extreme example, a hut erected by Sir Douglas Mawson's Australasian Antarctic Expedition (1911–1914) at Cape Denison was named "Home of Blizzard" because it frequently encountered katabatic winds topping up to the speed of 160 km per hour and an annual mean wind speed exceeding 70 kilometres per hour (Mawson 1915).

Temperature is, of course, an important element in the Antarctic weather. In general, variation in temperature is in accordance with latitudinal and altitudinal parameters. The polar plateau receives little solar energy due to high albedo, whereas dark bare ground, for example the Dry Valleys, coastal areas, and nunataks, can be heated up above freezing point relatively quickly (Campbell and Claridge 1987). The lowest temperature ever recorded was -89.3 °C at Vostok Station (78°28'00''S 106°48'00'') in July 1983.

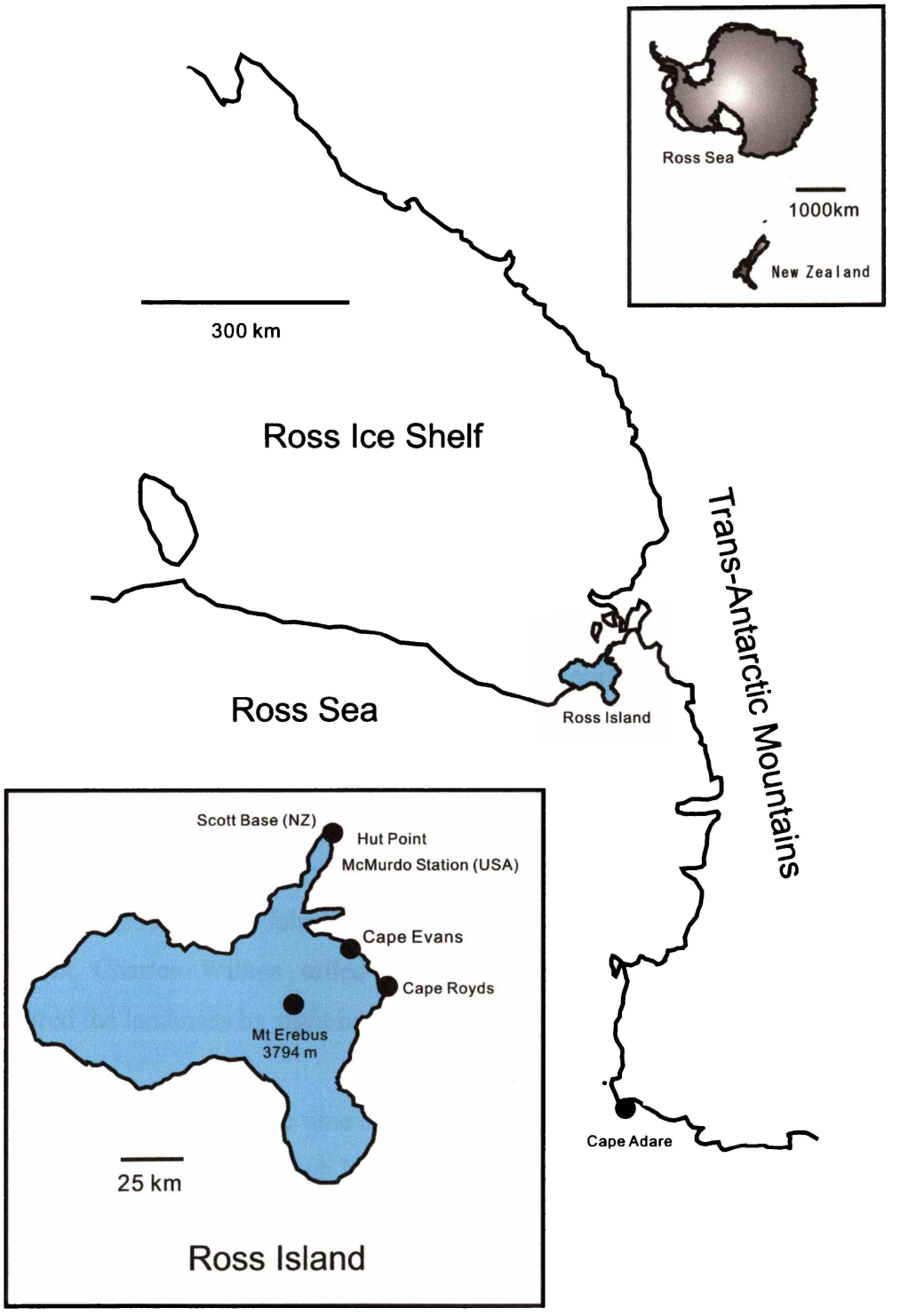

Figure 1.1 Map of Antarctica and New Zealand (top right), Ross Sea Region (middle) and Ross Island (left bottom) Historic huts of Ross Dependency are located at Hut Point, Cape Evans and Cape Royds on Ross Island, and at Cape Adare at the northern end of the Trans-Antarctic Mountains.

1.1.2. Discovery of Antarctica, history and relationship with New Zealand

1.1.2.1. Exploration before the Heroic Era

The existence of a large unknown continent, *Terra Australis Incognita*, at the southern extremity was believed since ancient times to balance the known land mass in the Northern Hemisphere. Abel Tasman, the Dutchman, and the first European to sight New Zealand considered the landmass as a part of the great unknown continent in 1642. It was later confirmed by Sir James Cook, an accomplished navigator, that the island Tasman had sighted previously was not a part of *Terra Australis Incognita* in 1771. Captain Cook was the first to cross the Antarctic Circle and circumnavigated in high southern latitudes, but was unable to find any landmass en route in 1773. Russian Naval Vice-Admiral Bellingshausen sighted the first land, named Peter I Island, on a chart in the south of the Antarctic Circle, but was also unsuccessful in finding the continent in 1821.

The first actual sighting of the continent was in the northern tip of the Antarctic Peninsula by men on the brig *Williams* under command of Captain Edward Bransfield in 1820. Ten months later, American sealer Nathaniel Palmer and his men witnessed the landmass in the same area. The other side of the continent, now called Enderby Land, was first recorded by an English sealer Captain John Biscoe in 1831. The Frenchman Jules-Sebastian Dumont D'Urville and the American Lieutenant Charles Wilkes sailed south of Australia and claimed to have discovered the landmass by sight in 1841.

A significant discovery of the time came later in 1841 when Sir James Clark Ross entered into an open sea through heavy ice packs, and as Ross and his men were travelling south they discovered mountain ranges in Victoria Island, an active volcanic mountain named after his ship, Mt Erebus, and a great white ice shelf, aptly named the Barrier. The names of the open sea, the island bearing Mt Erebus, and the Barrier are today named after his remarkable achievements, respectively Ross Sea, Ross Island and Ross Ice Shelf.

1.1.2.2. The Heroic Era (1895 – 1917)

The Ross Sea region was unexplored further because the main focus of the early Antarctic exploration was commercially orientated. The Antarctic Peninsula and neighbouring islands were frequently visited only by sealers who did not publish maps nor communicate their findings as their objective was to protect their trade. The first landings outside the Peninsula took place in the summer of 1895, when a party, including a Norwegian-born Australian settler, Carsten Borchgrevink, landed at Cape Adare. Six months later, the International Geographical Congress participated by several European countries voted Antarctica as a new exploration ground. Subsequently, a period of three decades was coined the "Heroic Era" from which many stories of human courage, endurance, and tragedy under the extreme nature of Antarctica have been colourfully described in the literature. For general references on the Heroic Era, see Quartermain (1967), Reader's Digest (1985) and McGonigal and Woodworth (2001).

The three expeditions relevant to this study in the Heroic Era are the British National Antarctic Expedition (Discovery Expedition) (1901–1904) and the British Antarctic Expedition (Terra Nova Expedition) (1910–1913) both led by Captain Robert F Scott, and Ernest Shackleton's British Antarctic Expedition (1907–1909). These three expeditions each established their base on Ross Island. Incidentally, their final major port before sailing down the Ross Sea was Lyttleton located south of Christchurch.

The Royal Geographical Society together with the Royal Society organised the National Antarctic Expedition (1902–1904) under command of Robert Falcon Scott. Geographical and scientific findings and the accounts of journey are described elsewhere (Huntford 1979; Scott 1905). The expedition members set up an Australian style pre-fabricated wooden hut at Hut Point (77°50'50''S, 166°38'30''E) serving as a barrack as well as a scientific laboratory. This hut functioned as a vital final shelter for those who set out to the South Pole and other southern journeys for Discovery and following expeditions.

Ernest Shackleton, who had been invalided to England in 1903 by Captain Scott, was determined to attain the highest southern geographical latitude, the South

Pole, in his own British Antarctic (Nimrod) Expedition (1907–1909) (Shackleton 1909). Having searched for a suitable landing site for a winter base in King Edward VII Land first, he reluctantly decided to set up a hut at Cape Royds (77°33'10.7''S, 166°10'06.4''E). His expedition was a mixture of success and failure (Huntford 1985). Although achieving the first successful ascent of Mt Erebus (3794 m) and the attainment to the Magnetic South Pole by his members were remarkable per se, his own party had to make a gut-wrenching decision to return within 97 miles of the South Pole in order to survive with shortage of food and fuel. This hut was re-visited by members of following expeditions.

Captain Robert Scott returned to Ross Island and built a hut at Cape Evans (77°38'10''S, 166°25'04''E) in the British Antarctic (Terra Nova) Expedition (1910–1913) for a bid to reach the South Pole for the first time, and to conduct a series of scientific and geographical investigations in the proximity of the Ross Sea region. The prime objective of the expedition was achieved but in an unexpected fashion, the party reached its final destination "without the reward of priority" (Scott 1913). The Norwegian polar explorer Ronald Amundsen had beaten them to the goal by a month. The remainder of the expedition including their deaths and the other aspects of the expedition became well-known through a number of books (Cherry-Garrard 1937; Huntford 1979; Quartermain 1967).

Undeterred by the previous failures, Sir Ernest Shackleton came back to Antarctica in the British Imperial Trans-Antarctic Expedition (1914–1917) with two parties. One party, led by himself, approached the Weddell Sea east of the Antarctic Peninsula in order to cross the continent from the unknown territory to Ross Island via the South Pole. The other was a supporting party laying depots for the crossing party. The supporting party used the hut at Cape Evans as a refuge after their ship was driven from her moorings before unloading sufficient supplies at Cape Evans. Although the expedition did not succeed in its prime objective, their survival was illustrated in an epic book "South" by Shackleton (1922).

After the rescue mission of the marooned men at Cape Evans in 1917, the three huts were rarely visited until the late 1950s. While the Ross Dependency, between the 160th degree of east longitude and the 150th degrees of west, situated south of

the 60th degree of south latitude, became under the jurisdiction of the Governor General of New Zealand by the United Kingdom Order in Council in 1923, the New Zealand government did not participate in activities in the area until two proposals of the Trans-Antarctic Expedition and the International Geophysical Year (IGY) were internationally recognised in 1955 (Quartermain 1967).

1.1.2.3. Conservation and preservation efforts to the historic sites and artefacts

During the IGY New Zealand's Scott Base was established at Fram Point south of Hut Point and the American McMurdo Station in 1957 (Helm and Miller 1964). During the Trans-Antarctic Expedition (1955–1958), minor maintenance was carried out (Quartermain 1963, 1964). The Antarctic Division of Department of Scientific and Industrial Research started a hut caretaker programme by excavation of ice inside the historic huts and restoration of artefacts at Cape Royds and Cape Evans in 1960–1961 and at Hut Point in 1963–1964 summer season (Quartermain 1963, 1964). In the following years, efforts were undertaken to prepare guidelines for the long-term preservation of these huts and plans for the preservation of historic huts were laid in 1979 (Turner 1979).

The Antarctic Heritage Trust (AHT) was founded in 1987 to perform restoration and preservation work on historic buildings and artefacts. The Trust administers historic interests in the Ross Dependency on behalf of the international community in accordance with the provisions of the Antarctic Treaty (1959) and the Protocol on Environmental Protection to the Antarctic Treaty, Madrid (1991). Conservators and specialists have been sent annually to the historic sites.

1.1.2.4. The K021 Event

A group of scientists from the University of Waikato, headed by Professor Roberta Farrell, and Professor Robert Blanchette and his associates from University of Minnesota have been carrying out research at the historic sites on Ross Island since 1997. The event, coded as K021 in the Antarctica New Zealand science events, has three major goals; to identify and characterise biological and non-biological deterioration processes in the historic buildings and artefacts; to investigate microbiological biodiversity at historic sites; and to test

conservationally suitable materials for future preservation work. The research presented here is the result of participation participated in the K021 event in the 2001-2002 summer season.

1.2. Goal, aims and structure of the thesis

The thesis research contributed to one of the K021 goals; investigation of microbial diversity at historic sites on Ross Island. The overall aim of the thesis was to qualitatively assess microbial diversity using classical morphological taxonomy and molecular techniques in the historic hut areas on Ross Island, Antarctica. Chapter one includes geographical characteristics of Antarctica and historical background of the Ross Sea region. Chapter two describes the screening for the presence of *B. anthracis*, a pathogenic and non-endemic organism in Antarctica, with use of molecular techniques in the stable areas of the historic hut at Cape Evans. Chapter three shows mycological diversity inside the historic hut at Hut Point. An emphasis was made in the thesis research to consider the relationship between human activity and microbial introduction.

CHAPTER TWO

2. Molecular Screening for *Bacillus anthracis* at Cape Evans, Ross Island, Antarctica

2.1. Introduction

2.1.1. *Bacillus anthracis* potentially in the historic hut area of Cape Evans

2.1.1.1. Cape Evans Hut

Captain Robert F Scott and his members of the British Antarctic (Terra Nova) Expedition (1910–1913) built a hut at Cape Evans (77°38'10''S, 166°25'04''E) on Ross Island. A large quantity of foodstuffs, coal, and provisions were unloaded at Cape Evans (Scott 1913). The hut was the biggest (15.2m x 7.6m) amongst others built in the Heroic Era (Pearson 1992) which comfortably accommodated 27 people and 17 ponies in the first year and 25 people and 7 Indian mules in the second year (Scott 1913). The hut was re-occupied by 10 members of the Ross Sea party of Shackleton's Trans-Antarctic Expedition (TAE) (1915–1917) after their ship was blown away from the mooring at Cape Evans. Three of the TAE expedition members perished on the way back from the southern depot-laying journey to the Cape Evans Hut. The remainder of the members were rescued by Shackleton in 1917.

The hut was essentially left untouched by human until the IGY (1957–1958). Early conservation efforts were made with regards to excavation of the ice inside the hut, documentation and maintenance of artefacts by New Zealand Antarctic

Society volunteers (Harrowfield 1995; Quartermain 1963). Major conservation work in the stores, annex and stables was carried out, including re-cladding the stables in the 1988–89 summer season (Harrowfield 1995). Today the hut appears to be intact at first sight, but the severe Antarctic weather conditions have contributed to the deterioration of the hut and artefacts at Cape Evans (Farrell et al. 1998a). These processes have been recently well-described, in particular the defibration of the structural wood due to chemical precipitation onto the surface (Blanchette et al. 2002).

2.1.1.2. Speculation of the presence of anthrax causing organism at Cape Evans

There was a report of potential anthrax disease in Captain Scott's expedition (1911 – 1913). Petty Officer Edgar Evans, who was in the Captain Scott's Polar Party, died at the foot of Beardmore Glacier on the way back to the headquarters on Ross Island. Scott (1913) recorded that his death was due to frost-bitten fingers, falls on Beardmore Glacier and loss of confidence in himself. Similarly, Rogers (1974) concluded the probable cause of his death is a combination of nutrient and vitamin deficiency and head injury, subsequently causing a fatal brainstem haemorrhage. In contrast, Falckh (1987) suggested that *Bacillus anthracis* spores were present in the Cape Evans hut and that Petty Officer Evans died from cutaneous infection of anthrax through hand wounds on the polar plateau with anthrax proliferating at a slow rate and consequently causing death. Flackh (1987) speculated that *B. anthracis* spores were brought to the hut at Cape Evans together with live animals such as Manchurian ponies and dogs and a large quantities of fodders and animal carcasses. Falckh's view was solely based on circumstantial and historical records with no supportive scientific evidence.

Figure 2.1 Pictures of the Cape Evans Hut Stable areas in front (top) and inside stables (bottom). (photos by Ryuji Minasaki, January 2002)

2.1.2. *Bacillus anthracis*

2.1.2.1. Bacteriology and Ecology

Bacillus anthracis is a gram-positive, non-motile, rod-shaped, aerobic, and facultatively anaerobic bacterium. It is the causative agent of disease anthrax whose word originated from the Greek word *anthrakos* meaning 'coal' referring to the eschar in the cutaneous form of the disease (Turnbull 2002). The disease has a long association with human activity in different geographic areas and anthrax epidemics have been historically well-recorded (Turnbull 2002). Humans played a major role in the worldwide dispersal of the organism through commercial activity and domesticated animals to areas where *B. anthracis* was previously unreported (Smith et al. 2000; Turnbull 2002).

The organism is an obligate pathogen and predominantly affects herbivorous animals. Occasionally omnivorous and carnivorous animals consuming contaminated meat develop symptoms of anthrax (Figure 2.2). It is widely believed that a complete life cycle, germination of spores, multiplication of bacilli, and sporulation upon encountering unfavourable conditions, is exclusively restricted to inside a host (Mock and Fouet 2001), although there is some speculation of propagation in soil under very specific conditions (Van Ness 1971).

Bacillus species form endospores capable of remaining in a dormant state for an extended period of time and able to resist physical and chemical treatments, such as heat, ultraviolet, and ionising radiation, disinfectants (Gould, 1977). *B. anthracis* spores play a central role in anthrax ecology (Dragon and Rennie 1995). Spores remain dormant in soil until an appropriate host becomes infected. Once the spores enter into the host, rapid germination of spores occurs followed by multiplication of vegetative cells. The host develops the symptoms associated with anthrax and may eventually die whilst vegetative cells start forming spores upon limitation of surrounding nutrients and exposure to free oxygen. The spores are released into the environment and await their next host, as long as it takes, possibly for decades.

With special significance for this study, viability of *B. anthracis* vegetative cells was demonstrated for 8 years after a rapid freezing to –5 ~ –10 °C (Stein 1943). In contrast, viability of the *B. anthracis* spores have been demonstrated much longer, for example, ~40 year-old viable *B. anthracis* spores were isolated in the soil on Gruinard Island, which had been contaminated by high concentrations of the *B. anthracis* spores after intentional release during the Second World War (Manchee et al. 1981). Wilson and Russell (1964) were able to demonstrate viable anthrax spores in dried soil which had been kept in a container for 60 years at room temperature. The limit of spore viability was tested when Redmond et al. (1998) successfully cultured ~80 year-old *B. anthracis* spores kept in a glass capillary tube (20 x 2 mm) embedded in a museum specimen. They found that the recovery of viable spores were difficult, only 4 colonies were isolated from the liquid (10–15 μl) in the capillary by rigorous enrichment procedures and that these colonies were extremely small indicating the *Bacillus anthracis* spores may not be as indestructible as generally perceived (Redmond 1998).

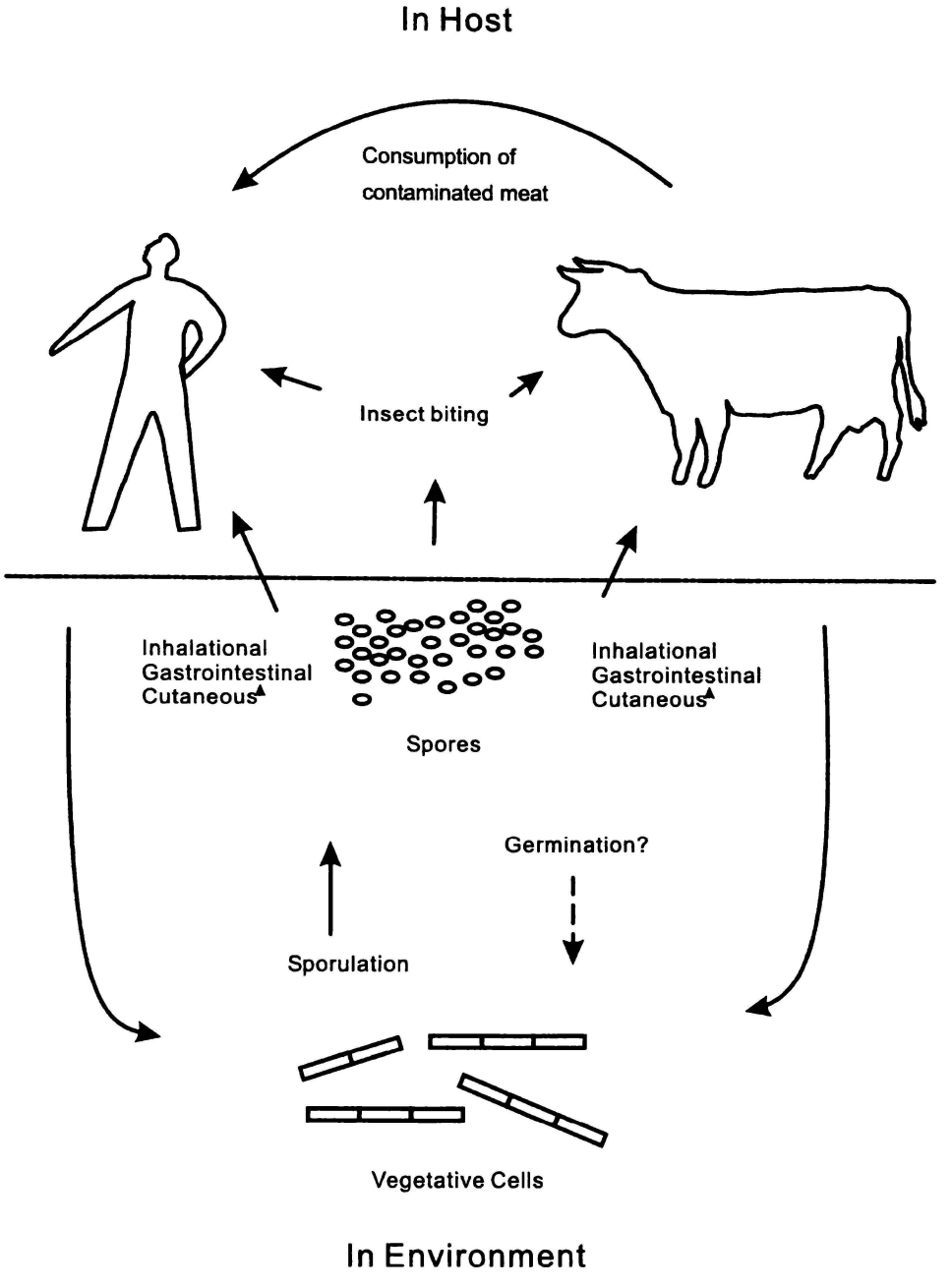

Figure 2.2 Life cycle of *B. anthracis*. Centre of infection route is the spore. Animals take up spores in three routes (inhalational, gastrointestinal, or cutaneous) directly from environment, transmission between animals by insect biting, and consumption of contaminated carcass and meat. Vegetative cells initiate sporulation upon release from the host to surrounding environment in the presence of free oxygen.

2.1.2.2. Anthrax epidemiology and pathology

Transmission of the disease is associated with the introduction of the spores to a host via cutaneous contact, ingestion and/or inhalation (Mock and Fouet 2001; Watson and Keir 1994). Cutaneous anthrax results from contact of *B. anthracis* spore or vegetative cells in wounds or cuts. Consumption of meat contaminated with *B. anthracis* can cause the gastrointestinal anthrax. Inhalation of the spores into lungs brings about the most serious and often fatal form of anthrax. Early diagnosis of cutaneous anthrax is straightforward as a black eschar develops at the point of infection, which can be treated with a variety of antibiotics. Gastrointestinal and inhalational anthrax is rather difficult to identify, since early symptoms of the disease are indistinguishable from flu symptoms of gastroenteritis and slight fever. By the time the disease is diagnosed, no treatment may be effective in saving the life of the patient.

Anthrax has a seasonal occurrence in the natural environment. The exact mechanism has not been fully elucidated but the temperature, rains, drought, soil type, climate, topology, and other factors in the locality are thought to be involved in anthrax transmission to a host (Dragon and Rennie 1995; Dragon et al. 2001; Turnbull 2002; Van Ness 1971). Sources of infections are soil, water, and carcasses which are contaminated by the spores. Anthrax disease transmits from one individual to another only in extremely rare circumstances (Turnbull 2002), except ingestion of anthrax-contaminated carcasses. Insects may also play a role in dispersal of the spores (Ebedes 1976) and a rare case of human cutaneous anthrax has been reported by insect bite (Bradrić and Punda-Polić 1992).

The disease manifests itself according to the health state of the animal and the type of infecting strain (Turnbull et al. 1992). Omnivores and carnivores have a high degree of resistance to anthrax infection, while herbivores are more likely to succumb to the disease (Watson and Keir 1994). People with occupational exposure to anthrax spores for a long period of time may develop some degree of immunity to anthrax infection (Watson and Keir 1994).

Three proteins, protective antigen, lethal factor, and oedema factor, make up the anthrax toxin in binary combinations (Mock and Fouet 2001). These proteins per

se do not elicit anthrax symptoms; combination of protective antigen and lethal factor aggravates death of the animal, and skin oedema is caused by the combinatorial effects protective antigen and oedema factor (Lacy and Collier 2002). The anthrax toxins act upon the endothelial cell membranes causing weakening of the membrane integrity and adhesion of leukocytes and platelets, with consequent vascular tissue damage; ultimately thrombosis, oedema and haemorrhage trigger the sudden death of a host (Hanna 1999; Turnbull 2002).

2.1.2.3. Evolution of the *Bacillus cereus* group

Bacillus anthracis belongs to the *Bacillus cereus* group together with *B. cereus*, *B. mycoides*, and *B. thuringiensis* (Figure 2.3). *B. cereus* and *B. thuringiensis* are also pathogenic microorganisms to humans and insect larvae, respectively. The group has been subjected to extensive taxonomic and phylogenetic investigation due to its genetic similarity and difficulties in identification of the organisms (Keim and Smith 2002). *B. anthracis* requires two plasmids, pX01 and pX02, for its virulence, and when the plasmids are cured (lost) from the host *B. anthracis* it is almost indistinguishable to *B. cereus* (Turnbull et al. 1992).

B. anthracis is thought to have evolved very recently from ancestral *B. cereus* or *B. thuringiensis* by acquisition of two plasmids essential for its virulence (Schupp et al. 2000). Vegetative growth in soil under specific conditions has been hypothesised (Titball et al. 1991; Van Ness 1971), however, it has not been conclusively demonstrated. Evolutionary processes in soil are limited since vegetative cells outside the host do not survive well (Turnbull et al. 1989). Explosive growth occurs upon infection of a host where evolutionary processes (mutation, recombination and selection) may be exerted on the pathogen until sporulation is completed. The evolutionary process is suspended in time in a resting spore state which could last many decades if no host is infected, therefore, *B. anthracis* evolves intermittently. There is very little genetic variability in the *B. cereus* group suggestive of recent divergence. *B. anthracis* strains have minor genetic variability owing to the unique evolution processes.

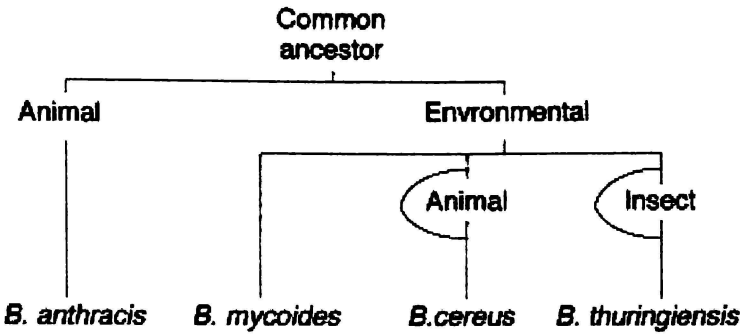

Figure 2.3 Hypothetical evolution of the *B. cereus* group adapted from Turnbull (1999).

2.1.2.4. Genetic similarity within the *B. cereus* group

Genetic evidence based on GC content and sequence similarities indicated that the *B. cereus* group had high similarity in comparison with other *Bacillus* species. Kaneko et al. (1978) compared the GC (%) content and DNA-hybridisation of *B. anthracis*, *B. cereus*, and *B. thuringiensis* and found that the three bacilli all possessed 36.4 ±0.6% (GC) and very high homology (hybridisation-based over 90%), between *B. anthracis* and *B. cereus*. Harrell et al. (1995) investigated sequences of the intragenic spacer region of 16S-23S rDNA and *gyrB-gyrA* of *B. anthracis*, *B. cereus* and *B. mycoides*. They found a single deletion between *B. anthracis* and *B. cereus* and 13 nucleotide changes between *B. anthracis* and *B. mycoides* in the intragenic spacer region of 16S-23S rDNA sequence. For the gyrase intragenic spacer region, *B. anthracis* showed one and two nucleotide differences from *B. cereus* and *B. mycoides*, respectively. An investigation to differentiate the *B. cereus* group by transfer DNA-PCR analysis failed to produce characteristic bands (Borin et al. 1997). Homoduplex-heteroduplex polymorphism analysis of 16S-23S internal transcribed spacers region revealed two groups in the *B. cereus* group, one with *B. anthracis*, *B. cereus*, and *B. thuringiensis* and the other with *B. mycoides* (Daffonchio et al. 2000).

Comparative studies of the 16S rRNA sequence between *B. anthracis* Stern strain and *B. cereus* NCTC11143 strain of 1446 bases showed the region to be identical (Ash et al. 1991). Similarly, 2889 base long 23S rRNA sequences of *B. anthracis* Stern strain and *B. cereus* NCTC11143 strain were almost indistinguishable, only containing a single nucleotide change and a single base insertion in *B. cereus* (Ash and Collins 1992). The results of a study by Helgason (2000) provide support for

the notion that *B. anthracis*, *B. cereus*, and *B. thuringiensis* represent a single species based on genetic information using multilocus enzyme electrophoresis and multiple locus sequence typing. Patra et al. (2002) also found similar results from rRNA ribotyping among the *B. cereus* group.

2.1.2.5. Genetic variability in *B. anthracis*

Bacillus anthracis is the most monomorphic microorganism ever examined (Keim and Smith 2002). Analysis of three *B. anthracis* strains, Ames, Vollume, and Sterne, by pulsed-field gel electrophoresis with restriction enzymes, *NotI*, *SfiI*, and *SmaI* resulted in identical patterns (Harrell et al. 1995). Likewise, total DNA from 40 different *B. anthracis* isolates (Ames, New Hampshire, Pasteur, Stern, Vollum strains) were individually digested with 18 restriction enzymes and all strains generated the exact same patterns from each restriction enzyme treatment (Henderson et al. 1994). However, PCR fingerprinting with arbitrary and specific oligonucleotide primers generated three different profiles, one of which is exclusively characteristic to vaccine strains (Henderson et al. 1994).

Arbitrarily primed PCR fingerprints of Ames, Sterne, and Vollum strains generated the first significant sequence of variable regions (designated as *vrrA* for variable region with repetitive sequence) of *B. anthracis*, in which the Ames and Sterne strains contained four consecutive repeats of CAATATCAACAA and the Ames strain showed one nucleotide deletion (Andersen et al. 1996). The Vollum strain was almost identical to the Sterne strain, except it had two repeats of CAATAACAACAA. Jackson et al. (1997) found that 198 *B. anthracis* isolates could be categorically differentiated by five polymorphisms in *vrrA* with the presence of two to six copies of the CAATAACAACAA sequence, giving rise to five alleles. This type of variable number tandem repeats (VNTR) can be derived from polymerase slippage and unequal crossing over (Keim and Smith 2002).

Keim et al. (1997) used amplified fragment length polymorphism (AFLP) for discrimination of *B. anthracis* isolates and identified two distinct lineages, the 'A' and 'B' lineages, representing non-African and African strains, respectively. This study found a highly polymorphic region termed *vrrB* which was characterised by sequence comparison of 28 diverse *B. anthracis* isolates (Schupp et al. 2000). The

presence of VNTR and 11 different alleles was confirmed, giving rise to 8 different lengths in the *vrrB* open reading frame (Schupp et al. 2000).

A comprehensive study to discriminate among different *B. anthracis* isolates was carried out by Keim et al. (2000) with the use of multi-locus variable number tandem repeats analysis (MLVA) at eight markers, six of which are located on the chromosome and one each on the pX01 and pX02 plasmids. They identified 89 distinct genotypes, which were clustered into six groups (A1, A2, A3, A4, B1, and B2) (Keim et al. 2000). The six groups are further categorically placed as A and B lineages congruent to the previous study (Keim et al. 1997).

Price et al. (1999) focused on genetic variability of the 2,292 base pair long protective antigen (*pag*) gene among 26 diverse *B. anthracis* strains. A total of five point mutations were identified and phylogenetic analysis of the *pag* gene corresponded to the previous study by Keim et al. (1997) indicating that little or no horizontal transfer among different strains had occurred during the plasmid evolution in *B. anthracis*.

Keim and colleagues hypothesised that the geographical origin of *B. anthracis* is southern Africa since the A and B lineages of the organism are present in the Southern Africa and the B branch is exclusively limited to the region (Keim et al. 1997). Smith et al. (2000) screened genotypes of 98 *B. anthracis* isolates from Kruger National Park, South Africa with the MLVA and found that the A and B lineages had individual geographical epicentres correlating to the calcium content and the pH in soil. The A lineage was shown to possess greater genetic variability than the B lineage (Keim et al. 2000), and the authors speculated that genotypic variability had resulted in greater phenotypic adaptability of the A lineages with worldwide dispersal (Smith et al. 2000).

2.1.2.6. *B. anthracis* diagnostics

Diagnostic procedures for the identification of *B. anthracis* are well established using both bacteriological (Logan et al. 1985, Sterne 1959, Turnbull et al. 1992) and molecular genetic techniques (Ellerbrok et al. 2002, Jackson et al. 1998) (Table 2.2 – Table 2.4). A virulent *B. anthracis* strain has a unique characteristic

colonial morphology on nutrient or blood agar characterised by a mat appearance, fairly flat, markedly tacky, white or grey-white and non-haemolytic on blood agar, often having curly tailing at the edges (Turnbull 2002). Cells are non-motile, sensitive to penicillin and gamma phage and capable of producing capsule in blood or on nutrient agar containing 0.7% bicarbonate following incubation in 5-20% carbon dioxide (Turnbull 2002). Identification from phenotypical characterisations was sometimes inconclusive since many *B. anthracis*-like colonies were recovered from necrotic and environmental samples (Turnbull 1999, Turnbull et al. 1992). There has been a report of a penicillin-resistant *B. anthracis* strain (Bradrić and Punda-Polić 1992), consequently creating need for universally accurate diagnostic procedures.

Anthrax pathogenesis requires two plasmids, pX01 and pX02 (Turnbull et al. 1992). The pX01 plasmid has been fully sequenced from the Sterne strain (AF065404) (Okinaka et al. 1999). It is 181,654 nucleotides long and carries the toxin genes *pagA*, *cya*, and *lef*, as well as regulatory elements, a resolvase, a transposase, and the germination operon *gerX*. Plasmid pX02 possesses genes responsible for capsule formation *capA*, *capB*, *capC*, and the gene *dep* for degradation. The plasmid is 96,231 base pair in size (AF188935). *B. anthracis* has been hypothesised to lose pX02 spontaneously in harsh natural environments giving rise to avirulent or attenuated *Bacillus anthracis* (Turnbull et al. 1992). In the laboratory, fully virulent strains are easily cured of either or both plasmids (Uchida et al. 1985). Detection of these plasmids enables investigators to obtain information regarding *B. anthracis* virulence. With the advent of PCR, definitive differentiation of *B. anthracis* and closely related species in the *B. cereus* group is possible with molecular markers on pX01, and pX02 (Table 2.1). The VNTR can differentiate *B. anthracis* genotypes (Jackson et al. 1997) and was used for molecular genotyping for many samples from anthrax epidemics (Jackson et al. 1998; Patra et al. 1998). Ultimately the MLVA analysis (Keim et al. 2000) was developed to reveal specific genotypes of *B. anthracis* strain from a variety of sources, most notably isolates derived from bioterrorism activities (Hoffmaster et al. 2002; Keim et al. 2001).

Table 2.1 List of published markers based on the PCR method for the detection of *B. anthracis*. Accession number is in accordance with the database in National Center for Biotechnology Information (NCBI) Bethesda, Maryland USA.

Location	Gene / Region	Accession number	Reference
Chromosome	*Ba813*	U46157	Patra et al. (1996)
	sap	Z36946	Etienne-Toumelin et al. (1995)
	rpoB	Not available	Qi et al. (2001)
	vrrA	L48553	Jackson et al. (1998)
	vrrB	AF238885	Schupp et al. (2000)
pX01	*pag*	AF306782	Price et al. (1999)
		M22589	Jackson et al. (1998)
		Not available	Ramisse et al. (1996)
	lef	M29081	Jackson et al. (1998)
		M29081	Sjöstedt et al. (1997)
		Not available	Ramisse et al. (1996)
	cya	M24074	Jackson et al. (1998)
		Not available	Ramisse et al. (1996)
pX02	*cap*	M24150	Makino et al. (1989)
		M24150	Jackson et al. (1998)
		M24150	Sjöstedt et al. (1997)
		Not available	Ramisse et al. (1996)

2.1.2.7. Microbial forensics

With the recent explosion of genetic information on *B. anthracis*, for example the *B. anthracis* genome project carried out by The Institute of Genomic Research (TIGR) Maryland USA, it has become feasible to trace probable origin(s) of anthrax outbreaks and to develop an epidemiological perspective. This so called 'microbial forensics' (Cummings and Relman 2002) first characterised the origin of anthrax spores used for bioaggression by the Aum Shinrikyo cult in Tokyo, Japan in 1993 (Keim et al. 2001). The MLVA method identified that the strain released by the cult was most likely the commercially available vaccine Sterne 34F2 strain (Keim et al. 2001).

B. anthracis isolates from a victim from a series of the anthrax-borne-letter bioterrorism acts in Florida in 2001 were rigorously investigated and the complete sequence was soon obtained by TIGR so as to pinpoint the origin of *B. anthracis* isolates and the prime suspect in this bioterrorism act (Read et al. 2002). The VNTR typing recognised these isolates were from the same source, an Ames

strain previously isolated from a dead cow in Texas in 1981 which was subsequently sent to the strain archives of U.S Army Medical Research Institute in Fort Detrick, Maryland. Sequence analysis of the complete genomes with a statistical model to distinguish between true genetic polymorphisms and random sequencing errors, which was tested with use of closely related Ames strains, revealed 11 undetected sequence differences by the VNTR typing between the Florida isolate and Porton isolate (plasmid-cured Ames strain). A Phylogenetic tree constructed from the Florida isolate, four Fort Detrick isolates, the only known Ames strain (Texas 1997 strain) other than Fort Detrick, and two non-Ames strains isolated in the United States showed that the Florida isolate clustered with the Fort Detrick isolates. MVLA confirmed the result of phylogenetic tree that all isolates from victims were indistinguishable to the Ames strain used in the laboratories (Hoffmaster et al. 2002). In conclusion, the Ames strain was used in the biological attach with use of *B. anthracis*, which most likely originated from Fort Detrick. Thus, although *Bacillus anthracis* is extremely monomorphic, it displays sufficient variability to be utilised for MLVA in search of the origin of anthrax epidemics. With MLVA, surveillance of anthrax epidemiology, both natural and artificial, can be achieved at high resolution.

Table 2.2 *B. anthracis* detection method from different matrices and its detection limit from published references.

Reference	Technique	Matrices	Selection/Enrichment	DNA Isolation	Detection limit	Comment
Beyer et al. (1995)	PCR (nested)	Soil from former tannery site	TSB for enrichment No selective media used	Lysozyme, Proteinase K, SDS	1 fg DNA with nested PCR 10 spores in 100 g soil	Targets were genes in pX01 and pX02.
Beyer et al. (1999)	PCR-ELISA	Soil from former tannery site	TSB, Blood agar	Easy DNA™ Kit	100 fg DNA without signal enhancement, 10 fg DNA with AmpliQ 10 spores in 100 g soil	Targets include chromosome, pX01 and pX02. Positive PCR results from uncharacterised sequences partly complementary to the primers
Bruno and Yu (1996)	Immunomagnetic Electrochemiluminescent	Soil	N/A	N/A	10^2 to 10^5 spores in buffer	Detection process is short (less than 1.5 hours). Detection levels were variable with strains, Ames > Sterne > Vollum
Carl et al. (1992)	PCR (nested)	N/A	N/A	Combinations of 15 min boiling, SDS, lysozyme, Proteinase K	2×10^4 spores (non-nested) 2 spores (nested)	Target was pX01. Qunantitated DNA, spore and vegetative cells were used.
Cheun et al. (2001)	PCR (nested)	Lymph node; pig meat	BCA, TSB, PLET	FastPrep™	1 cell of DNA in 1 g of sample	Chromosome, S-layer, pX01 and pX02 markers were amplified. Known amount of spores were inoculated into the matrices.
Dragon et al. (2001)	PLET	Heterogeneous environmental sample	Floating extraction, ethanol purification, PLET	N/A	2 spores per g of sample	Morphological determination after 48 hour incubation. MLVA was used for confirmation.

Abbreviations; BCA, *Bacillus cereus* selective agar; PBS, phosphate-buffered saline; PLET, polymyxin-lysozyme-ethylenediaminetetraacetic acid-thallium acetate plate; TSB, tryptic soy broth

Table 2.3 *B. anthracis* detection method from different matrices and its detection limit from published references. (Continued)

Reference	Technique	Matrices	Selection/Enrichment	DNA Isolation	Detection limit	Comment
Ellerbrok et al. (2002)	TaqMan™ Real Time PCR	N/A	N/A	Isolated from vegetative cells by boiling at 80 °C for 30 minutes	10 spores in 10^6 *B. cereus* or *B. megaterium* background	2-3 hours for confirmation. Targets were cloned into plasmids and used for detection. Successful detection of *B. anthracis* spores without DNA extraction by TaqMan PCR
Gatto-Menking et al. (1995)	Immunomagnetic Electrochemiluminescent	N/A	N/A	N/A	100 spores in buffer	Spore preparation obtained externally. Protocol required less than 45 minutes.
Jackson et al. (1998)	PCR (nested)	Formalin-fixed Human tissues	N/A	Xylene, ethyl alcohol, and acetone washes, then Tris-HCl, EDTA, Tween-20, proteinase K	N/A	DNA recovered from historical human tissues of anthrax victims. Nested primers improved detection levels.
Johns et al. (1994}	PCR	N/A	N/A	Boiling for vegetative cells, bead-beating for spores	200 fg DNA	Exogenous *B. anthracis* DNA may have increased detection level
Makino et al. (1993)	PCR	Blood and spleen in mice	N/A	Lysozyme, SDS, proteinase K	0.25 μg DNA 10^3 spore forming unit	DNA prepared from vegetative cells. Spores were seeded in blood and tissue samples in dilution.
Makino et al. (2001)	LightCycler™ real time PCR	Air	BCA	Heating at 95 °C for 15 minutes	1 spore by real time PCR 10 spores by PCR	Air sample inoculated with spore dilution before enrichment

Abbreviations; BCA, *Bacillus cereus* selective agar; PBS, phosphate-buffered saline; PLET, polymyxin-lysozyme-ethylenediaminetetraacetic acid-thallium acetate plate; TSB, tryptic soy broth

Table 2.4 *B. anthracis* detection method from different matrices and its detection limit from published references. (Continued)

Reference	Technique	Matrices	Selection/Enrichment	DNA Isolation	Detection limit	Comment
Qi et al. (2001)	LightCycler™ real time PCR	N/A	N/A	N/A	N/A	Target was the rpo gene in chromosome. All 144 *B. anthracis* strains resulted in positive reaction. One but all 175 closely related Bacilli did not react.
Manchee et al. (1981)	Nutrient Agar PLET	Soil	Heat treatment of vegetative cells, NA, PLET	N/A	3 spores per g of soil	Environmental sample contaminated with a high concentration of *B. anthracis* was systematically analysed for concentration and distribution.
Ramisse et al. (1996)	Multiplex PCR	N/A	N/A	N/A	N/A	Multiplex PCR was designed to amplify chromosomal marker, pX01 and pX02 simultaneously.
Reif et al. (1994)	PCR	N/A	PBS	Boiling for 30 minutes, or 10 minutes bead-beating	1 spores per PCR reaction	Untreated spores were directly added into PCR reaction mixture, and 100 spores were detectable.
Sjöstedt et al. (1997)	PCR (nested)	11 types of soil	PLET	Mutanolysin, lipase, SDS, proteinase K, CTAB	pX01 – 1 spore (PCR) pX02 – 10 spores (PCR) pX01, pX02 – 1 spore (southern blot)	Spore in dilution series were inoculated in soil before extraction of DNA. Some levels of PCR inhibition were observed. Nested primers improved sensitivity.

Abbreviations; BCA, *Bacillus cereus* selective agar; PBS, phosphate-buffered saline; PLET, polymyxin-lysozyme-ethylenediaminetetraacetic acid-thallium acetate plate; TSB, tryptic soy broth

2.1.3. Ancient DNA

It was hypothesised for this research thesis work that *B. anthracis* could be present in the form of viable spore, non-viable spore, and/or degraded spore in the historic hut areas, especially where ponies were accommodated. With the use of molecular markers to detect targets in samples of historic material, this type of study has been termed as ancient DNA research (Pääbo et al. 1989). Nucleic acids are preserved in a wide range of historic and prehistoric materials from which DNA has been extracted, amplified by PCR and characterised such as in the studies of the systematics of extinct species, human migration and phylogeny, historical epidemics of diseases, and rate of mutations (O'Rourke et al. 2000; Wayne et al. 1999). The time scale of the ancient DNA research ranges from several decades (Taubenberger et al. 1997) to millions of years ago (Cano et al. 1995; Vreeland et al. 2000). Geographical distributions of the ancient DNA samples are diverse; brown bear DNA in the permafrost in the Arctic regions (Barnes 2002), historic human remains from Middle East (Vernesi et al. 2001) and the last meal of a human remain found in a glacier in the Alps (Rollo et al. 2002).

Ancient Adélie penguin DNA isolated from permafrost layers of the penguin bones in the Ross Sea region were in an excellent condition (Lambert et al. 2002). It is however unknown how fast the quality of DNA in a top layer of the Antarctic soil deteriorates where physical (i.e. freeze and thaw) and chemical (i.e. salt concentration) parameters could dramatically change over time, although DNA could be protected by being surface bound. DNA degradation in the environment is brought about by DNA hydrolysis, DNA oxidation, and non-enzymatic DNA methylation (Lindahl 1993). In the extremely arid and cold antarctic conditions, residual DNA in the environment can be mistakenly perceived to be more stable than that in temperate regions, however, the DNA double helix does not retain its structural stability under extremely dry conditions, and DNA in a hygroscopic state is vulnerable to rehydration (Lindahl 1993). Amplification efficiency of ancient DNA is often compromised due to PCR inhibition (Wilson 1997) and DNA degradation (Lindahl 1993). Researches dealing with ancient DNA encounter many challenges in planning, sampling, experimental procedure, interpretation of data, and independent replication (O'Rourke et al. 2000; Wayne

et al. 1999). Failure to comply with rigorous quality control has resulted, in some cases, in heavy criticism (Pääbo et al. 1989; Lindahl 1993).

2.1.4. Scope of this study

This chapter aimed to screen for the presence of *B. anthracis* DNA with molecular diagnostic tools in the stables of the historic hut area at Cape Evans on Ross Island, Antarctica. Absolute care was taken to minimise cross-contamination of samples and introduction of positive *B. anthracis* DNA into the samples. The laboratories (C2.10 and C2.08) had never accommodated *B. anthracis* or *B. anthracis* DNA with the exception of the positive control DNA in the current study. DNA extraction, PCR preparation, and electrophoresis were carried out at different locations. DNA handling took place in a laminar flow cabinet and a Physical Containment Level 2 cabinet with aerosol resistant tips and dedicated pipettes. Appropriate positive and negative controls were used throughout the study as specified in the Materials and Methods.

The research described in this chapter was original as carried out by the author and was supervised by Professor Roberta Farrell and Dr Ron Ronimus. The samples were independently examined at National Centre for Disease Investigation, Wellington, New Zealand and were to be analysed by Dr Pamala Coker and Dr Kimothy Smith at Lawrence Livermore National Laboratory, California, USA for replication and for germination of viable *B. anthracis* spores.

2.2. Materials and Methods

2.2.1. Sampling

The area for sampling is located at Cape Evans (77° 38' 10"S, 166° 25' 04"E), Ross Island, Antarctica. Inside and outside of the stable area was extensively sampled by the candidate in January 2002 (Figure 2.1, Figure 2.4). A total of 74 samples were collected as aseptically as possible. All precautions were taken to minimise possibility of inhaling *B. anthracis* spores (gloves, mask, and glasses). The samples were stored in 50 ml sterile falcon tubes (Greiner bio-one, USA) and brought back to New Zealand under the Ministry of Agriculture and Fishery Permit Number 2001013830.

2.2.2. DNA extraction

DNA from environmental samples were prepared by a modified PSB (phosphate, SDS, and bead-beating) method (Miller et al. 1999) from approximately 0.5 – 1 g of samples. Samples were transferred into a 2 ml bead-beater vial (Porex Bio Products Inc, USA) containing 0.5 g of 0.1 mm zirconia/silica beads and 0.5 g of 2.5 mm glass beads (Biospec Products Inc, USA), and re-suspended in 300 µl of phosphate buffer (100 mM NaH_2PO_4), 300 µl of SDS lysis buffer (100 mM NaCl, 500 mM Tris pH 8.0, 10% SDS), and 300 µl of chloroform-isoamyl alcohol (24:1). The vials were shaken in a FastPrep® FP120 Cell Disrupter (BIO101 Thermo Savant, USA) at 4.5 m/s for 45 seconds and centrifuged at full speed for 5 minutes (Eppendorf Centrigufe 5415D, Hamburg, Germany) to pellet debris. After transferring the supernatants to new microfuge tubes, ammonium acetate was added to make a final concentration of 2.5 M, and the samples gently mixed and spun down for 5 minutes at maximum speed. The supernatants were transferred into new microfuge tubes and 0.54 volumes of isopropanol added. Pellets were formed after 15 minutes incubation at room temperature followed by centrifugation for 15 minutes at maximum speed. The supernatants were carefully removed and washed twice with 1 ml of 70% ethanol. The pellets were air-dried and re-suspended in 100 µl molecular grade TE buffer at pH 7.5 (USB Corporation, USA).

2.2.3. Optimisation of bead-beating application time

Spores are very resistant to physical and chemical agents (Gould 1977). Bead-beating application time for 10 minutes had previously been tested and shown to disrupt only *B. anthracis* spores without addition of environmental matrices (Johns et al. 1994; Reif et al. 1994). The efficiency of the PSB-bead-beating (PSB-BB) DNA extraction method on spores, however, has not been tested (Miller et al. 1999). Scoria and hay samples were used in comparison with the scoria sample inoculated with *B. licheniformis* spores to measure the efficiency of the PSB-BB method on spores and DNA quality for subsequent PCR. The three samples were individually subjected to 3/4, 3, 6, and 9 minutes of mechanical mixing, while the tubes were kept cold in ice at every 3/4 minutes. PCR was performed with the universal 16S rDNA gene primers (Johnson 1994).

2.2.4. DNA quantitation

The amount of DNA extracted from samples was measured spectrophotometrically. An aliquot, 10 µl, of original suspension was diluted into 90 µl Milli-Q prepared water (Milli-Q H_2O). For quantitation of double stranded DNA, an optical density (OD) of 1 corresponded approximately to 50 ng/µl at 260 nm. Purity of DNA was estimated from the ratio of OD_{260}/OD_{280} (Sambrook et al. 1989).

2.2.5. Positive *B. anthracis* DNA and negative control DNA

Dr Souichi Makino from Obihiro Veterinary University, Japan kindly provided *B. anthracis* DNA, Pasteur II strain (Uchida et al. 1993), which was used as a positive control DNA. *B. cereus* and *B. licheniformis* spores were kindly provided from Thermophile Research Unit, University of Waikato. The *B. cereus* strain type is not known. *B. licheniformis* is a strain isolated from a milk powder product plant and is similar to the type strain DSMZ8785 (Ronimus et al. in press). DNA was extracted from bacilli spores as described in Section 2.2.2.

2.2.6. Polymerase Chain Reaction optimisation

DNA templates from environmental samples are often problematic in PCR because many contaminants can affect the efficiency of *Taq* polymerase (Wilson

1997). This problem can sometimes be solved by increasing the amount of *Taq* polymerase in the reaction mixture (Sjöstedt et al. 1997). PCR reactions were carried out with DNA preparations of sample #65 and #74 which were spiked with 1 pg of *B. anthracis* DNA at four different concentrations of *Taq* polymerase to determine the minimum amount required for sufficient amplification. These concentrations ranged 0.5, 1.0, 1.5 and 2.0 units in a 25 µl reaction. For all other environmental DNA samples, the PCR reaction was carried out as in the Section 2.2.8.

2.2.7. Detection level

There are two advantages of using nested primers. Nested primers can increase the sensitivity of PCR when target DNA is in low concentration and can also give higher specificity in selection of target sequence among DNA from environmental samples. The use of a second PCR with an inner pair of primers improves the efficiency of the PCR (Beyer et al. 1995). Published nested primers were used in this study (Table 2.5).

2.2.8. Polymerase chain reaction

Polymerase chain reaction (Saiki et al. 1988) was performed as previously described (Jackson et al. 1998). The initial PCR was performed in a total volume of 25 µl with the following components: 10 mM Tris-HCl, 15 mM MgCl2, 50 mM KCl, (pH 8.3 at 20 °C) (Roche Diagnostics GmbH, Germany); 0.2 mM dNTPs (all four dNTPs) (Roche Diagnostics GmbH, Germany); 0.20 µM of each primer (Invitrogen NZ), 1.5 units of *Taq* DNA polymerase (Roche Diagnostics GmbH, Germany), and 10 – 100 ng of template DNA. The reaction mixture was denatured for 5 minutes at 95 °C, followed by 40 cycles (30 – 35 cycles for the nested PCR) of denaturation for 1 minute at 94 °C, annealing for 1 minute at 55 °C, and extension of primers for 1 minute at 72 °C. Normally the final incubation is to ensure all products have been amplified and become double-stranded, the reaction mixture was held at 72 °C for 5 minutes, then cooled to 5 °C. PCR was achieved by Eppendorf Mastercycler® (Eppendorf-Netheler-Hinz GmbH, Germany).

2.2.9. Fluorescence resonance energy transfer PCR

Fluorescence resonance energy transfer (FRET) PCR exploits the 5'-exonuclease activity of DNA polymerase which cleaves fluorescein-labelled oligonucleotide probes as extension stage of PCR progresses, resulting in fluorescence emission (Wittwer et al. 1997). Fluorescence is measured at each cycle and is related to the original target concentration. The technique requires designing primers and probes specific to the target sequence. Failure of annealing in either primers or probes during PCR will result in no amplification of PCR products, and thus no associated increase in fluorescence. FRET-PCR was carried out according to Qi et al. (2001). The PCR mixture contained; 1× TaqMan® Universal PCR Master Mix (Applied Biosystems, USA), 1μM each of rpoBF1 primer (CCACCAACAGTAGAAAATGCC) and rpoBR1 primer (AAATTTCACCAGTTTCTGGATCT) (Invitrogen NZ), 0.2 μM BaP1 probe (6FAM-TCCAAAGCGCTATGATTTAGCAAATGT-TAMRA) (Applied Biosystems, CA, USA), and 10-50 ng of the DNA extracts. The FRET-PCR was accomplished using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, USA) under the following conditions; initial denaturation for 30 seconds at 95 °C, followed by 50 cycles of denaturation for 5 seconds at 95 °C, annealing for 15 seconds at 63 °C, and extension of primers for 30 seconds at 72 °C.

2.2.10. Electrophoresis

PCR products were analysed by agarose gel electrophoresis through 1.8–2.2 % (wt/vol) SeaKem agarose gel (BMA Inc, USA) prepared with TAE buffer (40 mM Tris-acetate and 1 mM EDTA) in EASYCAST™ (Owl Separation Systems, USA) electrophoresis boxes. A 6× loading buffer (0.25% bromophenol blue and 40% glycerol in water) was mixed with PCR product and molecular markers (50 bp DNA ladder Invitrogen, USA) equivalent to 0.5 μg per lane were loaded into a gel. Electrophoresis was for 2–3 hours at 70 volts. Gels were stained for 30 minutes in water containing ethidium bromide (0.5 μg / ml) and de-stained in running tap water for 30 minutes before visualised using UV illumination.

2.2.11. Freeze and squeeze method for purification of amplicons

A method called "freeze and squeeze" was performed to remove primers and non-target PCR products before sequencing (Thuring et al. 1975). A sufficient amount of PCR product (approximately 300 µl) was loaded on a thick (8 mm) agarose gel (1.2–1.5 % wt/vol) and ran at 50 volts for 2-3 hours before staining and de-staining. The band of interest was cut with a microscope slide cover. The gel piece was placed in an appropriate sized piece of parafilm, folded and frozen overnight at –20 °C. The parafilm-wrapped gel piece was gently, but firmly squeezed using gloved hands so that droplets containing the DNA fragment of interest were collected into a microfuge tube leaving the agarose behind.

2.2.12. DNA clean-up

The microfuge tube containing fragments of agarose and DNA was spun for 1 minute at 5000 g to remove residual agarose. The supernatant was transferred without taking the agarose pellet into a new tube, then DNA was extracted with 1 volume of phenol:chloroform:isoamyl-alcohol (25:24:1). Phases were separated by centrifugation for 3 minutes at 5000 g, and the top layer transferred to a new tube. DNA was precipitated by addition of ammonium acetate to make a final concentration of 2.5 M and 1 volume of ice-cold isopropyl-alcohol. After incubation for 15 minutes at room temperature, the DNA pellet was obtained by centrifugation at maximum speed for 20 minutes and was washed twice with 70% ethanol. Residual ethanol in the pellet evaporated in an incubation oven at 35 °C for 1 hour and the DNA was re-suspended in 50 µl TE buffer (pH 7.5) or Milli-Q H_2O.

2.2.13. Sequencing

DNA sequences were obtained from the University of Waikato DNA Sequencing Facility using the ABI Prism 7700 Sequence Detection System and Amersham Biosciences MegaBACE DNA analysis system.

2.2.14. Sequence alignment analysis

Sequences were aligned by the Clustal W programme available at a website of European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw/).

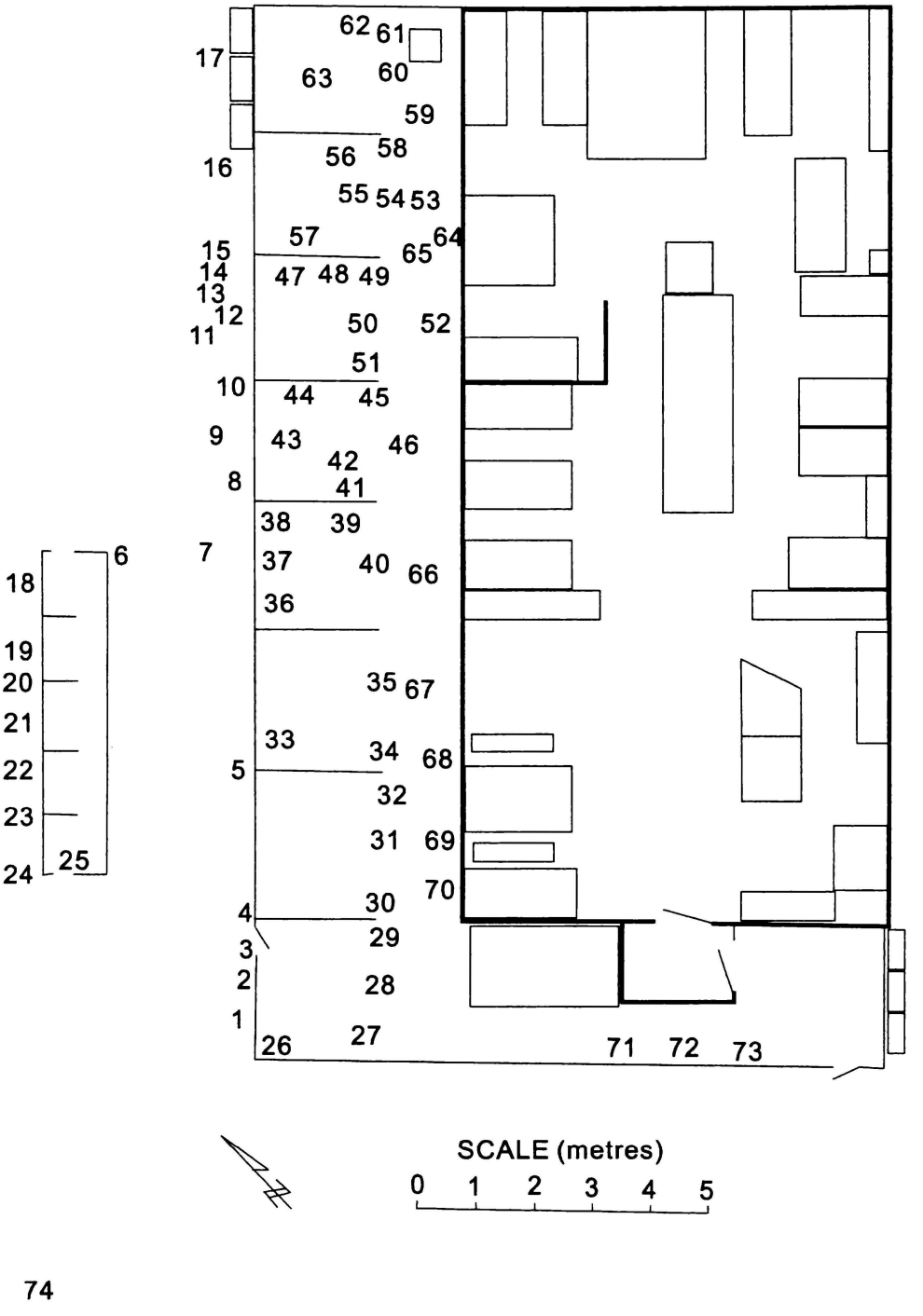

Figure 2.4 Sampling locations at Cape Evans Hut, Ross Island, Antarctica

Table 2.5 Primers used for *Bacillus anthracis* detection and sequencing. Primers are used according to Jackson et al. (1998), Price et al. (1999) and Cheun et al. (2001). Amplicon sizes are expressed in base pairs. 'P' indicates the primary primer sets while 'N' is for the nested primer sets. Accession numbers are for the respective gene target sequences.

Primer set	P / N	Accession no.	Location	Sequence (5'–3')	Amplicon sizes
GPR-4	P	L48553	C	ACA ACT ACC ACC GAT GGC	377-425
GPR-5	P			TTA TTT ATC ATA TTA GTT GGA TTC G	
EWA-1	N	L48553	C	TAT GGT TGG TAT TGC TG	142-190
EWA-2	N			ATG GTT CCG CCT TAT CG	
PA-1F	P	M22589	pX01	CCA GAC CGT GAC AAT GAT G	511
PA-1R	P			CAA GTT CTT TCC CCT GCT A	
PA-2F	N	M22589	pX01	CGA AAA GGT TAC AGG ACG G	409
PA-1R	N			CAA GTT CTT TCC CCT GCT A	
CAPA-F	P	M24150	pX02	CAG AAG CAG TAG CAC CAG TAA	329
CAPA-R	P			ATT TTC ACC AGC ACC CAC	
CAPA-Fnest	N	M24150	pX02	TGA CGA TGG TTG GTG ACA	305
CAPA-Rnest	N			CCT TAT TGT ATC TTT AGT TCC C	
vrrB-F	P	AF238895	C	ATA GGT GGT TTT CCG CAA GTT ATT C	352-424
vrrB-R	P			CCC AAG GTG AAG ATT GTT GTT GA	
PA-5F	P	M22589	pX02	ATC CTA GTG ATC CAT TAG AAA CGA C	330
PA-5R	P			CTT CTC TAT GAG CCT CCT TAA CTA CTG	
PA-5F-nest	N	M22589	pX02	AGT GAT CCA TTA GAA ACG AC	307
PA-5R-nest	N			TAA CTA CTG ACT CAT CCG C	
PA-Makino-F	P	M22589	pX02	GAGGTAGAAGGATATACGGT	596
PA-Makino-R	P			TCCTAACACTAACGAAGTCG	
PA-Makino-F-nest	N	M22589	pX02	ATCACCAGAGGCAAGACACCC	210
PA-Makino-R-nest	N			ACCAATATCAAAGAACGACGC	

2.3. Results

2.3.1. Sampling

A total of 74 samples of heterogeneous nature consisting mostly from scoria and hay materials were collected inside and outside the historic hut at Cape Evans (Figure 2.4). The sampling spots were arbitrarily chosen as some areas were permanently frozen preventing from sampling.

2.3.2. DNA extraction and quantitation

The amount of DNA recovered from the DNA extraction procedure varied significantly among samples. There was no apparent association between sample types (scoria and hay) and the amount of DNA recovered. Overall, the bead-beating method produced sufficient amount of DNA from environmental samples for PCR reactions (Table 2.6).

2.3.3. Bead-beading application time

The bead-beating method produced sufficient amount of DNA, and possibly RNA, as visualised (in reverse image) in a dark broad band at top of lanes (Figure 2.5). Increased application time for bead-beating generated, most likely was due to the sharing effect of matrices and DNA, smaller fragments. A bead-beating application time of 3/4 minute produced amplifiable DNA with the universal 16S rDNA gene primers (Johnson 1994) when extraction were from soil matrices. The hay sample did not produce amplifiable DNA with the universal primers possibly due to failure of the PCR inhibition of *Taq* polymerase by plant polysaccharides. When the samples were bead-beaten more than 3 minutes, none of the subsequent PCRs were successful. It was concluded that bead-beating for 3/4 minute would generate DNA which could be amplifiable with PCR. It is, however, unclear whether all spores were ruptured sufficiently from this procedure.

Table 2.6 Sample weight and DNA concentration. Samples were subjected to the PSC bead-beating method for DNA extraction. Ratio indicates visual approximation of sample contents between scoria and hay (S, scoria over 80%; S/H, scoria between 80 – 50%; H/S, hay between 80 – 50%; H, hay over 80%). Sample 74 is animal dung (D).

Sample number	Weight (g)	DNA conc. (ng /µl)	Ratio	Sample number	Weight (g)	DNA conc. (ng / µl)	Ratio
1	1.139	62.35	S	38	0.345	47.33	H/S
2	1.517	149.56	S/H	39	0.722	70.57	S/H
3	1.096	110.27	S	40	0.459	18.87	S
4	1.076	205.60	S/H	41	0.246	9.91	H
5	1.408	169.83	S	42	0.121	9.23	H
6	1.042	100.10	S	43	0.164	41.77	H
7	0.552	148.88	H/S	44	0.235	64.23	H
8	0.492	52.83	S/H	45	0.354	19.66	S/H
9	0.498	132.03	S	46	1.615	2.00	S
10	0.532	91.80	H/S	47	0.284	33.27	H
11	1.723	10.00	S	48	0.238	18.28	H
12	0.308	44.50	H/S	49	1.444	38.82	S/H
13	0.908	18.20	S/H	50	1.156	33.51	S
14	1.033	11.20	S/H	51	1.395	36.37	S/H
15	1.173	15.20	S/H	52	1.607	21.27	S/H
16	0.551	36.20	HS	53	1.607	12.01	S
17	0.854	29.70	S/H	54	1.643	18.37	S/H
18	1.052	8.66	S	55	0.956	5.48	H/S
19	0.955	58.50	S	56	0.419	207.90	H/S
20	0.593	11.10	S	57	0.219	210.10	H
21	0.752	31.00	S	58	0.487	14.70	S/H
22	0.842	19.30	S	59	1.562	15.48	S/H
23	1.048	39.80	S	60	0.353	4.46	H/S
24	1.140	46.60	S	61	0.363	3.85	H/S
25	0.854	52.60	S	62	0.964	30.23	S/H
26	1.063	16.20	S	63	0.566	18.06	S/H
27	1.887	42.79	S	64	0.155	26.03	H
28	1.505	26.90	S/H	65	1.702	43.00	S/H
29	1.751	93.30	S	66	1.674	36.93	H
30	1.005	15.20	S	67	1.347	30.93	S
31	0.751	41.40	S/H	68	1.508	18.12	S
32	1.444	242.73	S	69	1.642	3.00	S/H
33	0.430	139.36	H	70	1.604	27.77	S
34	0.252	24.80	H	71	2.455	0.56	S
35	2.593	27.54	S/H	72	2.533	13.02	S
36	0.253	34.45	H	73	1.900	1.40	S
37	0.177	7.29	H	74	0.266	163.30	D

Sample number	11				7				11			
Sample type	Scoria				Hay				Scoria spiked with *B. licheniformis* (2.0×10^8)			
Application time (min)	0.75	3	6	9	0.75	3	6	9	0.75	3	6	9

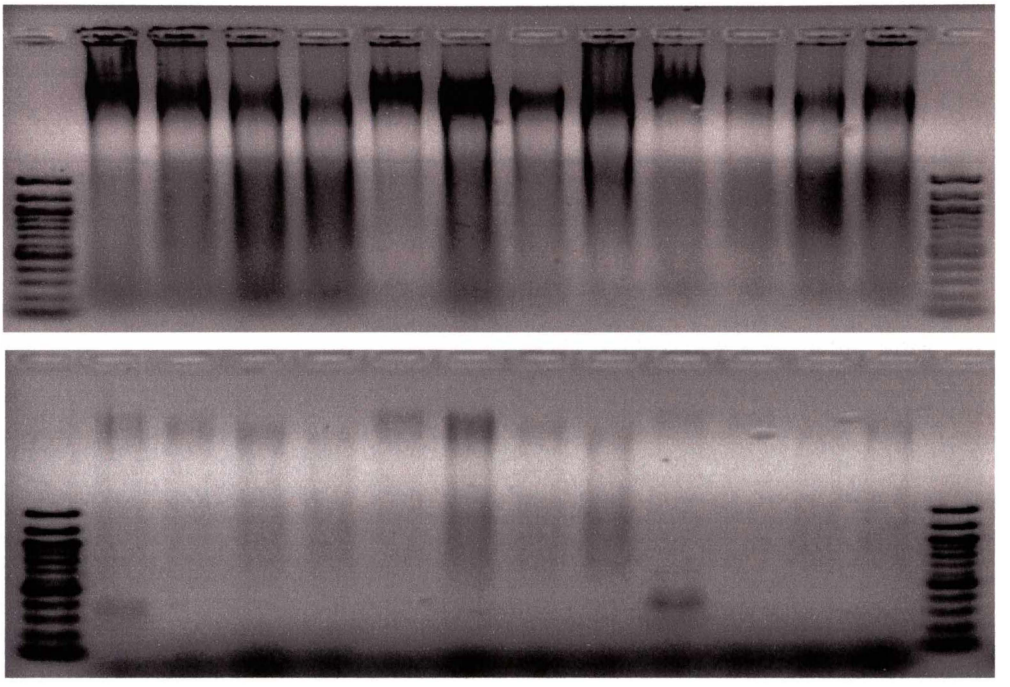

Figure 2.5 Effect of bead-beating application time on PCR. Samples # 11 and #7 were bead-beaten for 0.75, 3, 6, and 9 minutes. *B. licheniformis* spores were inoculated into sample #11 to observe the efficiency of the protocol on the bacilli spores. The DNA/RNA extracts (top) and PCR products (bottom) were run on the agarose gel (1.2%) for 2 hours at 70 volts. The 100 bp ladder (Invitrogen NZ) was run along the extracts and PCR products in the both end of the lanes with a thick band at 500 bp position.

2.3.4. PCR optimisation

DNA from environmental samples is known to contain a diversity of contaminants, and consequently efficiency of PCR can be greatly reduced (Wilson 1997). Figure 2.6 shows the efficiency of PCR in relation to the concentration of *Taq* polymerase. PCR did not amplify the expected amplicon 409 bp long from environmental samples (74 and 63) with CAPA-F/R primers at 0.5 units of *Taq* polymerase. The positive DNA was unable to be amplified with 0.5 units of *Taq* polymerase. With increasing concentration of *Taq* polymerase from 1.0 to 2.0, it

was evident that the intensity of the bands was enhanced for both environmental samples. The pure positive DNA preparation did not show much difference once the *Taq* concentration was increased above 1.0. Considering the effectiveness of the polymerase chain reaction to produce DNA amplicons and the cost of *Taq* polymerase, it was decided that 1.5 units of *Taq* polymerase per reaction with 10–50 ng DNA would be used for all subsequent PCR assays, unless otherwise stated.

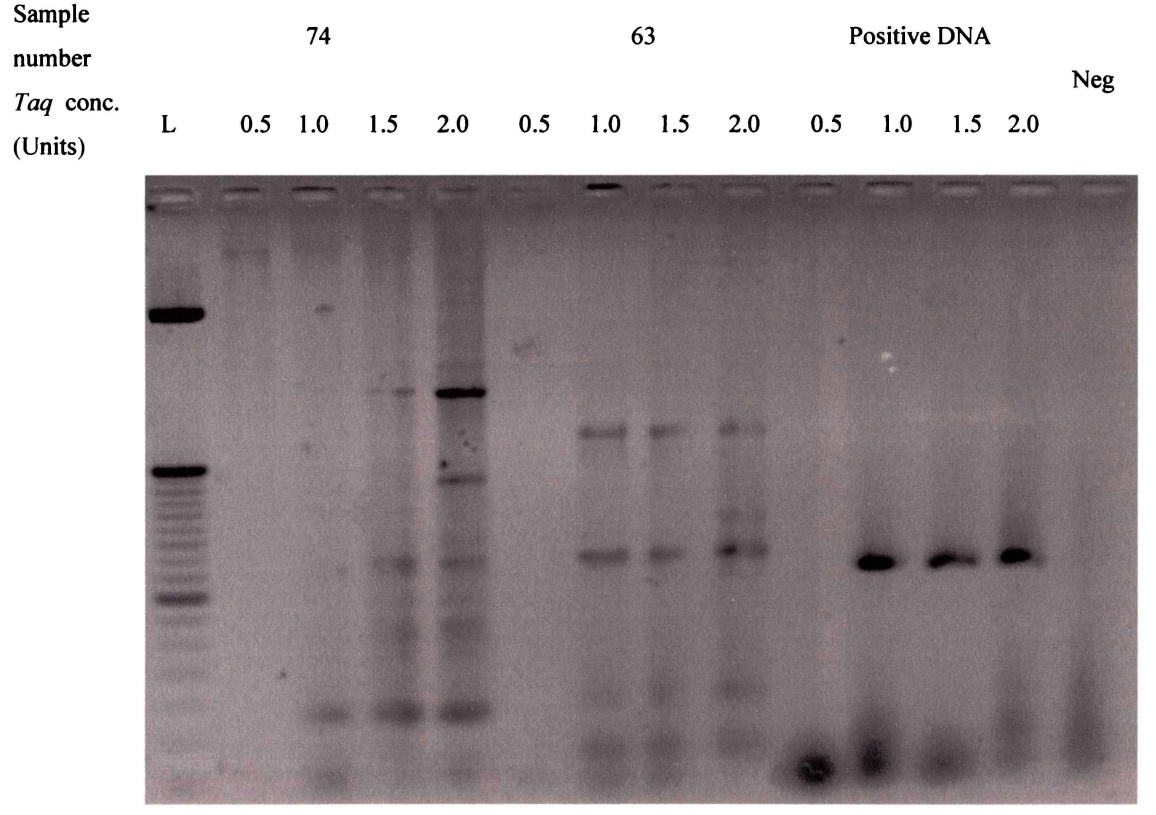

Figure 2.6 Effect of *Taq* polymerase concentration on PCR efficiency. The sample #74 and #63 are inoculated with 1pg of positive *B. anthracis* DNA. *Taq* polymerase concentration increased from left to right (0.5, 1.0, 1.5, and 2.0 unites). The CAPA-F/R primers were used to amplify 409 bp amplicons. Negative control (no DNA) is at extreme right lane. The 50 bp molecular marker (Invitrogen NZ) is on the extreme left lane.

2.3.5. Detection Level

Results of assessment in detection levels targeting different locations of *B. anthracis* with the positive *B. anthracis* DNA are summarised in Figure 2.7. The

marker in the *vrrA* gene on the chromosome amplified by GPR F/R primers had a lowest detection level at 100 pg. The plasmids, both pX01 and pX02, had higher detection levels than the chromosomal marker, as PA1 F/R and CAPA1 F/R primers amplified the targets from 1 pg and 10 fg positive DNA, respectively. The CAPA1 F/R primers were chosen for the initial screening of the 74 environmental samples owing to its highest level of detection. Thereafter samples resulting in a positive PCR with a band at approximate size (409 bp) were further analysed with six sets of primers.

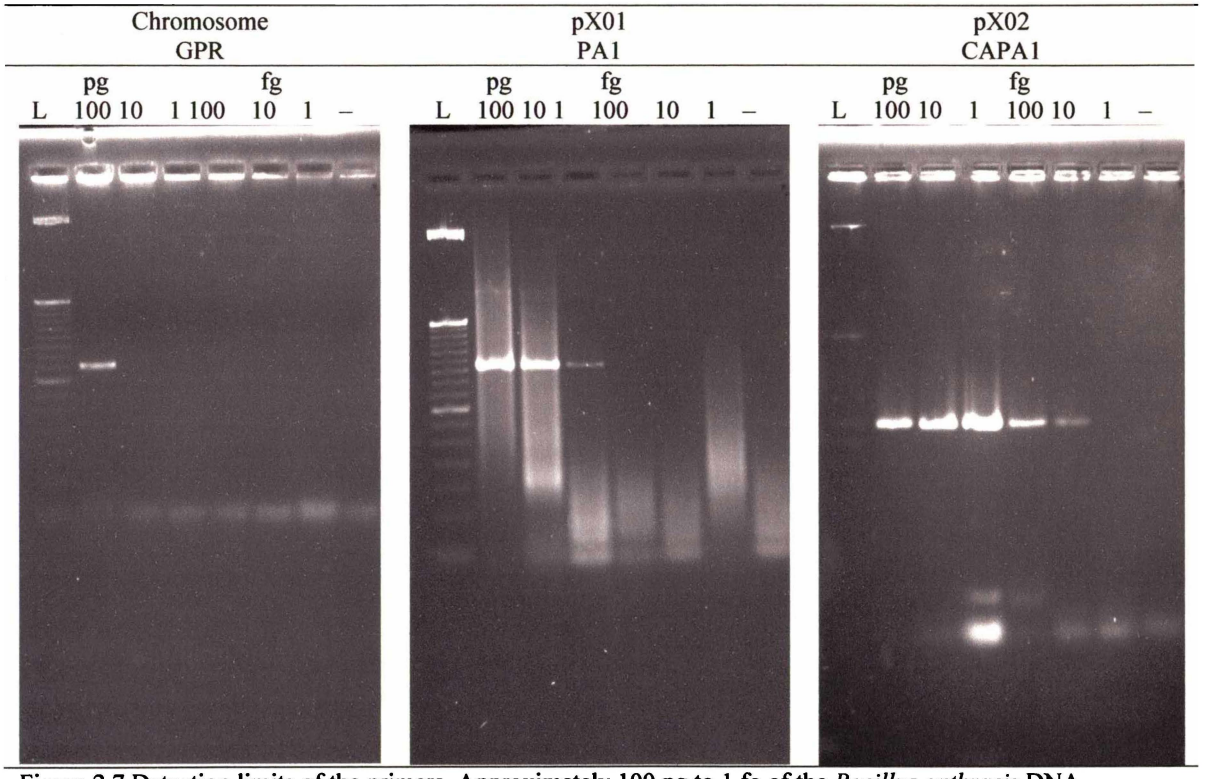

Figure 2.7 Detection limits of the primers. Approximately 100 pg to 1 fg of the *Bacillus anthracis* DNA (Pasteur II) was used for PCR with three markers, PCR products were then ran on the agarose gels. Negative control and 50 bp ladder were located at extreme right and left, respectively.

2.3.6. Screening with CAPA primers

The results of the initial screening of 74 environmental DNA preparations with CAPA F/R primers are tabulated (Table 2.7). All samples were tested with (spiked) and without positive *B. anthracis* DNA. A total of 25 environmental DNA samples without positive DNA spiking (10 pg) resulted in successful PCR with a band at approximate size of 409 bp. Many samples failed to produce a band in the gel profile, suggesting that there were no target sequences available for

primer binding or that there may have been contaminant(s) inhibiting the PCR reaction, or both. Samples in bold on Table 2.7 were selected for further analysis based on intensity of their bands at 409 bp with an additional 6 sets of primers.

Table 2.7 Results of PCR with CAPA primers. PCR results are categorised as following; positive PCR reaction with observation of amplicon at approximate size are expressed as positive (+), while no amplification or amplification without expected amplicon is marked as negative (-). Samples in bold were further analysed with an additional six sets of primers in Section 2.3.7.

Sample number	PCR result		Sample number	PCR result	
	Sample	Spiked		Sample	Spiked
1	-	-	38	-	-
2	-	-	**39**	+	+
3	-	-	**40**	+	+
4	-	-	41	+	+
5	-	-	42	-	+
6	-	-	43	-	-
7	-	-	44	-	-
8	-	-	45	+	+
9	-	-	**46**	+	+
10	+	+	47	-	-
11	+	-	**48**	+	+
12	-	-	49	-	-
13	-	-	50	-	-
14	-	-	51	-	-
15	+	-	52	+	-
16	-	-	**53**	+	-
17	-	-	54	-	-
18	-	-	55	+	-
19	-	-	56	-	-
20	+	-	57	-	-
21	+	+	**58**	+	+
22	-	-	**59**	+	+
23	+	+	**60**	+	+
24	+	+	61	-	+
25	-	-	**62**	+	+
26	-	-	63	+	+
27	-	-	64	-	-
28	-	-	65	-	-
29	-	-	66	-	-
30	-	-	67	-	-
31	+	-	68	+	+
32	-	-	69	+	+
33	-	-	**70**	-	-
34	-	-	**71**	+	+
35	-	-	72	-	+
36	-	-	73	-	-
37	-	-	74	-	-

2.3.7. Screening with primary and nested primers

The potentially positive environmental samples were further analysed with three sets of primers (nested primers) for a subsequent PCR (Table 2.5, Appendix A). The *vrrB* primers were obtained at this stage and included for strain characterisation (Schupp et al. 2000). The *vrrB* primers do not have the nested primer sets. The results were a mixture of successful and unsuccessful amplifications of the expected amplicons (Table 2.8, Appendix A). The nested primers increased the sensitivity of detection as is evident in the case with the *vrrA* gene using the EWA F/R primers. The first round of PCR with the GPR F/R could not amplify visible amplicons of the right size on the gel, but the EWA F/R primers produced a thick band (Appendix A). Similarly, the other nested primers gave positive results whereas the first primer sets could not. However, samples 39, 48, and 58 did not generate expected amplicons with the nested primers despite the first round of PCR with the CAPA1 F/R primer which showed signs of amplification. Sample 68 was the first sample which showed positive amplification with the expected size band for all six primers. At that time, the *vrrB* primers were not available and soon the DNA extract of sample 68 became limiting, and thus no data for the *vrrB* locus was obtained.

2.3.8. FRET

Fluorescence resonance energy transfer (FRET) PCR did not result in clear and sharp amplification in both positive control DNA (Pasteur II) and environmental samples. When positive control DNA (~400 pg) was analysed, exponential amplification started only after 45 cycles. Similarly, environmental samples (41, 45 and 68) with positive reactions in the initial screening with CAPA F/R primers showed a sign of amplification only after 60 cycles or failed to react at all.

2.3.9. Sequence analysis

DNA sequences of various regions on the chromosome and pX02 from 6 samples, as attained by the University of Waikato DNA Sequencing Unit, of specific PCR amplicons were aligned and compared to the positive *B. anthracis* DNA sequence data and sequences from the database at the National Center for Biotechnology Information (NCBI). The *vrrA* sequence of sample 32, 40, 53 and 70 were

obtained and aligned with the Clustal W program (Appendix B). The sequences of sample #40 and #53 were identical to the positive control DNA and the database on NCBI typical of Pasteur, Sterne, and Ames strains having four repeats of CAATATCAACAA. One nucleotide change (A → G) was found in sample #70 in the sequencing reaction (one forward and one reverse), but when sequencing was carried out with a fresh amplicon of sample #70 (one forward and one reverse), the sequence did not result in the same mutation (Figure 2.8). The sequence amplified by *vrrA* F/R primers from sample #32 revealed an interesting outcome. It has a sequence similarity to Vollum strain of *B. anthracis* with several nucleotide mutations. The sequence has close similarity to Vollum (e value – $4e^{-33}$), Sterne (e value – $2e^{-25}$), Ames (e value – $2e^{-25}$), *B. mycoides* (e value – $2e^{-7}$), and *B. cereus* (e value – $8e^{-7}$), respectively. The Expect value (e) is a parameter that describes the number of hits one can expect to see just by chance when searching a database of a particular size and the lower the e-value, or the closer it is to 0 the more significant the match is. These sequences were covered by four sequencing reactions (each forward and reverse sequenced twice).

The sequence amplified by PA2 F/R primers from sample #60 matched exactly with the positive control DNA. Similarly, the *vrrB* sequence of sample #53 was identical to the positive DNA and the most common profile G amongst 11 *vrrB* haplotypes (Schupp et al. 2000). The depth of coverage was two times (one forward and one reverse) for these sequences.

Figure 2.8 The *vrrB* sequences of sample #70. The consensus *vrrB* sequence of *B. anthracis* is 5′-GCATGATGCACA-3′. A Single nucleotide change was observed from A to G in the sequences obtained from separately prepared PCR products. .

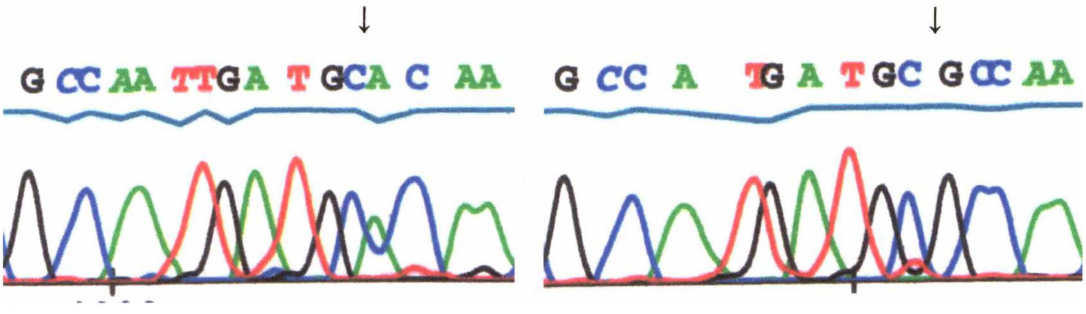

Table 2.8 Results of *Bacillus anthracis* environmental samples screened with the nested primers and the *vrrB* primers. 20 samples were investigated rigorously with seven sets of primers. Presence of a positive band at approximate size is designated as +, whereas – indicates no band at close proximity to the expected size. Sample #68 was not tested (n/a) with the *vrrB* primers.

	Primers				Primers (nested)		
Primers	GPR	PA1	CAPA1	*vrrB*	EWA	PA2	CAPA2
Amplicon size	377-425	511	409	352-424	142-190	329	302
Gene	*vrrA*	Protective Antigen	Capsule	*vrrB*	*vrrA*	Protective Antigen	Capsule
Sample							
10	-	-	-	-	-	-	+
11	-	+	+	-	+	+	+
15	-	-	-	-	-	-	-
23	-	-	-	-	-	-	+
24	-	-	+	+	-	-	+
31	-	-	-	-	-	-	+
32	-	+	-	-	+	+	-
39	-	+	+	-	-	+	-
40	-	-	-	+	+	-	+
46	-	-	-	-	+	-	-
48	-	-	+	+	+	-	-
53	-	-	+	-	+	-	+
58	-	-	+	-	-	+	-
59	-	+	+	+	+	+	+
60	-	+	+	-	-	+	+
62	-	+	+	-	+	+	+
68	-	+	+	n/a	+	+	+
70	-	-	+	-	+	+	+
71	-	-	+	-	-	-	+

2.4. Discussion

2.4.1. DNA extraction

DNA extraction was largely successful from scoria, hay and mixtures of organic materials taken from the stables area of the Historic Hut at Cape Evans, Ross Island Antarctica. A DNA extraction method was also developed which was shown to be effective in producing of DNA from *B. licheniformis* spores. In the environmental samples, it is not entirely clear whether spores were mechanically disrupted effectively to release optimal levels of *B. anthracis* DNA suitable for PCR. Johns et al. (1994) and Reif et al. (1994) applied bead mill homogenisation for 10 minutes to obtain *B. anthracis* DNA from spore preparations, but they did not include environmental matrices, only spores and 0.1 mm beads. In this study, bead-beating homogenisation was carried out in a polycarbonate tube containing 0.5 g of 0.1 mm zirconia/silica beads and 0.5 g of 2.5 mm glass beads and 0.5–1 g of environmental sample for 45 seconds at 4.5 m/s. When *B. licheniformis* spores (108) were inoculated into the environmental samples and bead-mill homogenised, only a 45 second application time produced amplifiable DNA. This result does not exclude the possibility that DNA of exogenous *B. licheniformis* DNA and non-spore forming bacteria DNA could have been excessively sheared after 45 seconds of bead-beating while simultaneously the spores could remained still intact, even after 9 minutes of bead-beating.

There are several possible scenarios regarding with the effects of environmental matrices and application time in relation to disruption of *B. anthracis* and fragmentation of any remaining vegetative cells. For example, inclusion of environmental matrices may have had a positive effect on DNA recovery and spore disruption by increasing the likelihood of collisions between spores and environmental matrices, or a negative effect due to inefficiencies in physical mixing in a tube. Two major drawbacks of the bead-beating method are known: that large amounts of contaminants can be recovered as well as DNA, and that recovered DNA can be fragmented by the shearing effect (Miller et al. 1999). Miller et al. (1999) found that high molecular weight DNA was recovered maximally with use of shorter application times (30–120 seconds) and

homogenisation at low speed. Therefore, the DNA obtained from the protocol employed in this study could have originated from exogenous *B. anthracis* DNA, and/or less intact (i.e. non-viable spores) *B. anthracis* spores, as well as other environmental DNA.

2.4.2. Detection level

The PCR technique exploits the presence of a target sequence homologous to the primers and can theoretically amplify a single template molecule (Saiki et al. 1988; Mullis et al. 1986). The number of target sequences present in a genome should be included for consideration in assessment of the detection level by PCR since the target sequence may occur in multiple numbers, such as ribosomal DNA (Johnson 1994) and plasmids (Ramisse et al. 1996; Reif et al. 1994). There are a number of primers designed for *B. anthracis* detection (Table 2.1). The primers targeting genes on the two plasmids, pX01 and pX02, are of importance because they are essential for virulent *B. anthracis* toxin and capsule production (Turnbull et al. 1992). The exact copy number of the two plasmids in a *B. anthracis* cell at a given time is unknown. It can be assumed that the copy number of the plasmids greatly reflects the physiological conditions of a *B. anthracis* cell at the time of DNA extraction.

Many investigators have assessed the detection level of *B. anthracis* molecular markers and diligently tried to increase the efficiencies of the various assays as summarised in Table 2.2 – Table 2.4 (Beyer et al. 1995; Carl et al. 1992; Johns et al. 1994; Reif et al. 1994; Sjöstedt et al. 1997). In general, there are two approaches to assess the level of detection. One is to use a known amount of total DNA extract from vegetative cells and determine the level of sensitivity in grams (μg, ng and pg) of DNA, then calculate per spores (Carl et al. 1992; Johns et al. 1994; Reif et al. 1994). The other is to prepare a known number of spores, from which DNA is extracted and the sensitivity measured (Beyer et al. 1995; Reif et al. 1994; Sjöstedt et al. 1997). The reliability of accurate detection levels of these investigations is limited since the molecular ratio of chromosome:pX01:pX02 were unavailable (Johns et al. 1994; Reif et al. 1994) and most importantly, exogenous *B. anthracis* DNA in spore preparation was not accounted for (Beyer et al. 1995; Carl et al. 1992; Johns et al. 1994; Reif et al. 1994; Sjöstedt et al.

1997), giving detection levels higher than 'realistic figures'. Reif et al. (1994) noted that positive results were obtained using PCR with a *B. anthracis* spore filtrate supernatant and concluded that the DNA had originated extracellularly.

Read et al. (2002) sequenced the entire genome of the *B. anthracis* Florida isolate and suggested that a molecular ratio of pX01:pX02:chromosome was 3:2:1 based on the sequence coverage ratio. It is most likely that they harvested vegetative cells for DNA preparation for the genome sequence. Molecular size of the chromosome is estimated about 5 – 6 mega base pairs (Mbp) (Keim and Smith 2002), and the final draft of the chromosomal DNA sequence will be available online soon. The two plasmids are completely sequenced, pX01 from Sterne strain (AF065404) (Okinaka et al. 1999) and pX02 from Pasteur strain (AF188935) sized at 181,654 bp and 96,231 bp, respectively. According to the molecular ratio and molecular sizes of the chromosome, pX01, and pX02, copy numbers of the two plasmids can be calculated. If the chromosomal size of *B. anthracis* is assumed as 5 Mbp in the Florida isolate, it should contain 15 and 10 Mbp of pX01 and pX02, respectively. The exact sizes of each plasmid are known, and therefore the copy number of the plasmids are deduced approximately as 83 (pX01) and 104 (pX02) copies, respectively. These figures indicate that the plasmid markers are approximately 100 times more sensitive than a chromosomal marker, if the marker does not have multiple copies. The above mentioned calculation may not be totally applicable universally, but the results obtained in this study (Table 2.7) showed congruent trends in detection level. The PA1 F/R primers targeting pX01 were more sensitive than the chromosomal marker. The marker on pX02 was even more sensitive than the pX01 marker, making it the most sensitive marker.

2.4.3. PCR optimisation with *Taq* polymerase

DNA preparation for PCR from the environmental samples is known to be problematic due to inefficiencies in DNA recovery (Miller et al. 1999) and the presence of many contaminants (Wilson 1997). Sjöstedt et al. (1997) used two different types of DNA polymerase (1 unit each of *Taq* and *Pfu* polymerase) to overcome inhibition of PCR during the detection of *B. anthracis* in a variety of soil samples. The current study encountered a similar situation where activity of the *Taq* polymerase was attenuated in PCR reactions. This was at least partially

overcome by increasing the concentration of *Taq* polymerase in the reaction tubes. Some DNA preparations failed to amplify at all, perhaps owing to the absence of complementary sequence to the primers in the DNA extract or high levels of PCR inhibition by uncharacterised contaminants, or both.

2.4.4. Bead-beating application time

For this study it is deemed to be relatively important to obtain DNA amplifiable for the subsequent PCR that is not overly sheared from *B. anthracis* spores in environmental samples. *Bacillus* species produce dormant endospores capable of resisting chemical, heat, desiccation, hydrostatic pressure, ultraviolet and ionizing irradiation (Gould 1977). The cortex of the spore exerts a high osmotic pressure to effectively squeeze water out of the core, but water content within the core is maintained at equilibrium (Gould 1977). Dragon and Rennie (1995) hypothesised that *B. anthracis* spores require high levels of calcium content in their dormant environment for extended viability of spores based on two facts. First, calcium and dipicolinic acid immobilise internal components of a cell by forming an extensive salt lattice (Gould 1977). Second, anthrax epidemic areas have been closely associated to high calcareous content in soil (Hugh-Johns and Hussaini 1975; Van Ness 1971). They suggested that water diffusion from the core could result in the leaching of calcium cations and the weakening of the salt lattice; consequently, the spores are less viable in a soil with low calcareous content (Dragon and Rennie 1995). The sampling site at Cape Evans is of igneous origin with little calcium content (McCraw 1967). It could be possible that the *B. anthracis* spore integrity in the samples was relatively low due to numerous freeze–thaw cycles in Antarctica in comparison with the laboratory cultured and sporulated *B. anthracis*, which required 10 minutes of bead-beating homogenisation (Johns et al. 1994; Reif et al. 1994). It was unclear whether the positive results from PCR indicated that the DNA template was originally from either exogenous *B. anthracis* DNA, DNA from spores, frozen vegetative cells, or degraded spores.

2.4.5. Screening with CAPA primers

The CAPA F/R primers targeting the *cap* gene of pX02 plasmid were used for the initial screening of 74 environmental samples based on the assumption and results that these primers were the most sensitive. Some samples appeared to have not amplified after PCR even when the concentration of *Taq* polymerase had been increased to 1.5 units per reaction mixture. These samples might have had a higher content of inhibitors, which could not be overcome by the given *Taq* polymerase concentration in these reactions or had no amplifiable target DNA.

2.4.6. Screening with all primers (primary and nested sets)

Agarose gel results of detailed examination of samples with 7 different sets of primers (GPR, CAPA1, PA1, EWA, CAPA-nested, PA-nested, and *vrrB*) are shown in Appendix A. The nested sets increased the sensitivity of PCR, which was particularly evident with the EWA primers (Table 2.8). The GPR F/R primers did not amplify a visible band on the gel, but subsequent PCR with the nested primers, EWA F/R, showed a successful amplification of the correct size for *B. anthracis* in samples 11, 32, 40, 46, 53, 59, 62, 68 and 70. On the other hand, samples 39, 48, and 58 did not generate expected amplicons with the nested primers despite the first round of PCR with CAPA F/R which showed signs of amplification. This can perhaps be explained that the analysed DNA was of environmental origin with an extremely heterogeneous and degraded nature and very low target sequences numbers, which may contain DNA sequence compatible with the CAPA1 F/R primers resulting in amplification of the amplicon at expected size. These amplicons did not have internal sequence homologous to the nested primers, CAPA-nested F/R, thus no observation of amplification with the nested primers. Another potential, and more likely scenario, is that the second round of PCR with the nested primers failed due to a high concentration of templates generated by the first round of PCR.

The detection levels of the chromosomal markers, *vrrA* (amplified by GPR F/R) and *vrrB* are different as the amplification of *vrrB* was successful without nested sets of primers. No *vrrA* gene was amplified with the first round of PCR with GPR F/R primers. These two markers, *vrrA* and *vrrB*, are located in the chromosome (Andersen et al. 1996; Schupp et al. 2000), and the exact copy

number of these genes in the chromosome is not available until the final draft of the genome sequence is released. The results might be quite plausible if the *vrrB* marker is present in the chromosome in multiple copies.

2.4.7. FRET

Fluorescence resonance energy transfer (FRET) PCR with environmental DNA was unable to convincingly show successful positive reactions. Several studies have attempted to use this method for a quick screening method for *B. anthracis*, but none of these studies used *B. anthracis* DNA together with environmental DNA, rather enrichment of environmental samples and isolation of *B. anthracis* before DNA preparation (Ellerbrok et al. 2002; Lee et al. 1999; Makino et al. 2001; Qi et al. 2001). In this study, the amplification of the *rpoB* marker from environmental DNA was trialed despite the known possibility that they may have contained potential inhibitors for PCR reactions. It was not as successful as the positive PCR reactions on electrophoresis gels most probably due to the fact that FRET PCR is more sensitive to inhibitors than the conventional PCR (Wittwer et al. 1997). Usefulness of the *rpoB* marker was doubted in the recent study as *B. cereus* and *B. megaterium* were shown to generate weak amplification signals after 30 cycles (Ellerbrok et al. 2002). Thus, FRET PCR targeting of the *rpo* marker of *B. anthracis* could not produce reliable results, most likely because DNA directly extracted from environmental samples caused inhibition of amplification. The weak amplifications could also indicate the presence of the *rpo* marker from the members of *B. cereus* group or closely related *Bacillus* species. For a rapid *B. anthracis* diagnostic purpose, DNA extracted directly from environmental samples may need thorough DNA clean-up procedures otherwise FRET PCR could result in inconclusive results, although DNA clean-up may compromise speed of the entire experiment. When an immediate diagnosis of *B. anthracis* in non-clinical samples is not required, such as this study, historical specimens (Jackson et al. 1998) and bioterrorism epidemiology (Hoffmaster et al. 2002), the MLVA analysis (Keim et al. 2000) is useful as it is capable of picking up *B. anthracis* markers more reliably and pin-pointing a particular type of *B. anthracis* strain from the samples, thus giving information on *B. anthracis* at higher resolution.

2.4.8. Sequence analysis

Sequences of the regions amplified by the *vrrA* and *vrrB* primers were comparatively analysed to the positive *B. anthracis* DNA (Pasteur II) and database at NCBI (Appendix B). The *vrrA* sequence of samples #40 and #53 were found to have four repeats of CAATATCAACAA which matches that of the Sterne, Pasteur, and Ames strain profile (Jackson et al. 1997). The positive *B. anthracis* DNA (Uchida et al. 1993) is of Pasteur origin and samples #40 and #53 have exactly the same sequence as the positive control DNA. The four repeat is the most common *B. anthracis* profile; nearly 60% of 198 isolates analysed by Jackson et al. (1997) possessed this profile. The #70 *vrrA* sequence initially showed one nucleotide change (A → G) at position 446. Sample #70 was re-examined with a new preparation of the extracted environmental DNA and PCR for subsequent sequencing, which resulted in no nucleotide change (i.e. the A → G was not observed). This contradiction could be explained by two possible scenarios. First, the nucleotide change may have occurred from an incorporation error due to *Taq* polymerase infidelity as an average error rate of *Taq* Polymerase in a PCR reaction is known to occur about 8.0 x 10-6 mutation frequency per base pair per duplication (Cline et al. 1996). Second, it may also be possible that the DNA template was damaged from DNA extraction and/or exogenous DNA was already degraded in the environment. Amplification of ancient DNA has been reported to have a higher rate of nucleotide change (Hofreiter et al. 2001; Hoss 1996). In general, the depth of sequence coverage is generally above 10 times (i.e. 5 forward and reverse sequences each) before a final annotation of a genome database is published to minimise inclusion of sequence artefacts and ambiguities. It is still unclear whether the #70 *vrrA* nucleotide change represents a true property of sample #70 or due to PCR infidelity and poor DNA template quality.

The #32 *vrrA* sequence did not match up exactly with any sequences in the database at NCBI (Appendix C). It had the closest sequence similarity to *B. anthracis* Vollum strain, and lesser to Sterne strain, Ames strain, *B. mycoides*, and *B. cereus*, indicating that the sequence is likely to have originated exclusively from a member of the *B. cereus* group. The electrophoresis gel profile of sample #32 showed positive results PCR reactions with markers on *vrrA* and *pagA* genes (Appendix A). The *pagA* gene is located on pX01 which is an essential

component for anthrax toxin (Turnbull et al. 1992). An indication of the presence of pX01 component suggests *B. anthracis* DNA was present in #32 sample. Alternatively, the #32 sequence could potentially be of *B. thuringiensis* or of an unknown member of the *B. cereus* group since another member of the *B. cereus* group, *B. thuringiensis*, has not been sequenced for *vrrA* as well as a novel strain of *B. anthracis* (Schupp et al. 2000).

2.4.9. Validation with other primers (PA 5 and PA - Makino primers)

Four sets of primers were used for an additional PCR analysis. The protective antigen gene is known to contain a variable region at the 3' end (Price et al. 1999). The primers, PA5 F/R and PA5 nested F/R, designed to amplify this variable region could not amplify the expected amplicons from 18 positive samples, even when the nested sets were used. Similarly, another set of primers (Cheun et al. 2001), PA-Makino and PA-Makino-nested, which amplifies the region overlapping the PA1 F/R amplicon, did not amplify the expected amplicon amongst 18 positive samples either. Although Hoffmaster et al. (2002) noted difficulties in amplification of the markers on the *pagA* gene, this result poses a question whether the positive results are a consequence of contamination of positive DNA (Pasteur II) and/or positive amplicons (*vrrA*, PA1, CAPA1), or of the *B. anthracis* DNA being degraded and/or the recovered DNA being sheared. It is of importance to identify any possibility of false contamination; three scenarios are described below.

Case 1: Likelihood of contamination of positive DNA evaluated

Positive DNA (Pasteur II) contamination in the samples could be remotely possible since the arrival of the positive DNA sample from Dr Makino was concurrent with the timing of DNA extraction from the environmental samples. If the positive *Bacillus anthracis* DNA had contaminated one of the master mixes in the process of DNA extraction or DNA re-suspension in TE buffer, all the tests would have turned up as positive results, or at least many more samples would have resulted in being positive considering that the DNA preparations might have contained variable quantities and types of inhibitors of PCR. If the positive DNA had contaminated all the samples, PCR with the PA5 and PA-Makino primers to

the protective antigen gene would have given positive results. Thus, it is highly unlikely that the positive results were a mere contamination of positive DNA (Pasteur II).

Case 2: Contamination with positive amplicons evaluated

Another source of contamination could have been from amplicons (*vrrA*, EWA, PA, PA-nest CAPA, and CAPA-nest) of the positive *B. anthracis* DNA. The remainder of the primers (*vrrB*, PA5, and PA-Makino) were obtained after DNA extraction and DNA re-suspension. If these amplicons (*vrrA*, EWA, PA1, PA2 CAPA1, and CAPA2) had collectively or individually contaminated one of the master mixes in the process of DNA extraction or DNA re-suspension in TE buffer, there would have been many more positives, considering that these amplicons would have been introduced into samples in relatively large quantities. Although the PA5 and PA-Makino primers did not show any sign of amplifications amongst 18 samples, the *vrrB* F/R primers successfully amplified the expected amplicon from sample #59, from which the sequence was obtained and identified as a *B. anthracis* sequence of *vrrB* (Appendix E). The genotype of the sample #59 gave the most common profile among a diversity of *B. anthracis* strains (Schupp et al. 2000). The positive DNA (Pasteur II) has the same repeats profile as well. It is, however, important to note that *B. cereus* has the same sequence, but about 50 base pairs shorter at 5' end of *vrrB* open reading frame (Schupp et al. 2000). The 5' end of the ORF was not covered by the *vrrB* F/R primers, so it is inconclusive if the sequence of the sample #59 is truly of *B. anthracis*. Despite the ambiguity over the true identity of the *vrrB* sequence from sample #59, it is not possible to have collective contaminations of the amplicons into individual samples.

Case 3: *B. anthracis* DNA present but degraded or sheared

The last possibility is that the *B. anthracis* DNA in the samples was sheared from the DNA extraction procedure, and/or the spores and DNA were preserved in a partially degraded state. Many samples showed that the markers on pX01, pX02 and chromosome were often not detected altogether and that varying degrees of band intensity were found. Mechanical extraction of DNA can sometimes be problematic as it may shear DNA (Miller et al. 1999). Failure in PCR reactions

could have been due to the fact that template DNA was in a poor condition for PCR, leading subsequently to the primers being unable to bind to target sequences, and thus no amplification in some samples. It is also evident with ancient DNA that the recovered sequence sometimes may contain artefact change(s) (Lindahl 1993; Pääbo et al. 1989). It could also be assumed that the *B. anthracis* spores present in samples may have been degraded, as it has been reported that *B. anthracis* spores did not germinate well and grew in a small colony after 80 years of dormancy (Redmond et al. 1998). These factors may have contributed to the results of inconsistent PCR amplification and nucleotide change in sequence, especially when in combination with extremely low target sequences.

It would essentially negate the above three scenarios of false positive PCR reactions due to positive control DNA contamination if the sequences of positive samples have convincing nucleotide change(s) or different profiles in the variable regions of *vrrA* and *vrrB* between positive sample and positive *B. anthracis* DNA. Thus, future research should include obtaining a full length sequence for the open reading frame #59 *vrrB* sequence to differentiate that the #59 was not *B. cereus*, even though positive reactions of PCR on the pX01 and pX02 plasmids were obtained.

2.4.10. Viability tests and validation by external organisations

It is of essential importance to have an independent organisation to confirm results when dealing with extraction of DNA from environmental samples, in particular ancient DNA (O'Rourke et al. 2000; Wayne et al. 1999). Six environmental samples (18, 41, 45, 68, 73 and 74) were sent to the National Centre for Disease Investigation (NCDI), Upper Hut, New Zealand for culturing viable spores. NCDI were unable to isolate *B. anthracis* since there was an outgrowth by other microorganisms, although *Bacillus* species were isolated. Dr Pamala Coker and Dr Kimothy Smith at Lawrence Livermore National Laboratory, California, USA are currently (January – May 2003) analysing 40 environmental samples from Cape Evans. Their world-leading expertise in *B. anthracis* research should bring detailed accounts of *B. anthracis* presence and type strain with a likely detection level of 1 – 3 spores per gram of sample (Coker et al. 2002; Dragon, et al. 2001; Keim and Smith 2002; Read et al. 2002).

2.5. Conclusion

The aim of this chapter was to screen for the presence *B. anthracis* in environmental samples collected at the stables of the historic hut area at Cape Evans on Ross Island, Antarctica with molecular diagnostic tools. A total of 74 samples were collected in the vicinity of suspected areas of *B. anthracis* contamination at Cape Evans. These samples were extracted using the optimised PSB method to obtain amplifiable DNA for subsequent PCR and FRET analysis specifically designed for *B. anthracis* screening. PCR efficiency was somewhat compromised in the extracted environmental DNA when *Taq* polymerase concentration was at 0.5 U, but significantly improved at a higher *Taq* polymerase concentration of 1.5 U. When a known amount of *B. anthracis* DNA was tested for assessment of the detection level, a marker on pX02 gave the highest detection level in comparison with markers on pX01 and the chromosome. The marker on the pX02 plasmid was, therefore, used for an initial screening before selecting candidate samples for further analysis with markers on pX01, pX02 and the chromosome. Nested primer sets were used to attain increased sensitivity and specificity, which was often seen on the chromosomal marker *vrrA* in many samples. Only samples 11, 59, 62, 68, and 70 showed positive PCR reactions with all three sets of the nested primers while other samples showed positive reactions on one or two markers. FRET was not successful to amplify the target in environmental DNA samples as the extracted DNA from environmental samples likely contained inhibitors of PCR reactions. When the partial sequences of *vrrA* and *vrrB* genes were obtained, it was hoped that these sequences could provide an insight into the origin of *B. anthracis* DNA in comparison with the database on NCBI. Discrimination of the sequences between positive DNA (Pasteur II) and positive samples were unable to be established, except for one nucleotide change found in one occasion in the *vrrA* sequence from sample #70. Contamination of the positive DNA (Pasteur II) was highly unlikely, although NCDI could not culture in their initial trial viable *B. anthracis* from the samples (Appendix D). With respect to the quantity and quality of template garget DNA, it was thus concluded: that *B. anthracis* intact spores in the environmental samples were close to a non-viable and degraded state and originally in low numbers; that *B. anthracis* DNA in samples from frozen vegetative cells and/or degraded spores

was in poor condition: and that the mechanical DNA extraction was partially destructive, particularly for exogenous DNA already released by vegetative cells or degraded spores, for subsequent PCR reactions. Despite the severe experimental constrains of century-old target sequence, and probably also at low levels combined with the associated problems of template deterioration and PCR inhibitors, indications of *B. anthracis* DNA in the environs of Cape Evans Hut was demonstrated in this chapter. However, future research should aim to carry out replication of the results at an independent laboratory with both bacteriological and molecular diagnostic techniques. Currently (January – May 2003) Dr Pamala Coker and Dr Kimothy Smith of Lawrence Livermxore National Laboratory (LLNL), California, USA are analysing 40 environmental samples from the University of Waikato collection and 8 samples from University of Minnesota.

Finally, *B. anthracis* decontamination with 5% formaldehyde as has been performed for decontamination of Gruinard Island (Manchee et al. 1981; 1994) in the vicinity of the hut at Cape Evans is impossible since the chemical treatment would inevitably cause further deteriorations of historical artefacts as well as hut structure. No official scientific report of the anthrax infection has been reported in one hundred years of human activity in the area and apparent risk may seem minimal, however it is necessary to assess any potential risks to the health of conservators and visitors to the hut.

CHAPTER THREE

3. Mycological diversity inside the Discovery Hut, Ross Island, Antarctica

3.1. Introduction

3.1.1. Antarctic Mycology

Investigations of mycological flora in Antarctica often focus on diversity and abundance in relation to various factors affecting their survival in the Antarctic environment (Vishniac 1996). The geographical distributions of fungi are diverse. In the sub-Antarctic islands, macro-fungi are present as well as numerous filamentous micro-fungi and yeasts (Vishniac 1993). Many filamentous fungi and yeasts were isolated from the soils of the ice-free and coastal areas of Antarctic continent. An extensive search of filamentous micro-fungi isolated in the Antarctica and sub-Antarctic islands was carried out in this chapter. Filamentous micro-fungal diversity, the members of the phycomycetes, ascomycetes, coelomycetes and deuteromycetes, associated with the historic materials at Discovery Hut at Hut Point (77°50'50''S, 166°38'30''E) on Ross Island, Antarctica, was described. For the reviews of the Antarctic mycology in the context of the general Antarctic microbiology, see Wynn-Williams (1990) and Vincent (1988). In the area of the Antarctic mycology, including yeast as well as filamentous micro-fungi, Vishniac (1993; 1996) are useful references.

3.1.2. Biodiversity of filamentous micro-fungi in Antarctica

The diversity of filamentous micro-fungi, the members of the phycomycetes, ascomycetes, coelomycetes and deuteromycetes, isolated from Antarctica and sub-Antarctic Islands are documented in the literatures (Table 3.1 – Table 3.7).

These micro-fungi were isolated from various geographical locations; northern Victoria Land (Broady et al. 1987; Del Frate and Caretta 1990; Fenice, et al. 1997; Mercantini et al. 1989; Onofri et al. 1994; Zucconi et al. 1996), southern Victoria Land (Baublis et al. 1991; Cameron et al. 1974; 1976), Ross Island (Aislabie et al. 2001; Boyd and Boyd 1963; Greenfield 1981; 1983; Meyer et al. 1962; Meyer et al. 1963; Tubaki and Asano 1965; Ugolini and Starkey 1966), Windmill Islands (Azmi and Seppelt 1997; 1998; Heatwole et al. 1989; Kerry 1990a; McRae and Seppelt 1999), Vestfold Hills (Ellis 1980; Line 1988), Mirny (Meyer et al. 1967), Mac. Robertson and Enderby Lands (Fletcher et al. 1985; Kerry 1990b), Ongul Islands (Tubaki 1961; Tubaki and Asano 1965), Antarctic Peninsula (Caretta and Piontelli 1977; Möller and Dreyfuss 1996), sub-Antarctic islands (Bostelmann 1976; Cameron and Nenoit 1970; Möller and Dreyfuss 1996), such as Signy Island (Latter and Heal 1971; Marshall 1998; Pugh and Allsopp 1982; Weinstein et al. 1997, 2000), King George Island (Hurst et al. 1983), Ballany Islands (Ellis 1980), Macquarie Island (Bunt 1965; Ellis 1980; Kerry 1990a). A total of 252 taxa were recognised in the literature search and these filamentous micro-fungi were isolated from pristine soil, oil contaminated soil, geothermally heated soil, soils influenced by mosses and lichens, mosses and lichens, mummified seals and penguins, colonies of seals and penguins, soils in the vicinity of research stations, inside the research stations, and historic materials (summarised in Table 3.1 – Table 3.7).

3.1.3. Biodiversity of filamentous micro-fungi in the geothermal soils

Geothermally heated soils in the Antarctic environment provide a very unique oasis for organisms since the primary energy source can be obtained from the heated soils (Broady 1993). Establishment of microorganisms was observed to be very fast on newly formed islands from volcanic activity in the harbour of Deception Island (62°57S, 60°38'W) where *Penicillium*, *Fusarium*, *Tritirachium*, *Glicocladium*, *Cephalosporium* were isolated, however, it was not determined that the introduction of these microorganisms was due to natural processes or human activity (Cameron and Nenoit 1970). Mt Melbourne (2733m, 74°21'S, 164°42'E) is situated in the northern Victoria Land. Thermotolerant and thermophilic micro-fungi *Aspergillus*, *Chaetomium*, *Malbranchea pulchella* var. *sulfurea*, *Mucor*, *Myceliophthora thermophile*, *Paecilomyces* and *Penicillium* were reported

by Broady et al. (1987). *Chaetomium* species were commonly found in the soils of Mt Melbourne (Fenice et al. 1997; Onofri et al. 1994; Zucconi et al. 1996). Additionally, cosmopolitan genus such as *Acremonium* (Zucconi et al. 1996) and *Verticillium* (Fenice et al. 1997) were also reported from Mt Melbourne. Mycoflora of Mt Erebus (3794m, 77°32'S, 167°10'E) on Ross Island were reported including *Penicillium*, *Aspergillus* and *Neurospora* (Ugolini and Starkey 1966). Biological diversity of the geothermally heated soils is well-documented; for the review see Broady (1993).

3.1.4. Biodiversity of filamentous micro-fungi associated with biological matters

Colonisation of microorganisms is more pronounced where organic matters, such as animal dung, shed skins, feathers, mosses and lichens, exist in the Antarctic environment since microorganisms experience less exposure to the severe Antarctic climate and more available nutrients within the environment of these matters.

Many mummified penguins and seals are found in the Dry Valleys as animals presumably lost direction to the coastline and consequently died. The carcasses were then essentially freeze-dried in the environments of Antarctica with very slow decomposition rate. These mummified carcasses later served as an oasis in the nutrient depleted polar desert and provide a source of great nutrients for microorganisms in the soils of adjacent areas and within the mummified carcasses. Boyd et al. (1996) noted that filamentous fungi counts were, in general, low in the Wright Valley, except for the samples collected at areas next to mummified seals. Similarly, highest fungal density was recorded together with *Aspergillus paradoxus* and *Penicillium jansenii* in the mummified penguin and associated soils in Taylor Valley (Baublis et al. 1991). Mummified seals contained potentially pathogenic fungi *Phialophora fastigiata*, *Phialophora dermatitidis* and *Phialophora gougerotii* capable of causing skin lesions at Cape Bird, Ross Island (Greenfield and Wilson 1981).

Bird nests and seal colonies are also a home to many filamentous micro-fungi. Kerry (1990b) found that *Thelebolus microsporus* was widespread at localities

where seals and birds are associated in Vestfold Hills. *Chrysosporium* was exclusively present in the bird nests in the Northern Victoria Land (Mercantini et al. 1989). Similarly, *Chrysosporium pannorum* was more frequently isolated from soils with strong influence of animal activity and debris (Pugh and Allsopp 1982). Azmi and Seppelt (1998) found a diverse range of micro-fungi such as *Aspergillus*, *Chrysosporium*, *Mortierella*, *Penicillium*, *Phialophora malorum*, *Phoma*, *Thelebolus microsporus* from seal, penguin, snow petrel, fulmar and skua and their nests. Thermophilic fungi, *Scytalidium thermophilum* and *Thermomyces lanuginosus*, were isolated from petrel feathers and dung (Del Frate et al. 1990).

Many studies showed that plant materials such as mosses and lichens are associated with cosmopolitan species. Meyer et al. (1967) reported *Cladosporium* only in association with mosses and lichens, while Pugh and Allsopp (1982) recovered *Cladosporium cladosporioides* almost exclusively from mosses. *Phoma herbarum* was isolated from the plant materials in Mac. Robertson Land (Fletcher et al. 1985). McRae and Seppelt (1999) isolated only cosmopolitan species (*Phoma*, *Trichoderma*, *Penicillium*, *Thelebolus*, *Geomyces*) from two moss species, *Grimmia antarctici* and *Bryum pseudoteriquetrum*. Greenfield (1983) reported a pathogenic *Rhizopus* species associated with moss (*Bryum antarcticum*) at Cape Bird, Ross Island.

3.1.5. Biodiversity of filamentous micro-fungi at high human activity areas

In general, a higher diversity of filamentous micro-fungi is reported in the areas where biotic (mosses and lichens) and anthropogenic (human) influences are pronounced (Cameron et al. 1976; Sun et al. 1978; Vishniac 1996). In the study of qualitative measurement of filamentous micro-fungi, twenty two genera were isolated in the immediate vicinity of Casey Station (Azmi and Seppelt 1998). In contrast, soils that originated from the areas where human activities were comparatively lower than the Casey Station area showed a reduction in the number of isolated genera to 15 (Azmi and Seppelt 1998). Boyd and Boyd (1963) suggested that the soils near the McMurdo Station contained *Penicillium* and *Aspergillus* as a result of human activities. Cameron et al. (1974) also noted that previously undetected *Penicillium* species were recorded in the environmental

impact study of the Dry Valley Drilling Project spanning for eight years in the southern Victoria Land.

Pathogenic filamentous micro-fungi are also detected and believed to have originated from human activities. Mercantini et al. (1989) suspected the presence of two pathogenic filamentous fungi, *Microsporum gypseum* and *Trichophyton terrestre* was due to human activity in the Northern Victoria Land. Mercantini et al. (1993) additionally isolated another pathogen *Trichophyton mentagrophytes* in the same region. *Trichophyton* was also isolated from the tissue sample of a sledge dog which was put down due to severe skin infection (Bostelmann 1976).

Oil spills are a serious human impact to the Antarctic environment, in particular, to the soil microorganism community. Dominant species shifted from *Chrysosporium* to *Phialophora* at oil contaminated areas in the McMurdo Sound (Aislabie et al. 2001). Occurrence frequencies decreased in *Geomyces pannorum* and *Thelebolus microsporus* populations in the petroleum contaminated soils, whereas *Phialophora fastigiata* was isolated from petrol contaminated soils at David and Mawson stations (Kerry 1990b). It has not been demonstrated whether *Phialophora* has an ability to degrade petroleum.

3.1.6. Mycological investigation at the Historic Huts on Ross Island

Previous investigations of mycoflora in the Historic Huts areas on Ross Island, Antarctica focused on long-term survival of microorganisms. Meyer et al. (1962) demonstrated the viability of filamentous micro-fungi *Absidia corymbifera* and *Rhizopus arrhizus* from a sealed bottle of yeast from Cape Evans hut. They also isolated *Mucor* sp. from tinned barley from Shackleton's hut at Cape Royds and *Penicillium* sp., and unidentified dematiaceous fungi from hay at Cape Evans (Meyer et al. 1963). Nedwell et al. (1994) recovered only small numbers of unidentified filamentous fungi from historic materials at Cape Evans and Cape Royds. Tubaki and Asano (1965) isolated *Botryotrichum piluliferum* from horse dung collected at Cape Royds.

3.1.7. Discovery Hut

The Discovery Hut was erected at Hut Point (77°50'50''S, 166°38'30''E) in 1902 by the members of the National Antarctic Expedition (1902–1904) led by Captain Robert F Scott (Scott 1905). The hut was extensively used by the other expeditions in the Heroic Era as a key stepping stone to the southern latitudes and a shelter for those who returned from the south (Quartermain 1967). The hut was briefly re-visited by the members of the Operation High Jump in 1947 (Quartermain 1963). The American McMurdo Station was built nearby in the preparation for the International Geophysical Years. New Zealand's Scott Base was also established approximately 4 km south of McMurdo Station at that time. The hut has been subjected to ongoing conservation and preservation work for forty years while the surrounding areas were modified from the development of McMurdo Station (Harrowfield 1995; Turner 1979). The hut attracts many tourists as well as visitors from the nearby stations. The hut and the environs, therefore, are the most affected areas by decades of human activities in the Antarctica.

3.1.8. Scope of this study

This chapter describes the identification of filamentous micro-fungi isolated from the historic materials at Discovery Hut (77°50'50''S, 166°38'30''E) on Ross Island. It is the first report of viable filamentous fungi isolated from the foodstuffs and the internal environment of Discovery Hut. Isolation of the filamentous fungi was carried out by the author. Identification of these isolates was kindly carried out by Dr Margaret di Menna, AgResearch Ruakura, Hamilton. Discussions on human activity and micro-fungi diversity are included in this chapter. This chapter contains original research material carried out by the author unless otherwise acknowledged and was supervised by Professor Roberta L Farrell.

Table 3.1 Filamentous micro-fungi isolated in the Antarctic region.

Fungal species identified	References, Locations and Notes
Absidia corymbifera	30Aadh
Acremonium sp.	25Caf, 34Bbd
Acremonium butyric	33Had
Acremonium cerealis	33Had
Acremonium charticola	34Bbd, 42Bbdg
Acremonium crotociningenum	3De, 3Dd, 3Da, 23Faf
Acremonium psychrophilum	33Had
Acremonium rutilum	33Had
Acremonium strictum	14Bbd, 42Bbd
Acremonium terricola	21Iad
Acremonium zonatum	22Kad
Acrodyctis sp.	34Bbd
Alternaria sp.	16Bbe, 21Iad, 26Gbd, 34Bbd, 42Bbe
Alternaria alternate	2Dcf, 3Da, 18Bad, 23Faf, 33Had, 36Lcf
Alternaria cf. *chlamydospora*	33Had
Alternaria citri	4Baf,
Alternaria tenuis	10Bcf, 11Bcf, 18Bad,
Aphanoascus fulvescens	29Bad,
Arachniotus citrinus	10Bcf, 36Lcf
Arthrobotrys sp.	3Dd
Arthrobotrys ferox	16Bbd, 34Bbd, 42Bbd
Arthroderma cf. *cuniculi*	33Had
Ascochyta sp.	33Had
Ascochyta stilbocarpae	22Kad
Aspergillus sp.	3Dd, 6Aad, 7Bbdg, 12Iad, 19Bbfg, 28Bad, 29Bad, 39Abdg
Aspergillus flavus-oryzae	13Iad
Aspergillus fumigatus	4Baf, 13Iad, 15Cbd, 15Dbd, 15Jbd, 18Bad
Aspergillus heteromorphus	4Baf
Aspergillus koningii	18Bad
Aspergillus nidulans	3De, 3Dd, 3Da
Aspergillus niger	13Iad, 18Bad
Aspergillus paradoxus	4Baf
Aspergillus repens	10Bcf, 36Lcf, 38Aad
Aspergillus restrictus	38Aad
Aspergillus spinulosus	4Baf
Aspergillus sydowi	4Baf, 8Kbd
Aspergillus ustus	4Baf
Aspergillus versicolor	10Bcf, 11Bcf, 16Bbd, 18Bad, 26Gbd, 34Bbd, 36Lcf, 42Bbd
Aureobasidium sp.	10Bcf, 11Bcf, 16Bbd, 25Caf, 34Bbd, 42Bcdh
Aureobasidium pullulans	4Baf, 18Bad, 21Iad, 22Kad, 23Faf, 36Lcf

Keys for references: **1**, Aislabie et al. (2001); **2**, Azmi and Seppelt (1997); **3**, Azmi and Seppelt (1998); **4**, Baublis et al. (1991); **5**, Bostelmann (1976); **6**, Boyd et al. (1996); **7**, Broady et al. (1987); **8**, Bunt (1965); **9**, Cameron et al. (1970); **10**, Cameron et al. (1974); **11**, Cameron et al. (1976); **12**, Caretta and Piontelli (1977); **13**, Corte and Daglio (1962); **14**, Del Frate and Caretta (1990); **15**, Ellis (1980); **16**, Fenice et al. (1997); **17**, Fletcher et al. (1985); **18**, Greenfield (1981); **19**, Greenfield (1983); **20**, Heatwole et al. (1989); **21**, Hurst et al (1983); **22**, Kerry (1990a); **23**, Kerry (1990b); **24**, Latter and Heal (1971); **25**, Line (1988); **26**, Marshall (1998); **27**, McRae and Seppelt (1999); **28**, Mercantini et al. (1989); **29**, Mercantini et al. (1993); **30**, Meyer et al. (1962); **31**, Meyer et al. (1963); **32**, Meyer et al. (1967); **33**, Möller. et al. (1996); **34**, Onofri et al. (1994); **35**, Pugh and Allsopp (1982); **36**, Sun et al. (1978); **37**, Tubaki (1961); **38**, Tubaki and Asano (1965); **39**, Ugolini and Starkey (1966); **40**, Weinstein et al. (1997); **41**, Weinstein et al. (2000); **42**, Zucconi et al. (1996).
Keys for locations: ***ROSS SEA REGION*** **A**, Ross Island; **B**, Victoria Land; ***EAST ANTARCTICA*** **C**, Vestfold Hills; **D**, Windmill Islands; **E**, Ongul Islands; **F**, Other locations; ***ANTARCTIC PENINSULA AND NEIGHBOURING ISLANDS*** **G**, Signy Island; **H**, King George Island; **I**, Other locations; ***SUBANTARCTIC ISLANDS*** **J**, Ballany Islands; **K**, Macquarie Island; ***OTHER LOCATIONS*** **L**, Other locations.
Keys for notes: **a**, human activity high; **b**, human activity low; **c**, human activity unknown; **d**, strong biotic influence; **e**, little or no biotic influence; **f**, biotic influence unknown; **g**, isolated at geothermal areas; **h**, isolated from the historic materials;

Table 3.2 Filamentous micro-fungi isolated in the Antarctic region. (Continued)

Fungal species identified	References, Locations and Notes
Beauveria sp.	29Bad
Blodgettia borneti	37Ead, 37Ebe
Botryotrichum sp.	12Iad
Botryotrichum piluliferum	38Aad
Botrytis cinerea	3Da, 21Iad
Byssochlamys nivea	34Bbd
Camarosporium sp.	8Kbd, 33Had
Camarosporium metableticum	22Kad
Cephalosporium sp.	7Bbdg, 9Ifg, 35Gbd
Cephalosporium acremonium	4Baf
Chaetomium sp.	7Bbdg, 16Bbfg, 19Bbfg, 34Bbd, 35Gbd, 42Bbeg
Chaetomium gracile	15Jbd
Chaetophoma sp.	21Iad
Chalara sp.	33Had
Chalara constricta	33Had
Chaunopycnis alba	33Had
Chaunopycnis ovalispora	33Had
Chromelosporium ollare	33Had
Chrysosporium sp.	1Aa, 3De, 3Dd, 25Caf, 28Bad, 34Bbd, 35Gbd
Chrysosporium carmichaelii	29Bad,
Chrysosporium indicum	12Iad
Chrysosporium keratinophilum	12Iad
Chrysosporium merdarium	10Bcf, 11Bcf, 36Lcf
Chrysosporium pannorum	2Dcf, 3De, 3Dd, 3Da, 4Baf, 10Bcf, 11Bcf, 17Fad, 18Bad, 20Dcd, 21Iad, 22Kad, 22Dad, 29Bad, 35Gbd, 36Lcf, 37Ebe, 38Aad 10Bcf, 14Bbd, 34Bbd, 36Lcf, 37Ebe, 38Aad
Chrysosporium verrucosum	3Da, 13Iad, 21Iad, 32Fad, 33Had, 34Bad
Cladosporium sp.	16Bbd, 18Bad, 20Dcd, 23Faf, 35Gbd, 42Bbd
Cladosporium cladosporioides	14Bbd, 16Bbd, 17Fad, 17Fbd, 18Bad, 23Faf, 33Had, 42Bbd
Cladosporium herbarum	36Lcf
Cladosporium phaerospermum	25Caf
Cladosporium resinae	10Bcf, 11Bcf, 18Bad
Cladosporium sphaerospermum	4Baf
Cladosporium ulatum	12Iad
Cunninghamella antarctica	12Iad
Cunninghamella echinulata	37Ebe
Cylindrium griseum	33Had
Cylindrocarpon sp.	8Kbd
Cylindrophora sp.	

Keys for references: **1**, Aislabie et al. (2001); **2**, Azmi and Seppelt (1997); **3**, Azmi and Seppelt (1998); **4**, Baublis et al. (1991); **5**, Bostelmann (1976); **6**, Boyd et al. (1996); **7**, Broady et al. (1987); **8**, Bunt (1965); **9**, Cameron et al. (1970); **10**, Cameron et al. (1974); **11**, Cameron et al. (1976); **12**, Caretta and Piontelli (1977); **13**, Corte and Daglio (1962); **14**, Del Frate and Caretta (1990); **15**, Ellis (1980); **16**, Fenice et al. (1997); **17**, Fletcher et al. (1985); **18**, Greenfield (1981); **19**, Greenfield (1983); **20**, Heatwole et al. (1989); **21**, Hurst et al (1983); **22**, Kerry (1990a); **23**, Kerry (1990b); **24**, Latter and Heal (1971); **25**, Line (1988); **26**, Marshall (1998); **27**, McRae and Seppelt (1999); **28**, Mercantini et al. (1989); **29**, Mercantini et al. (1993); **30**, Meyer et al. (1962); **31**, Meyer et al. (1963); **32**, Meyer et al. (1967); **33**, Möller. et al. (1996); **34**, Onofri et al. (1994); **35**, Pugh and Allsopp (1982); **36**, Sun et al. (1978); **37**, Tubaki (1961); **38**, Tubaki and Asano (1965); **39**, Ugolini and Starkey (1966); **40**, Weinstein et al. (1997); **41**, Weinstein et al. (2000); **42**, Zucconi et al. (1996).
Keys for locations: ***ROSS SEA REGION*** **A**, Ross Island; **B**, Victoria Land; ***EAST ANTARCTICA*** **C**, Vestfold Hills; **D**, Windmill Islands; **E**, Ongul Islands; **F**, Other locations; ***ANTARCTIC PENINSULA AND NEIGHBOURING ISLANDS*** **G**, Signy Island; **H**, King George Island; **I**, Other locations; ***SUBANTARCTIC ISLANDS*** **J**, Ballany Islands; **K**, Macquarie Island; ***OTHER LOCATIONS*** **L**, Other locations.
Keys for notes: **a**, human activity high; **b**, human activity low; **c**, human activity unknown; **d**, strong biotic influence; **e**, little or no biotic influence; **f**, biotic influence unknown; **g**, isolated at geothermal areas; **h**, isolated from the historic materials;

Table 3.3 Filamentous micro-fungi isolated in the Antarctic region. (Continued)

Fungal species identified	References, Locations and Notes
Dematiaceae sp.	29Bad
Dendryphiella salina	10Bcf, 11Bcf, 16Bbd, 34Bbd, 36Lcf, 38Aad, 38Ead, 38Ebd, 42Bbd 12Iad
Diheterospora catenulata	3Da, 8Kbd
Diplodia sp.	21Iad
Doratomyce nanus	
Epicoccum nigrum	23Faf, 33Had
Epicoccum purpurascens	20Dcd
Exophiala sp.	26Gbd, 33Had
Fusarium sp.	9Ifg, 10Bcf, 11Bcf, 12Iad, 29Bad, 33Had
Fusarium fusarioides	4Baf
Fusarium lateritium	21Iad
Fusarium oxysporum	3Da
Geomyces sp.	3De, 3Dd, 3Da, 24Gbd, 34Bbd
Geomyces pannorum	23Faf, 23Fbe, 26Gbd, 27Dbd, 34Bbd, 41Gbe, 42Bbd
Geomyces pannorum var. *pannorum*	14Bbd, 16Bbd, 29Bad, 33Had, 42Bbd
Geomyces pannorum var. *vinaceus*	29Bad, 33Had
Geomyces vulgare	24Gbd
Geotrichum sp.	1Aa, 3Da
Geotrichum candidum	4Baf
Gliocladium sp.	9Ifg, 10Bcf, 11Bcf, 35Gbd
Gliomastix murorum	4Baf
Helminthosporium sp.	13Iad
Helminthosporium anomalum	10Bcf, 11Bcf, 36Lcf
Hormoconis resinae	23Faf
Humicola marvinii	40Gbe, 41Gbe
Hyalopus sp.	8Kbd
Leptosphaeria sp.	21Iad
Libertella sp.	33Had
Lichenoconium sp.	33Had

Keys for references: **1**, Aislabie et al. (2001); **2**, Azmi and Seppelt (1997); **3**, Azmi and Seppelt (1998); **4**, Baublis et al. (1991); **5**, Bostelmann (1976); **6**, Boyd et al. (1996); **7**, Broady et al. (1987); **8**, Bunt (1965); **9**, Cameron et al. (1970); **10**, Cameron et al. (1974); **11**, Cameron et al. (1976); **12**, Caretta and Piontelli (1977); **13**, Corte and Daglio (1962); **14**, Del Frate and Caretta (1990); **15**, Ellis (1980); **16**, Fenice et al. (1997); **17**, Fletcher et al. (1985); **18**, Greenfield (1981); **19**, Greenfield (1983); **20**, Heatwole et al. (1989); **21**, Hurst et al (1983); **22**, Kerry (1990a); **23**, Kerry (1990b); **24**, Latter and Heal (1971); **25**, Line (1988); **26**, Marshall (1998); **27**, McRae and Seppelt (1999); **28**, Mercantini et al. (1989); **29**, Mercantini et al. (1993); **30**, Meyer et al. (1962); **31**, Meyer et al. (1963); **32**, Meyer et al. (1967); **33**, Möller. et al. (1996); **34**, Onofri et al. (1994); **35**, Pugh and Allsopp (1982); **36**, Sun et al. (1978); **37**, Tubaki (1961); **38**, Tubaki and Asano (1965); **39**, Ugolini and Starkey (1966); **40**, Weinstein et al. (1997); **41**, Weinstein et al. (2000); **42**, Zucconi et al. (1996).
Keys for locations: ***ROSS SEA REGION*** **A**, Ross Island; **B**, Victoria Land; ***EAST ANTARCTICA*** **C**, Vestfold Hills; **D**, Windmill Islands; **E**, Ongul Islands; **F**, Other locations; ***ANTARCTIC PENINSULA AND NEIGHBOURING ISLANDS*** **G**, Signy Island; **H**, King George Island; **I**, Other locations; ***SUBANTARCTIC ISLANDS*** **J**, Ballany Islands; **K**, Macquarie Island; ***OTHER LOCATIONS*** **L**, Other locations.
Keys for notes: **a**, human activity high; **b**, human activity low; **c**, human activity unknown; **d**, strong biotic influence; **e**, little or no biotic influence; **f**, biotic influence unknown; **g**, isolated at geothermal areas; **h**, isolated from the historic materials;

Table 3.4 Filamentous micro-fungi isolated in the Antarctic region. (Continued)

Fungal species identified	References, Locations and Notes
Malbranchea sp.	28Bad
Malbranchea gypsea	29Bad
Malbranchea pulchella var. *sulfurea*	7Bbdg
Microsporum gypseum	
Monascella sp.	28Bad
Monocillium sp.	33Had
Monodictys austrina	33Had
Monosporium sp.	10Bcf, 11Bcf, 23Faf, 38Aad
Mortierella sp.	8Kbd
Mortierella alpine	3Dd, 3Da, 21Iad, 22Dad, 23Faf, 24Gbd, 35Gbd
Mortierella antarctica	24Gbd, 34Bbd
Mortierella elongata	14Bbd, 34Bbd, 42Bbd
Mortierella gamsii	41Gbe
Mortierella minutissina	3De, 3Dd, 3Da, 22Kad, 33Had
Mortierella parvispora	27Dbd
Mortierella pusilla	24Gbd
Mortierella ramanniana	18Bad
Mortierella turficola	18Bad
Mucor sp.	24Gbd
Mucor circinelloides	7Bbdg, 12Iad, 19Bbfg, 23Faf, 31Aadh
Mucor hiemalis	13Iad
Mucor jansseni	17Fad, 21Iad
Mucor mucedo	10Bcf, 11Bcf, 18Bad, 36Lcf
Mucor piriformis	38Aad
Mucor plumbeus	3Da
Mucor racemosus	17Fad
Mucor spinescens	13Iad, 18Bad
Myceliophthora sp.	13Iad
Myceliophthora thermophile	33Had
Myrioconium sp.	7Bbdg, 15Dbd 33Had
Nectria inventa	29Bad
Nectria peziza	2Dcf, 3De, 3Da
Ovadendron sulfureo-ochraceum	33Had

Keys for references: **1**, Aislabie et al. (2001); **2**, Azmi and Seppelt (1997); **3**, Azmi and Seppelt (1998); **4**, Baublis et al. (1991); **5**, Bostelmann (1976); **6**, Boyd et al. (1996); **7**, Broady et al. (1987); **8**, Bunt (1965); **9**, Cameron et al. (1970); **10**, Cameron et al. (1974); **11**, Cameron et al. (1976); **12**, Caretta and Piontelli (1977); **13**, Corte and Daglio (1962); **14**, Del Frate and Caretta (1990); **15**, Ellis (1980); **16**, Fenice et al. (1997); **17**, Fletcher et al. (1985); **18**, Greenfield (1981); **19**, Greenfield (1983); **20**, Heatwole et al. (1989); **21**, Hurst et al (1983); **22**, Kerry (1990a); **23**, Kerry (1990b); **24**, Latter and Heal (1971); **25**, Line (1988); **26**, Marshall (1998); **27**, McRae and Seppelt (1999); **28**, Mercantini et al. (1989); **29**, Mercantini et al. (1993); **30**, Meyer et al. (1962); **31**, Meyer et al. (1963); **32**, Meyer et al. (1967); **33**, Möller. et al. (1996); **34**, Onofri et al. (1994); **35**, Pugh and Allsopp (1982); **36**, Sun et al. (1978); **37**, Tubaki (1961); **38**, Tubaki and Asano (1965); **39**, Ugolini and Starkey (1966); **40**, Weinstein et al. (1997); **41**, Weinstein et al. (2000); **42**, Zucconi et al. (1996).
Keys for locations: ***ROSS SEA REGION*** **A**, Ross Island; **B**, Victoria Land; ***EAST ANTARCTICA*** **C**, Vestfold Hills; **D**, Windmill Islands; **E**, Ongul Islands; **F**, Other locations; ***ANTARCTIC PENINSULA AND NEIGHBOURING ISLANDS*** **G**, Signy Island; **H**, King George Island; **I**, Other locations; ***SUBANTARCTIC ISLANDS*** **J**, Ballany Islands; **K**, Macquarie Island; ***OTHER LOCATIONS*** **L**, Other locations.
Keys for notes: **a**, human activity high; **b**, human activity low; **c**, human activity unknown; **d**, strong biotic influence; **e**, little or no biotic influence; **f**, biotic influence unknown; **g**, isolated at geothermal areas; **h**, isolated from the historic materials;

Table 3.5 Filamentous micro-fungi isolated in the Antarctic region. (Continued)

Fungal species identified	References, Locations and Notes
Paecilomyces sp.	7Bbdg, 8Kbd, 10Bcf, 11Bcf, 19Bbfg, 28Bad
Paecilomyces cf. *clavisporus*	17Fad,
Paecilomyces farinosus	14Bbd,
Paecilomyces vaiotii	33Had
Penicillium sp.	3De, 3Dd, 3Da, 6Aad, 7Bbdg, 8Kbd, 9Ifg, 12Iad, 17Fad, 19Bbfg, 20Dcd, 21Iad, 23Faf, 28Bad, 29Bad, 31Aadh, 34Bbd, 39Abdg
Penicillium adametzi	10Bcf, 11Bcf, 18Bad, 36Lcf, 38Aad
Penicillium brevicompactum	13Iad, 17Fbd, 22Kad, 33Had
Penicillium canescens	10Bcf, 11Bcf, 36Lcf, 38Ebe
Penicillium charlesii	38Aad, 38Ead, 38Ebe
Penicillium chrysogenum	3De, 3Dd, 10Bcf, 11Bcf, 12Iad, 13Iad, 18Bad, 36Lcf
Penicillium citreo-viride	10Bcf, 11Bcf, 36Lcf
Penicillium commune	13Iad, 27Dbd,
Penicillium corylophilum	10Bcf, 11Bcf, 27Dbd, 36Lcf, 38Aad, 38Ebe
Penicillium crustosum	38Aad
Penicillium cyclopium	8Kbd, 17Fad, 17Fbd, 20Dcd, 22Kad, 23Faf,
Penicillium decumbes	13Iad
Penicillium echinulatum	20Dcd
Penicillium expansum	3Dd, 13Iad, 18Bad,
Penicillium frequentans	8Kbd, 18Bad,
Penicillium funiculosum	18Bad,
Penicillium glabrum	33Had
Penicillium granulatum	8Kbd
Penicillium janthinellum	8Kbd, 13Iad, 33Had
Penicillium jensenii	4Baf, 22Dad,
Penicillium lilacinum	12Iad, 18Bad,
Penicillium notatum	4Baf
Penicillium oxalicum	13Iad
Penicillium palitans	3Dd
Penicillium ramigena	13Iad
Penicillium roqueforti	13Iad
Penicillium solitum	27Dbd
Penicillium soppi	4Baf
Penicillium cf. *spinulosum*	17Fbd
Penicillium spinulosum	26Gbd
Penicillium verrucosa	4Baf
Penicillium viridicatum	8Kbd
Penicillium waksmanii	35Gbd

Keys for references: **1**, Aislabie et al. (2001); **2**, Azmi and Seppelt (1997); **3**, Azmi and Seppelt (1998); **4**, Baublis et al. (1991); **5**, Bostelmann (1976); **6**, Boyd et al. (1996); **7**, Broady et al. (1987); **8**, Bunt (1965); **9**, Cameron et al. (1970); **10**, Cameron et al. (1974); **11**, Cameron et al. (1976); **12**, Caretta and Piontelli (1977); **13**, Corte and Daglio (1962); **14**, Del Frate and Caretta (1990); **15**, Ellis (1980); **16**, Fenice et al. (1997); **17**, Fletcher et al. (1985); **18**, Greenfield (1981); **19**, Greenfield (1983); **20**, Heatwole et al. (1989); **21**, Hurst et al (1983); **22**, Kerry (1990a); **23**, Kerry (1990b); **24**, Latter and Heal (1971); **25**, Line (1988); **26**, Marshall (1998); **27**, McRae and Seppelt (1999); **28**, Mercantini et al. (1989); **29**, Mercantini et al. (1993); **30**, Meyer et al. (1962); **31**, Meyer et al. (1963); **32**, Meyer et al. (1967); **33**, Möller. et al. (1996); **34**, Onofri et al. (1994); **35**, Pugh and Allsopp (1982); **36**, Sun et al. (1978); **37**, Tubaki (1961); **38**, Tubaki and Asano (1965); **39**, Ugolini and Starkey (1966); **40**, Weinstein et al. (1997); **41**, Weinstein et al. (2000); **42**, Zucconi et al. (1996).
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Table 3.6 Filamentous micro-fungi isolated in the Antarctic region. (Continued)

Fungal species identified	References, Locations and Notes
Pestalotia sp.	34Bbd
Peyronellea sp.	21Iad
Phaeoseptoria sp.	33Had
Phaeosphaeria eustoma	33Had
Phaeosphaeria microscopica	33Had
Phialophora sp.	1Aa, 21Iad
Phialophora cf. *alba*	33Had
Phialophora dermatitidis	10Bcf, 11Bcf, 36Lcf
Phialophora fastigiata	14Bbd, 18Bad, 23Faf, 23Fbe, 34Bad, 36Lcf, 42Bbd
Phialophora gougerotii	10Bcf, 11Bcf, 36Lcf
Phialophora hyaline	33Had
Phialophora lagerbergii	4Baf, 10Bcf, 11Bcf, 36Lcf
Phialophora malorum	3Dd, 3Da, 33Had
Phialophora melinii	33Had
Phoma sp.	3De, 3Dd, 16Bbd, 22Kad, 27Dbd, 32Fad, 33Had, 34Bbd, 35Gbd, 42Bbd
Phoma exigua	22Kad
Phoma cf. *herbarum*	2Dcf
Phoma herbarum	3Dd, 3Da, 14Bbd, 17Fad, 22Dad, 23Faf, 23Fbe, 27Dbd, 42Bbd
Phoma hibernica	10Bcf, 11Bcf, 36Lcf
Phoma nebulosa	25Caf,
Phoma sorghina	16Bbd, 42Bbd
Phomopsis sp.	33Had
Phyllosticta sp.	8Kbd
Pleospora vegans	33Had
Polycoccum sp.	34Bbd
Polyscytalum sp.	8Kbd
Pycnoporus coccineus	4Baf
Pycnostysanus sp.	33Had
Rhizopus sp.	19Bad, 35Gbd
Rhizopus arrhizus	30Aadh
Rhizopus nigricans	13Iad
Rhizopus nodusus	10Bcf, 11Bc, 36Lcf f
Rhizopus stolonifer	3Dd, 3Da,
Rhodesiopsis gelatinosa	22Kad

Keys for references: **1**, Aislabie et al. (2001); **2**, Azmi and Seppelt (1997); **3**, Azmi and Seppelt (1998); **4**, Baublis et al. (1991); **5**, Bostelmann (1976); **6**, Boyd et al. (1996); **7**, Broady et al. (1987); **8**, Bunt (1965); **9**, Cameron et al. (1970); **10**, Cameron et al. (1974); **11**, Cameron et al. (1976); **12**, Caretta and Piontelli (1977); **13**, Corte and Daglio (1962); **14**, Del Frate and Caretta (1990); **15**, Ellis (1980); **16**, Fenice et al. (1997); **17**, Fletcher et al. (1985); **18**, Greenfield (1981); **19**, Greenfield (1983); **20**, Heatwole et al. (1989); **21**, Hurst et al (1983); **22**, Kerry (1990a); **23**, Kerry (1990b); **24**, Latter and Heal (1971); **25**, Line (1988); **26**, Marshall (1998); **27**, McRae and Seppelt (1999); **28**, Mercantini et al. (1989); **29**, Mercantini et al. (1993); **30**, Meyer et al. (1962); **31**, Meyer et al. (1963); **32**, Meyer et al. (1967); **33**, Möller. et al. (1996); **34**, Onofri et al. (1994); **35**, Pugh and Allsopp (1982); **36**, Sun et al. (1978); **37**, Tubaki (1961); **38**, Tubaki and Asano (1965); **39**, Ugolini and Starkey (1966); **40**, Weinstein et al. (1997); **41**, Weinstein et al. (2000); **42**, Zucconi et al. (1996).
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Table 3.7 Filamentous micro-fungi isolated in the Antarctic region. (Continued)

Fungal species identified	References, Locations and Notes
Sclerotinia sclerotirum	3Da
Scolecobasidium salinum	14Bbd, 23Faf
Scopularopsis sp.	29Bad
Scopulariopsis brevicaulis	28Bad, 29Bad
Scytalidium sp.	33Had
Scytalidium thermophilum	14Bbd,
Sepedonium chrysospermum	10Bcf, 11Bcf, 36Lcf
Septofusidium elegantulum	26Gbd
Sporotrichum carnis	13Iad
Sporotrichum roseum	13Iad
Sporotrix cf. *ramosissima*	17Fad
Stagonospora ischmaemi	22Kad
Stemphylium sp.	12Iad
Stemphylium botryosum	23Faf
Stephanosporium cerealis	24Gbd
Syncephalostrum racemosum	13Iad
Thelebolus microsporus	2Dcf, 3De, 3Dd, 3Da, 14Bbd, 16Bbd, 17Fad, 22Dad, 23Faf, 23Fbe, 27Dbd, 33Had, 34Bbd
Thermomyces lanugionosus	14Bbd, 15Kad
Tolypocladium nubicola	33Had
Tricellula cf. *aquatica*	33Had
Trichocladium opacum	33Had
Trichoderma sp.	25Caf,
Trichoderma glaucum	13Iad, 18Bad,
Trichoderma harzianum	3Dd, 3Da, 10Bcf, 11Bcf, 18Bad, 36Lcf
Trichoderma koningi	13Iad
Trichoderma lignorum	13Iad
Trichoderma psedokoningi	3Da
Trichoderma viride	18Bad, 23Faf, 27Dbd,
Trichophyton sp.	5Gad
Trichophyton mentagrophytes	29Bad
Trichophyton terrestre	28Bad, 35Gbd
Trichosporon beigelii	4Baf
Trichurus spiralis	33Had
Tritirachium sp.	9Ifg
Tritirachium album	9Ifg, 10Bcf, 11Bcf
Tritirachium roseum	9Ifg, 10Bcf, 11Bcf
Verticillium sp.	12Iad, 14Bbd, 17Fbd, 24Gbd, 25Cbf, 34Bbd, 35Gbd
Verticillium cf. *lecanii*	16Bbd
Volucrispora graminea	33Had
Wardomyces sp.	35Gbd

Keys for references: **1**, Aislabie et al. (2001); **2**, Azmi and Seppelt (1997); **3**, Azmi and Seppelt (1998); **4**, Baublis et al. (1991); **5**, Bostelmann (1976); **6**, Boyd et al. (1996); **7**, Broady et al. (1987); **8**, Bunt (1965); **9**, Cameron et al. (1970); **10**, Cameron et al. (1974); **11**, Cameron et al. (1976); **12**, Caretta and Piontelli (1977); **13**, Corte and Daglio (1962); **14**, Del Frate and Caretta (1990); **15**, Ellis (1980); **16**, Fenice et al. (1997); **17**, Fletcher et al. (1985); **18**, Greenfield (1981); **19**, Greenfield (1983); **20**, Heatwole et al. (1989); **21**, Hurst et al (1983); **22**, Kerry (1990a); **23**, Kerry (1990b); **24**, Latter and Heal (1971); **25**, Line (1988); **26**, Marshall (1998); **27**, McRae and Seppelt (1999); **28**, Mercantini et al. (1989); **29**, Mercantini et al. (1993); **30**, Meyer et al. (1962); **31**, Meyer et al. (1963); **32**, Meyer et al. (1967); **33**, Möller. et al. (1996); **34**, Onofri et al. (1994); **35**, Pugh and Allsopp (1982); **36**, Sun et al. (1978); **37**, Tubaki (1961); **38**, Tubaki and Asano (1965); **39**, Ugolini and Starkey (1966); **40**, Weinstein et al. (1997); **41**, Weinstein et al. (2000); **42**, Zucconi et al. (1996).
Keys for locations: ***ROSS SEA REGION*** **A**, Ross Island; **B**, Victoria Land; ***EAST ANTARCTICA*** **C**, Vestfold Hills; **D**, Windmill Islands; **E**, Ongul Islands; **F**, Other locations; ***ANTARCTIC PENINSULA AND NEIGHBOURING ISLANDS*** **G**, Signy Island; **H**, King George Island; **I**, Other locations; ***SUBANTARCTIC ISLANDS*** **J**, Ballany Islands; **K**, Macquarie Island; ***OTHER LOCATIONS*** **L**, Other locations.
Keys for notes: **a**, human activity high; **b**, human activity low; **c**, human activity unknown; **d**, strong biotic influence; **e**, little or no biotic influence; **f**, biotic influence unknown; **g**, isolated at geothermal areas; **h**, isolated from the historic materials;

3.2. Materials and Methods

3.2.1. Sampling

Human and animal foodstuffs, swab samples and visible micro-fungi on the floor were collected aseptically by Professor Roberta L Farrell at Discovery Hut (77°50'50''S, 166°38'30''E), Ross Island Antarctica during the Antarctica New Zealand's Event K021 in December 2000 (Table 3.8). All samples were kept frozen and were brought back to University of Waikato, Hamilton, New Zealand under the Ministry of Agriculture and Fishery permit number 200010576. The samples and fungal isolates are currently held at the MAF transitional quarantine facility (C.2.10), The University of Waikato.

3.2.2. Isolation of micro-fungi

A small amount of each sample (~1g) was transferred into liquid growth media (yeast extract 0.2%, malt extract 1.5%) (Farrell et al. 1998b). These were incubated with horizontal shaking movement at three different temperatures of 4, 15, and 25 °C for one week before plated out onto the yeast malt extract (YM) agar (yeast extract 0.2%, malt extract 1.5%, agar 1.8%) (Farrell et al. 1998b), the basidiomycete agar (east extract 0.2%, malt extract 1.5%, agar 1.5%, benlate 0.06g, streptomycin 0.01g after autoclaving) (Worrall 1991), and Media 4 (yeast extract 0.2%, malt extract 1.5%, agar 1.8%, chloramphenicol 0.2g, streptomycin 0.1g after autoclaving) (Harrington 1981). The YM media is designed to encourage both microbial and fungal growth whereas the basidiomycete media and Media 4 contain antibiotics to suppress microbial over-growth before filamentous fungi can establish themselves. Following four weeks, all plates were daily observed to sub-culture filamentous fungi. Sub-cultures were isolated on the same media as their parental plates. All isolates were sub-cultured until colonies of uniform physical appearance were achieved.

3.2.3. Identification

Dr Margaret di Menna at AgResearch, Ruakura, Hamilton identified fungal isolates according to classical taxonomic morphological features.

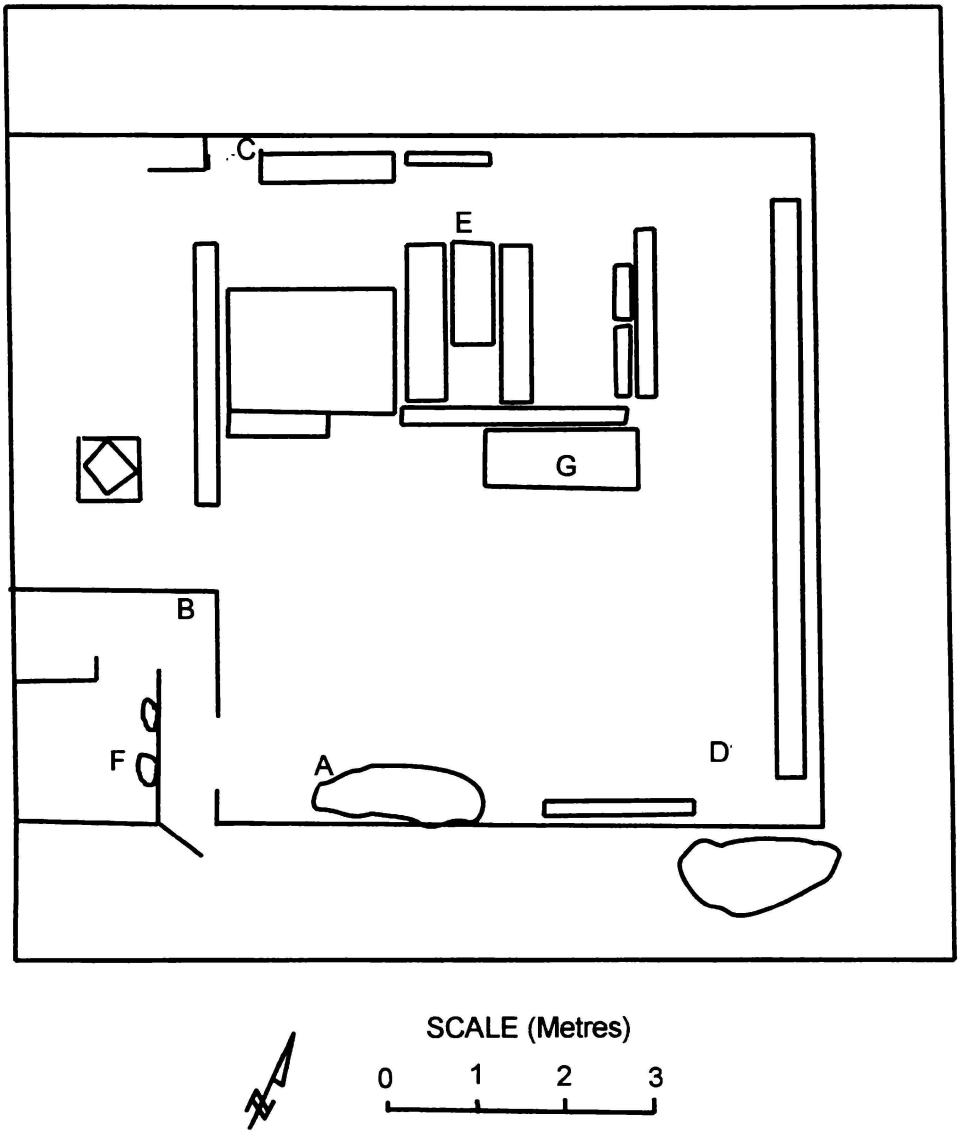


Figure 3.1 Sampling locations (A – G) at Discovery Hut, Ross Island, Antarctica.

Table 3.8 Sample description and fungal isolates. Keys; Y – isolates on yeast malt agar; 4 – isolates on Media 4; B – isolates on Basidiomycete agar.

Sample description	Fungal Isolates	Isolation Temperature		Media used for isolation	
		15 °C	25 °C	15 °C	25 °C
A. Straw underneath blubber	*Acremonium bacillisporum*	+		Y	
	Aphanocladium album	+	+	Y	Y
	Aspergillus versicolor		+		Y
	Cladosporium cladosporioides	+		Y	
	Geotrichum sp.	+		Y	
	Malbranchea sp.	+		Y	
	Penicillium canescens	+		Y	
	Penicillium minioluteum		+	Y	Y, 4
	Penicillium spinulosum	+		Y	
	Rhinocladiella sp.	+		Y	
B. Straw across from door way	*Aphanocladium album*	+		Y	
	Beauveria sp.	+		Y	
	Cladosporium cladosporioides	+		Y	
	Gliocladium sp.	+		Y	
	Penicillium griseoroseum	+		Y	
	Penicillium minioluteum	+	+	Y	Y
	Penicillium spinulosum	+		Y	
	Rhinocladiella sp.	+		Y	
C. Fungal colony inside of exterior western wall	*Botrytis cinerea*	+		Y	
	Cladosporium cladosporioides	+		Y	
	Myceliophthora sp.	+		Y	
	Penicillium brevicompuctum	+		Y	
	Penicillium fellutanum	+		Y	
	Penicillium minioluteum	+		Y	
	Tritirachium sp.	+		Y	
D. Fungal colony on floor	*Cladosporium cladosporioides*	+		Y	
	Penicillium minioluteum	+		Y	
	Penicillium cf. *verrucosum*	+		Y	
E. Molasses seeping near the stove	*Aphanocladium album*		+		B
	Geotrichum sp.	+		Y	
	Malbranchea sp.	+		Y	
	Penicillium minioluteum	+		Y	
F. Mutton	*Mucor* sp.		+		B
	Penicillium inflatum	+		Y	
	Penicillium minioluteum	+		Y	
G. Biscuit (dog/human consumption)	*Aphanocladium album*		+		B
	Cladisporium cladosporioides	+		Y	
	Myceliophthora sp.	+		Y	
	Penicillium brevicompactum	+		Y	
	Penicillium minioluteum	+		Y	

3.3. Results

3.3.1. Diversity

Filamentous micro-fungi were isolated and identified from the human and animal foodstuffs and the internal environment of the Discovery Hut at Hut Point, Ross Island, Antarctica (Table 3.8). There were 22 taxa and 14 genera recorded from this study. *Mucor* sp. (zygomycetes) was the only non-hyphomyces micro-fungi isolated at Hut Point. *Penicillium* species were most frequently isolated. In particular, *Penicillium minioluteum* was present in all the samples. *Cladosporium cladosporioides* was another common isolate amongst others. The straw samples contained a variety of fungi; *Acremonium bacillisporum*, *Aphanocladium album*, *Aspergillus versicolor*, *Beauveria* sp., *Cladosporium cladosporioides*, *Geotrichum* sp., *Gliocladium* sp., *Malbranchea* sp., *Penicillium canescens*, *Penicillium griseoroseum*, *Penicillium minioluteum*, *Penicillium spinulosum*, *Rhinocladiella* sp., *Acremonium bacillisporum*, *Aspergillus versicolor*, *Penicillium canescens*, *Penicillium griseoroseum*, *Penicillium spinulosum* and *Rhinocladiella* spp. were isolated only from the straw samples. Visible fungal colonies on the wall and floor also contained many filamentous micro-fungi; *Botrytis cinerea*, *Penicillium fellutanum*, and *Tritirachium* sp. which were not found in the other samples. The foodstuff remains (molasses, mutton and biscuit) in the hut had fewer fungal isolates than the straw and environmental samples. *Mucor* sp. was only recovered from mutton carcass. Seven previously unreported filamentous micro-fungi were isolated from this study as follows; *Acremonium bacillisporum*, *Aphanocladium album*, *Penicillium fellutanum*, *Penicillium griseoroseum*, *Penicillium inflatum*, *Penicillium minioluteum*, and *Rhinocladiella* sp.

3.3.2. Isolation temperatures

There was no isolation of filamentous micro-fungi at 4 °C. Majority of fungal isolates were obtained at 15 °C. *Aphanocladium album* isolates were more frequently isolated at 25 °C (three times) than 15 °C (once). *Aspergillus versicolor* and *Mucor* sp. were obtained only at 25 °C.

3.3.3. Use of the selective media

Penicillium minioluteum isolated from the straw underneath blubber grew on the media 4 in the presence of chloramphenicol and streptomycin. *Aphanocladium album* from the molasses and biscuit samples at 25 °C and *Mucor* sp. isolated from mutton at 25 °C was able to grow in the presence of benlate and streptomycin.

3.4. Discussion

3.4.1. Filamentous micro-fungi diversity inside Discovery Hut

The origins of filamentous fungi are diverse in Antarctica. They can be introduced to Antarctica by three main pathways; long distance transport by wind, animals and birds, and human (Vincent 2000). Many cosmopolitan genus, such as *Acremonium*, *Aspergillus*, *Cladosporium*, *Mucor* and *Penicillium*, were isolated from the samples collected inside Discovery Hut. They have been previously isolated in many occasions at different locations (see Table 3.1 – Table 3.7). The genus *Rhinocladiella*, and *Acremonium bacillisporum* and *Aphanocladium album* have not been reported in Antarctica. Four *Penicillium* species isolated in this study, *Penicillium fellutanum*, *Penicillium griseoroseum*, *Penicillium inflatum*, and *Penicillium minioluteum*, were also newly recorded from Antarctica. Food crates made of wood material had the highest diversity of filamentous micro-fungi in comparison with environmental samples at Mawson station (Kerry 1990b). Mercantini et al. (1993) isolated many fungal species from dust samples collected inside the Italian research station including the same genus isolated in this study, such as *Aspergillus*, *Beauveria*, *Malbranchea* and *Penicillium*. In particular, *Penicillium* species were prevalent in their study (Mercantini et al. 1993). Present study showed the same trend that *Penicillium* species were associated with human habitation areas of present and past. *Botrytis cinerea*, *Geotrichum*, *Gliocladium*, *Myceliophthora*, *Tritirachium* were occasionally isolated at different locations.

3.4.2. Human activity and fungal diversity

Microbial diversity and human activities are positively correlated in the Antarctic environment (Onofri et al. 1994; Vishniac 1996). Microbial introductions at Antarctic research stations are well-documented. Microbial dispersal by human activities in the Ross Sea region, in particular McMurdo Station and the Dry Valleys was noted as early as in the 1970s by Cameron and colleagues (see review in Cameron et al. 1972). The pathogenic fungi *Phialophora dermatidis* and *Phialophora gougerotii* near the McMurdo Station were recorded (Sun et al. 1978). Broady and Smith (1994) identified two non-indigenous algal species in the soil attached onto the imported fresh vegetables received at Scott Base directly

from New Zealand. Mercantini et al (1993) isolated many filamentous fungi from dust samples inside the Italian base at Terra Nova Bay, Northern Victoria Land and suggested human activity plays an important role in fungal dispersion. On the other side of the continent, highest fungal counts were recorded in the soils within the radius of one hundred metres from the main building, and gradual decrease in the fungal count was observed as the soil samples were taken at further localities at Showa Station (69°00'25"S, 39°35'01"E) (Toyoda et al. 1986). Similar trends were observed in the study of microbial dispersal determined with the use of PCR as well as plating method at Halley Station (S75°35'54", W26°32'28") which stands on Brunt Ice Shelf (Upton et al. 1997).

Fungal manifestation was common in a hut of the Heroic Era. For example, McLean (1918), the pioneer in Antarctic microbiology who carried out microbial investigations at Cape Denison in the Sir Douglas Mawson's Australasian Antarctic Expedition (1911 – 1914), noted 'it appeared that the Hut was infested by the spores of moulds which would doubtless have infected the culture tubes' and that 'fungi, represented by moulds of many species, grew luxuriantly along the rime-filled cracks between the inner lining boards of the hut'. Discovery Hut was extensively used in the four separate expeditions of Scott and Shackleton in the early 20th century. Mycoflora of Discovery Hut originated most likely from the human activities spanning for one hundred years. Scott (1905) brought sheep from New Zealand to Antarctica and slaughtered them for food. Sheep carcasses still remain in the hut. Ponies and mules were used for transportation, later for food in the Heroic Era (Scott 1913; Shackleton 1909). The hut was rarely visited until 1950s when the establishment of McMurdo Station and Scott Base prompted conservation work. The hut received many visitors every year including scientists, conservators, staffs from nearby stations and tourists. Many isolates from the foodstuffs at Discovery Hut presumptively originated from historic materials brought by explorers, and/or later dispersal from conservators and visitors.

The Discovery Hut provides a safe refuge for microorganisms today. Carbon, phosphorous, and nitrogen sources are plentiful in the ambient environment. Exposure to harmful UV light level is relatively small inside the hut. Water availability and temperature are restricting factors for the microbial growth most

of the year (Wynn-Williams 1990). However, relative humidity and temperature inside the hut rises above 65% and 0 °C respectively for about a month in summer (Minasaki et al. 2001). The internal climate is suitable for fungal growth, which was visually observed while the candidate was sampling in January 2002. It is possible to assume that many filamentous fungi may have already spread outside the hut or may have been brought into the hut due to a heavy human traffic of Discovery Hut, although they may not be able to survive under harsh and nutrient depleted environments (Upton et al. 1997).

3.4.3. Fungal isolation temperatures and antibiotic resistance

Almost all the isolates were achieved by incubation of growth media agar plates at 15 °C, except for *Aphanocladium album*, *Aspergillus versicolor*, *Mucor* sp., and *Penicillium minioluteum* (Table 3.8). *Penicillium minioluteum* was prevalent in the internal environment of the hut, but has not been reported in the literature from isolations in the McMurdo region or in the southern Victoria Land. This fungus was repeatedly isolated at both 15 °C and 25 °C and was possibly unable to establish outside the hut, thus no report of isolation of *Penicillium minioluteum*. Similarly, *Aphanocladium album* was not isolated in the literature and was possibly viable only inside Discovery Hut. A mesophic *Aspergillus versicolor* was isolated in the northern Victoria Land (Zucconi et al. 1996). *Mucor hiemalis* showed temperature adaptation to the Antarctic environment in comparison with the same species isolated in a temperate region (Line 1988). Two *Aphanocladium album* isolates, *Penicillium minioluteum*, and *Mucor* sp., showed growth in the presence of antibiotic compounds. These fungi may be parasitic fungi capable of tolerating antibiotics produced by their host or symbiont.

3.5. Conclusion

Human activities for one hundred years resulted in a significant effect on mycoflora of the hut. In this study, a total of twenty one filamentous micro-fungi taxa were isolated from the oldest historic hut on Ross Island. Seven taxa have not been reported in the previous investigation in mycological diversity on the entire Antarctic continent. Almost all the isolates were obtained at 15 °C. Future studies should be addressed as to whether fungi isolated in this study are mesophilic, psychrotolerant or psychrophilic from the growth rate relative to temperature (Zucconi et al. 1996). Antarctic microbiology has been traditionally developed in an ecological context with an analogy with the Martian environment (Cameron et al. 1976). However, little is known about the mechanisms in which filamentous fungi can survive numerous freeze-thaw cycles (Vishniac 1996) and how introduced organisms can adapt themselves in the Antarctic climate regime (Vincent 2000). Historic areas at Hut Point, Cape Evans and Cape Royds on Ross Island provide an excellent opportunity to collect samples which may contain various filamentous micro-fungi to test these unanswered questions.

An unexploited research area exists in Antarctic mycology. It has not been addressed to assess genetic variability among Antarctic isolates of the same species originated from different locations in Antarctica. Cosmopolitan species, such as *Cladosporium cladosporieides*, together with restriction fragment length polymorphism, allozyme analysis, or sequence comparison of the internal transcribed spacer regions, can be used for this purpose. Filamentous micro-fungi are widely known to possess extracellular activities which can selectively be isolated for a specific purpose. Relatively few authors investigated this subject (Bradner et al. 1999a, 1999b, Fenice et al. 1997, Hurst et al. 1983) in comparison with many ecological studies in Antarctic mycology.

CHAPTER FOUR

4. General Conclusions

The overall objective of this thesis was to qualitatively assess microbial diversity in relation to human activity in historic areas at Cape Evans and Hut Point on Ross Island, Antarctica. With use of both classical morphological taxonomy and molecular diagnostic markers, human influence on the microbial flora at historic areas was evaluated to be significant.

While an attempt to culture viable spores from environmental samples by NCDI was unsuccessful and additional confirmation tests at LLNL are being carried out, the experimental results indicated that *B. anthracis* DNA was present in the DNA obtained by the PSB-BB method from environmental samples collected in the stable areas of the historical hut at Cape Evans. Extracted environmental DNA contained non-specific targets as well as PCR inhibitors, which decreased the efficiency in PCR and particularly in FRET PCR. *B. anthracis* specific primers on three individual targets combined with the nested primers amplified bands at expected sizes on agarose gels from several samples. Sequence analyses of the *vrrA* gene could not distinguish between the *B. anthracis* positive DNA (Pasteur II) and the sequences from several positive samples, except for the sample #32. Possibility of the positive DNA contamination was discussed, but concluded it was highly unlikely. Future research should focus to address the extent of *B. anthracis* distribution in the vicinity of the hut and identification of the type strain with MLVA which undoubtedly would give possible geographical origin(s) of *B. anthracis* spores.

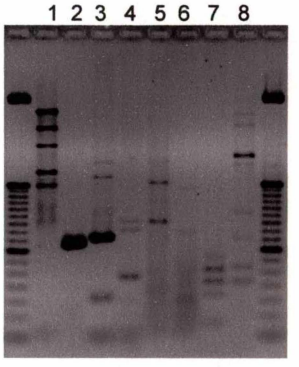
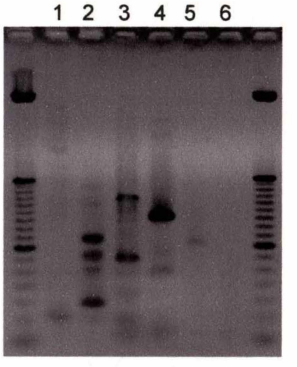
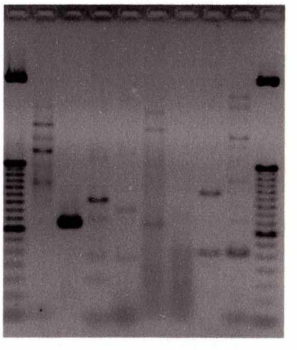
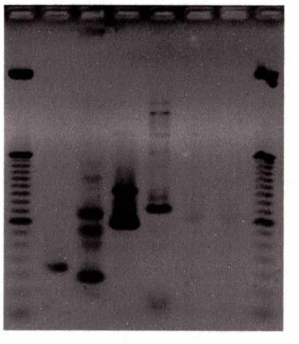
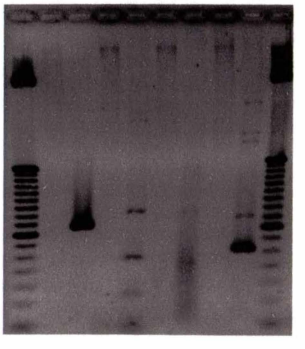
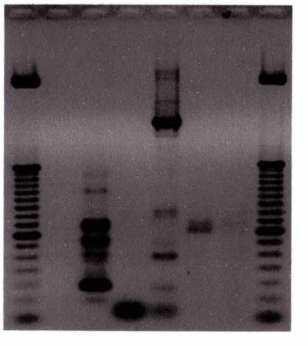
A diverse range of filamentous micro-fungi were isolated from the foodstuffs and internal environment at Discovery Hut at Point. *Penicillium* species were most frequently isolated in the samples, while cosmopolitan genus such as *Acremonium*, *Aspergillus*, *Cladosporium*, *Mucor* were also isolated. A thorough literature

search for filamentous micro-fungi identified seven new taxa in the continental Antarctica. Many isolates were obtained at 15 °C and some showed growth in the presence of antibiotic substances. Physiological adaptations and antibiotic resistances of these isolates were not well fully characterised nor understood, thus there is a necessity for the future investigations. Mycological investigation of the other historic areas at Capes Evans and Royds on Ross Island should also be carried out to enable for comparative study in mycological diversity.

Legacy of the human exploration in the early 20th century on Ross Island is not limited to historical interests and visible artefacts, but many unseen microorganisms introduced by the human activity prolonged for one hundred years. This thesis demonstrated that a wealth of introduced microorganisms existed at historic sites and that a generalised picture was drawn in the direct relationship between human activities and microbial diversity. *B. anthracis* was never reported scientifically as it is not an endemic microorganism in Antarctica. Although the connection with the death of Petty Officer Evans remained unsolved, *B. anthracis* DNA most likely originated from activities of the Captain Scott's expedition (1911-1913). Isolation of many filamentous micro-fungi suggested that they were brought into Discovery Hut through the expeditions and conservation activities. Molecular techniques should be employed as well as the conventional morphological identification methods for future research in the ecological study of Antarctic microbiology where unculturable microorganisms can be detected.

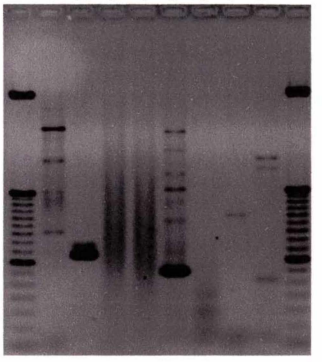
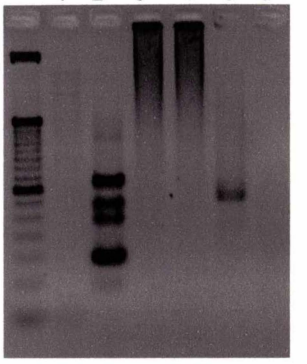
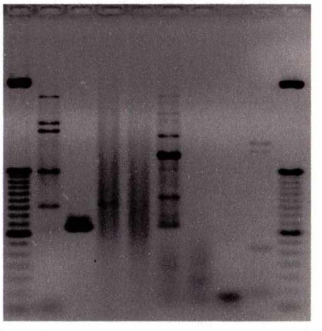
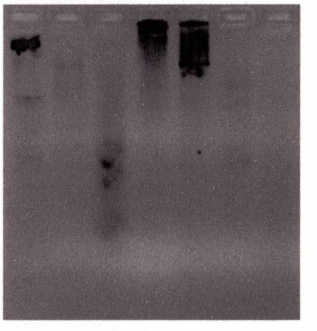
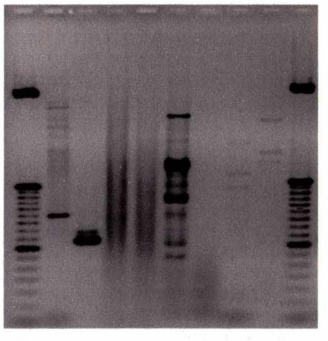
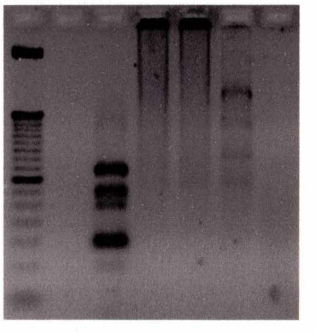
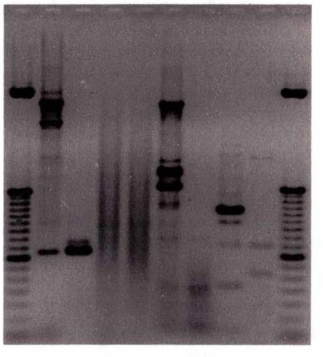
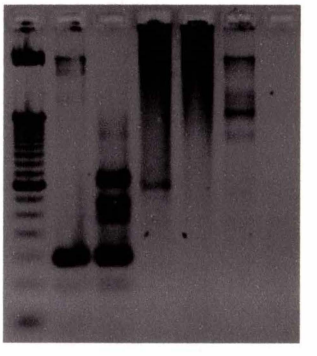
APPENDICES

Appendix A Profiles of electrophoresis gel (in reverse image). Environmental DNA samples were screened with the primary and nested primers. The 50 bp ladders are visible at both lanes and either extreme left or right lane with thicker bands at 350, 800 and 2653 bp positions. Sample 68 was not tested with the *vrrB* primers.

Sample	Primary PCR	Nested PCR
10		
11		
15		

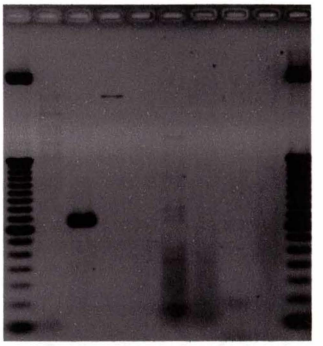
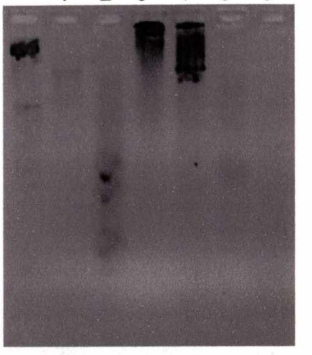
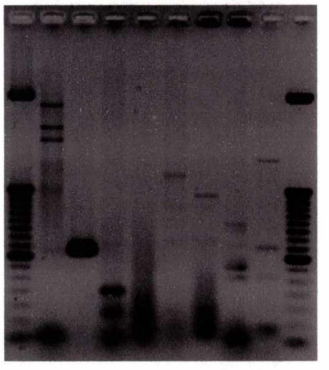
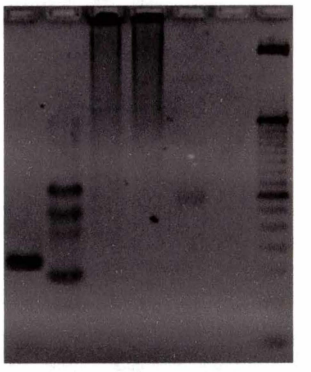
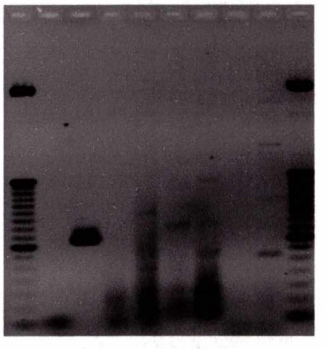
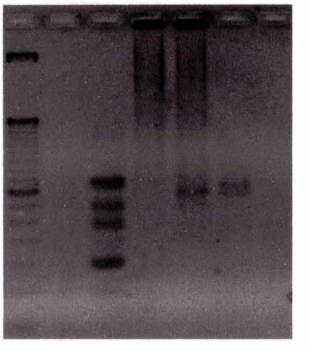
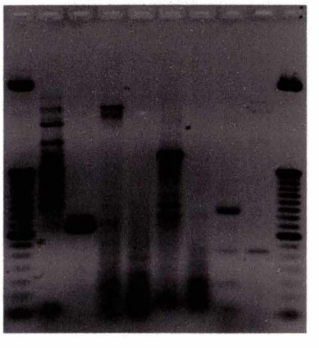
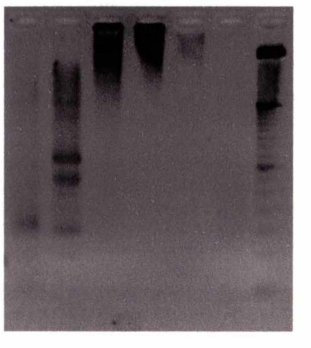
1 Environmental DNA + GPR primers
2 *B. licheniformis* DNA + GPR primers
3 Environmental DNA + PA primers
4 *B. licheniformis* DNA + PA primers
5 Environmental DNA + CAPA primers
6 *B. licheniformis* DNA + CAPA primers
7 Environmental DNA + *vrrB* primers
8 *B. licheniformis* DNA + *vrrB* primers

1 PCR product + EWA primers
2 PCR product + EWA primers
3 PCR product + PA-nested primers
4 PCR product + PA-nested primers
5 PCR product + CAPA-nested primers
6 PCR product + CAPA-nested primers

Sample	Primary PCR	Nested PCR
23		
24		
31		
32		

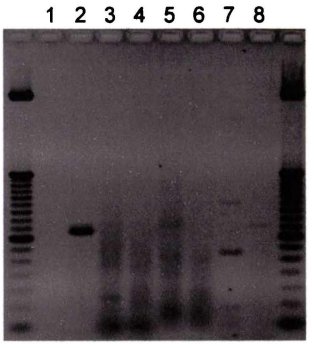
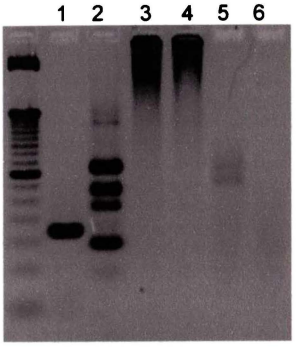
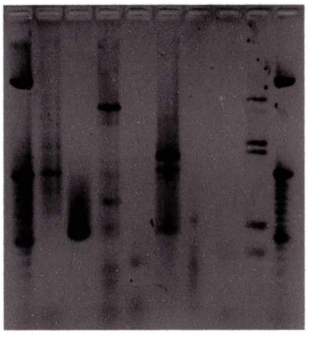
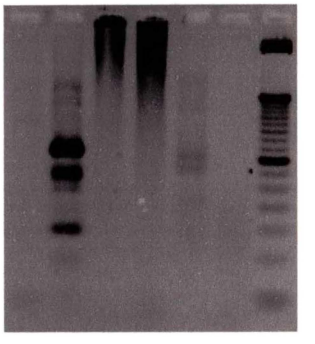
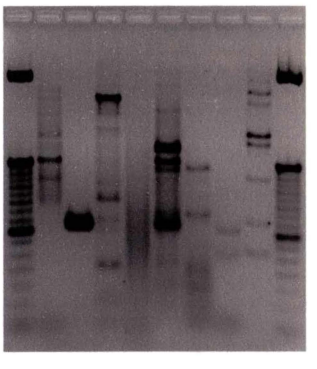
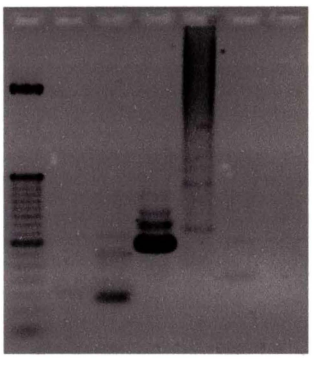
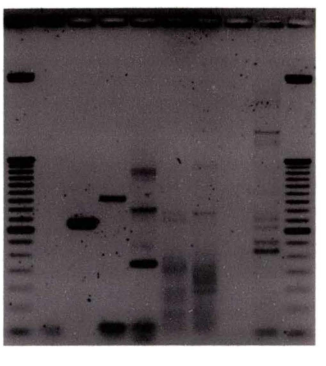
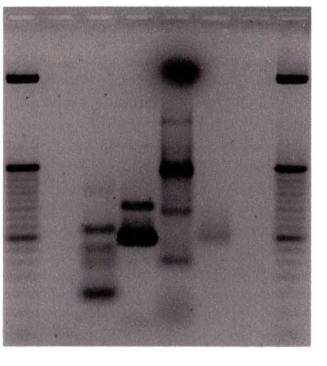
1 Environmental DNA + GPR primers
2 *B. licheniformis* DNA + GPR primers
3 Environmental DNA + PA primers
4 *B. licheniformis* DNA + PA primers
5 Environmental DNA + CAPA primers
6 *B. licheniformis* DNA + CAPA primers
7 Environmental DNA + *vrrB* primers
8 *B. licheniformis* DNA + *vrrB* primers

1 PCR product + EWA primers
2 PCR product + EWA primers
3 PCR product + PA-nested primers
4 PCR product + PA-nested primers
5 PCR product + CAPA-nested primers
6 PCR product + CAPA-nested primers

Sample	Primary PCR	Nested PCR
39		
40		
46		
48		

1 Environmental DNA + GPR primers
2 *B. licheniformis* DNA + GPR primers
3 Environmental DNA + PA primers
4 *B. licheniformis* DNA + PA primers
5 Environmental DNA + CAPA primers
6 *B. licheniformis* DNA + CAPA primers
7 Environmental DNA + *vrrB* primers
8 *B. licheniformis* DNA + *vrrB* primers

1 PCR product + EWA primers
2 PCR product + EWA primers
3 PCR product + PA-nested primers
4 PCR product + PA-nested primers
5 PCR product + CAPA-nested primers
6 PCR product + CAPA-nested primers

Sample	Primary PCR	Nested PCR
53		
58		
59		
60		

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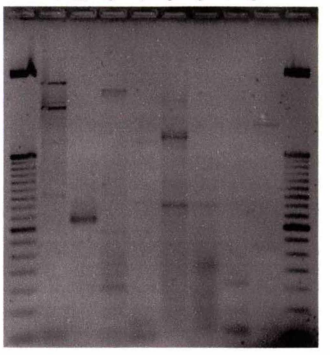
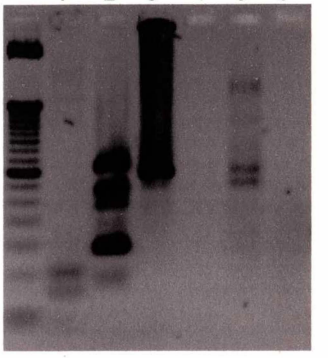
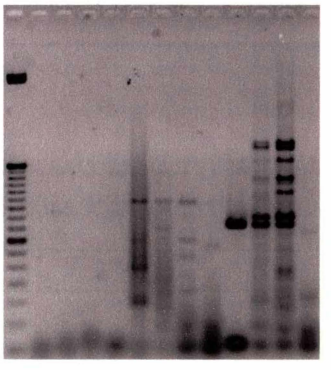
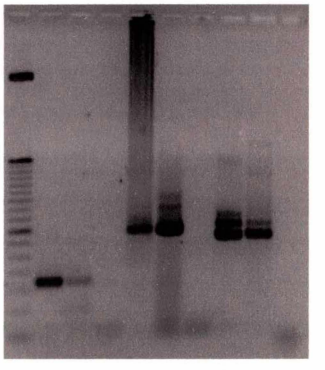
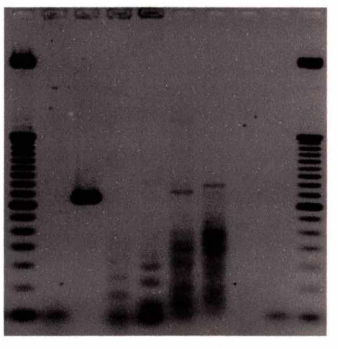
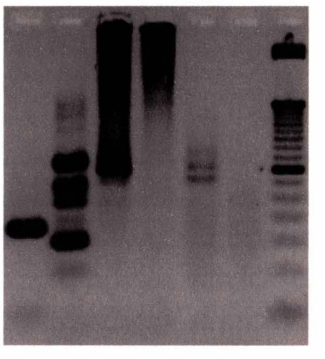
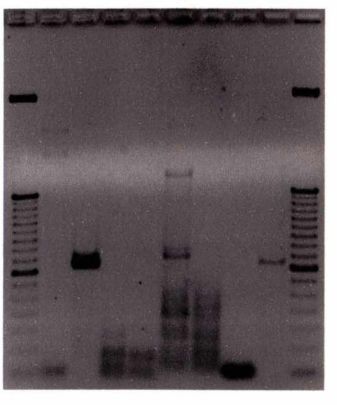
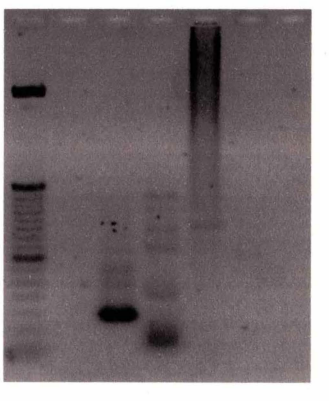
2 *B. licheniformis* DNA + GPR primers
3 Environmental DNA + PA primers
4 *B. licheniformis* DNA + PA primers
5 Environmental DNA + CAPA primers
6 *B. licheniformis* DNA + CAPA primers
7 Environmental DNA + *vrrB* primers
8 *B. licheniformis* DNA + *vrrB* primers

1 PCR product + EWA primers
2 PCR product + EWA primers
3 PCR product + PA-nested primers
4 PCR product + PA-nested primers
5 PCR product + CAPA-nested primers
6 PCR product + CAPA-nested primers

Sample	Primary PCR	Nested PCR
62		
68		
70		
71		

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5 Environmental DNA + CAPA primers
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7 Environmental DNA + *vrrB* primers
8 *B. licheniformis* DNA + *vrrB* primers

1 PCR product + EWA primers
2 PCR product + EWA primers
3 PCR product + PA-nested primers
4 PCR product + PA-nested primers
5 PCR product + CAPA-nested primers
6 PCR product + CAPA-nested primers

Appendix B Alignment of the *vrrA* gene (5′–3′) Sample #32, #40, #53, #70 and positive control DNA *vrrA* sequences were aligned with a consensus sequence (L48553) by Clustal W programme.

```
CLUSTAL W (1.82) multiple sequence alignment

Positive    ACAACTACCACCGATGGCACAAAAAAAGAAAGGGTTCCTTGCTAAACTCTTTAAAAAACA 60
Consensus   ACAACTACCACCGATGGCACAAAAAAAGAAAGGGTTCCTTGCTAAACTCTTTAAAAAACA 60
70          ------------------------------------------------------------
40          ------------------------------------------------------------
53          ------------------------------------------------------------
32          ------------------------------------------------------------


Positive    CGATCCAACCGAACCTTTCATGCAAATGGTTCCGCCTTATCGACAAATGGAAGGACCACC 120
Consensus   CGATCCAACCGAACCTTTCATGCAAATGGTTCCGCCTTATCGACAAATGGAAGGACCACC 120
70          -------------------------ATGGTTCCGCCTTATCGACAAATGGAAGGACCACC 35
40          -------------------------ATGGTTCCGCCTTATCGACAAATGGAAGGACCACC 35
53          -------------------------ATGGTTCCGCCTTATCGACAAATGGAAGGACCACC 35
32          -------------------------ATGGTTCCGCCTTATCGACAAATGGAAGGACCAC- 34
                                      **********************************


Positive    GCCAATGATGCACCAACAACAGCAACCGCCACCCCAATATCGACAGCA-ATATCAACAAC 179
Consensus   GCCAATGATGCACCAACAACAGCAACCGCCACCCCAATATCGACAGCA-ATATCAACAAC 179
70          GCCAATGATGCGCCAACAACAGCAACCGCCACCCCAATATCGACAGCA-ATATCAACAAC 94
40          GCCAATGATGCACCAACAACAGCAACCGCCACCCCAATATCGACAGCA-ATATCAACAAC 94
53          GCCAATGATGCACCAACAACAGCAACCGCCACCCCAATATCGACAGCA-ATATCAACAAC 94
32          --CAATGATGCACCAGCACCAGCAACCGCCACCTCAATATCGACAGCATAAATCAGCAAC 92
              ********* *** ** ************** ************** * **** ****


Positive    AATATCAACAACAATATCAACAACAATATCNACAACAATACCCGCAACAATACTCACAGC 239
Consensus   AATATCAACAACAATATCAACAACAATATCAACAACAATACCCGCAACAATACTCACAGC 239
70          AATATCAACAACAATATCAACAACAATATCAACAACAATACCCGCAACAATACTCACAGC 154
40          AATATCAACAACAATATCAACAACAATATCAACAAC---------------------- 130
53          AATATCAACAACAATATCAACAACAATATCAACAACAATA------------------- 134
32          AATACCAACACCAATACCCGCAACAATATCGACAGCAATACCAAC--CAATA-------- 142
            **** ***** ***** *  ********** *** *
```

```
Positive    AATACCAACCATACATGCAGCATCATCCCGAGCAAATGATCCCTCCTCAAATGTATGAAT 299
Consensus   AATACCAACCATACATGCAGCATCATCCCGAGCAAATGATCCCTCCTCAAATGTATGAAT 299
70          AATACCAACCATA---------------------------------------------- 167
40          ------------------------------------------------------------
53          ------------------------------------------------------------
32          ------------------------------------------------------------


Positive    CAAACGAAACGCGCGGCGGTGCAGCAACTACAGCAGCATCAAGTAGCGGCATCGGTAGT 358
Consensus   CAAACGAAACGCGCGGCGGTGCAGCAACTACAGCAGCATCAAGTAGCGGCATCGGTAGT 358
70          ------------------------------------------------------------
40          ------------------------------------------------------------
53          ------------------------------------------------------------
32          ------------------------------------------------------------
```

Appendix C Sample #32 *vrrA* gene BLAST search (5′–3′) The sequence obtained from sample #32 by the primers specifically amplifies *vrrA* gene was searched by standard nucleotide-nucleotide BLAST programme.

Bacillus anthracis (strain Vollum) vrrA gene, complete cds

Length = 1456

Score = 147 bits (74), Expect = 5e-33

Identities = 126/142 (88%), Gaps = 4/142 (2%)

Strand = Plus / Plus

```
#32    : 1   atggttccgccttatcgacaaatggaaggacca---ccaatgatgcaccagcaccagcaa 57
             ||||||||||||||||||||||||||||||||   |||||||||||||| || ||||||
Vollum: 410 atggttccgccttatcgacaaatggaaggaccaccgccaatgatgcaccaacaacagcaa 469

#32    : 58  ccgccacntcaatatngacagcataaatcagcaacaataccaacaccaatacccgcaaca 117
             |||||||  |||||| |||||||  | ||||  |||||||| |||||  ||||||||||||||
Vollum: 470 ccgccaccccaatatcgacagca-atatcaacaacaatatcaacaacaatacccgcaaca 528

#32    : 118 atatcgacagcaataccaacca 139
             |||   ||||||||||||||||
Vollum: 529 atactcacagcaataccaacca 550
```

Appendix D NCDI Report Sample 18, 41, 45, 68, 73 and 74 were tested for viable *B. anthracis* spores at National Centre for Disease Investigation.

2.AUG.2002 13:18 NCDI NO.467 P.2

NATIONAL CENTRE FOR DISEASE INVESTIGATION

NCDI
P.O. Box 40742
Upper Hutt
Phone (04) 526-9600
Fax (04) 526-9601

CASE NO : NZ0202282

Submitter: ROBERTA FARRELL
DEPARTMENT OF BIOLOGICAL SCIENCES
UNIVERSITY OF WAIKATO
HAMILTON

Species: Soil Sample **Age:** Unknown
Breed: ANTARTICA STABLSex: Unknown

Date Sent: 23 Jul 2002
Date Received: 24 Jul 2002 01:48 pm
Date Tested: 02 Aug 2002

Submitter Reference:

Owner: DR HUGH MORGAN
UNIVERSITY OF WAIKATO

Notification: Fax
Fax Number: 07 838 4976

Test Requested: 6 x Soil - Anthrax Culture,

MICROBIOLOGY - Project Cost Recovery

18	Soil (1)	No Anthrax Isolated
41	Soil (1)	No Anthrax Isolated
45	Soil (1)	No Anthrax Isolated
68	Soil (1)	No Anthrax Isolated
73	Soil (1)	No Anthrax Isolated
74	Soil (1)	No Anthrax Isolated

24 colonies were isolated on Blood and Plet agar, all these bacterial colony isolates were negative for plaque formation with gamma and w phage lysis.

Quality control of B.anthracis on Blood and Plet agar, showed growth +++ and plaque formation with both gamma and w phage.

(Note. Results apply only to samples received, on an as found basis. Precision data will be supplied upon request. H = High result. L = Low result. Reference ranges are standard AHL reference rang

Signed Michele Styles **Michele Styles (Technician)**

Signed Gary Horner **Gary Horner (TEAM LEADER)**

Report Date: 02 Aug 2002 **Final Report - MICROBIOLOGY** **Report Fee:** $210.00

02-AUG-2002 12:13 64 4 5265601 96% P.02

Appendix E Following DNA sequences were obtained from the University of Waikato DNA Sequencing Facility using the Amersham Biosciences MegaBACE DNA analysis system.

1025_32(EWA-R): Sample #32 *vrrA* forward sequence

1025_40(EWA-R): Sample #40 *vrrA* forward sequence

1025_40(EWA-F): Sample #40 *vrrA* reverse sequence

1025_53(EWA-R): Sample #53 *vrrA* forward sequence

1025_53(EWA-F): Sample #53 *vrrA* reverse sequence

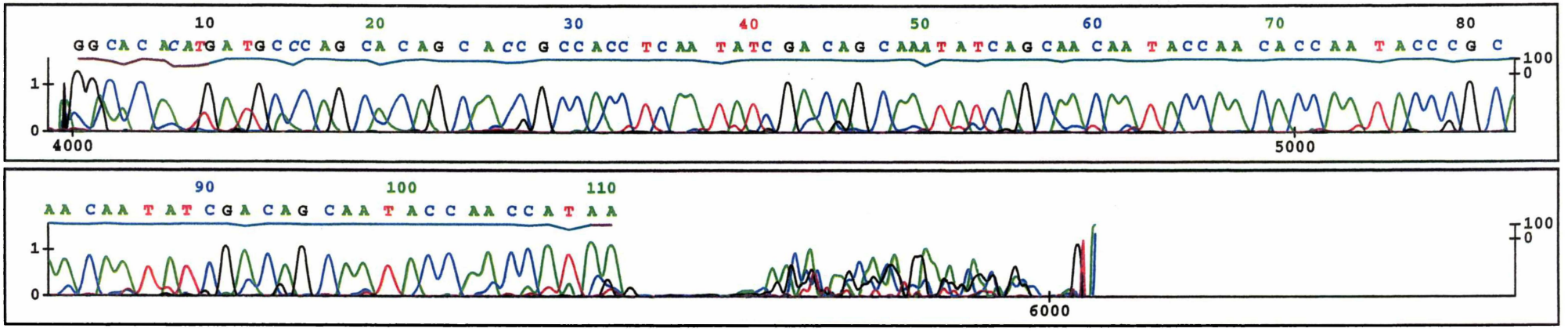
1025_70(EWA-R): Sample #70 *vrrA* forward sequence

1025_70(EWA-F): Sample #70 *vrrA* reverse sequence

1092_70EWA(EWAR): Sample # 70 *vrrA* forward sequence (repeat)

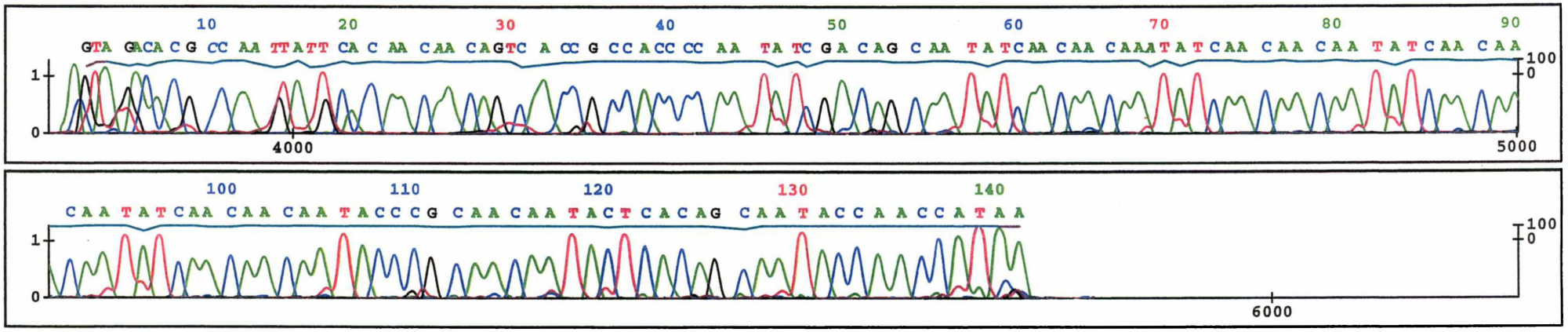
MegaBACE

MegaBACE 1030	020906A	Cimarron 3.12	Injection: 2.0 kV, 50.0 s
ICM ver. 2.4		Read length: 99	Run: 5.5 kV, 180.0 min
1030-13833	1025_32(EWA-R)	Overall quality: 92.0	Started Fri Sep 06 12:23:56 2002
BIO-MEGABACE	H02		ET Terminators



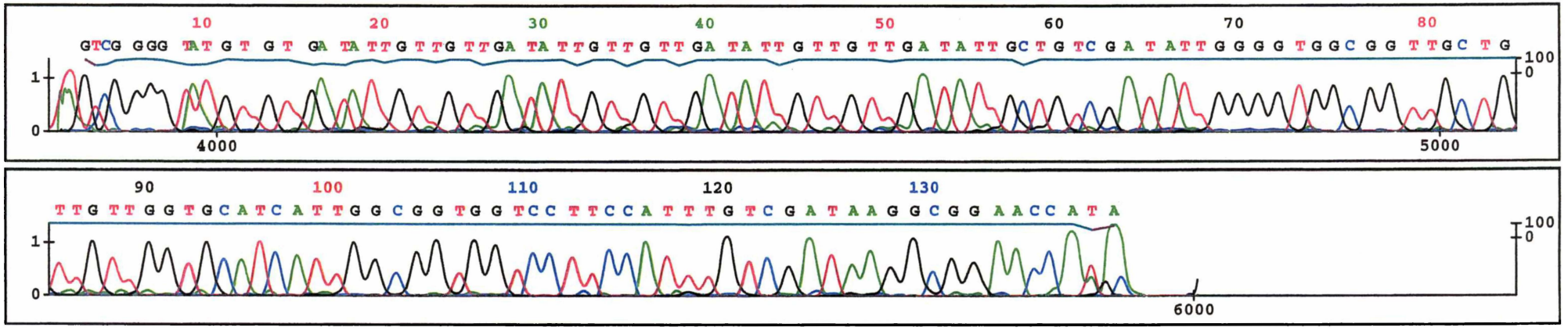
MegaBACE

MegaBACE 1030	020906A	Cimarron 3.12	Injection: 2.0 kV, 50.0 s
ICM ver. 2.4		Read length: 137	Run: 5.5 kV, 180.0 min
1030-13833	1025_40(EWA-R)	Overall quality: 91.0	Started Fri Sep 06 12:23:56 2002
BIO-MEGABACE	B03		ET Terminators



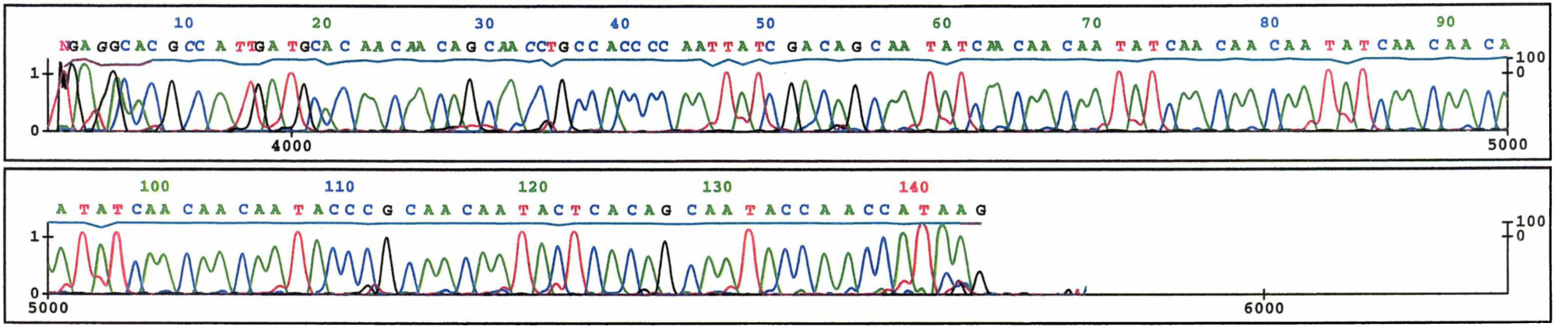
MegaBACE

MegaBACE 1030	020906A	Cimarron 3.12	Injection: 2.0 kV, 50.0 s
ICM ver. 2.4		Read length: 136	Run: 5.5 kV, 180.0 min
1030-13833	1025_40(EWA-F)	Overall quality: 93.0	Started Fri Sep 06 12:23:56 2002
BIO-MEGABACE	A03		ET Terminators



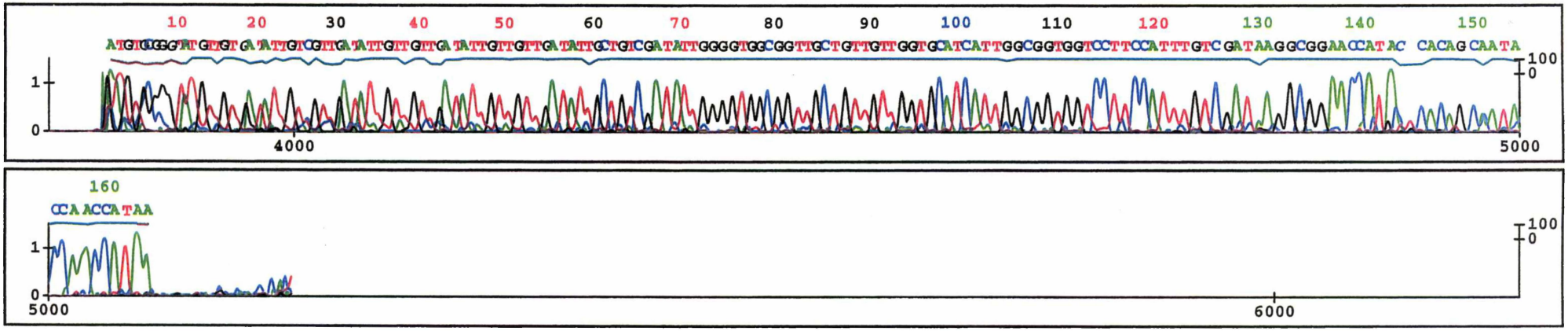
MegaBACE

MegaBACE 1030	020906A	Cimarron 3.12	Injection: 2.0 kV, 50.0 s
ICM ver. 2.4		Read length: 134	Run: 5.5 kV, 180.0 min
1030-13833	1025_53(EWA-R)	Overall quality: 92.0	Started Fri Sep 06 12:23:56 2002
BIO-MEGABACE	D03		ET Terminators



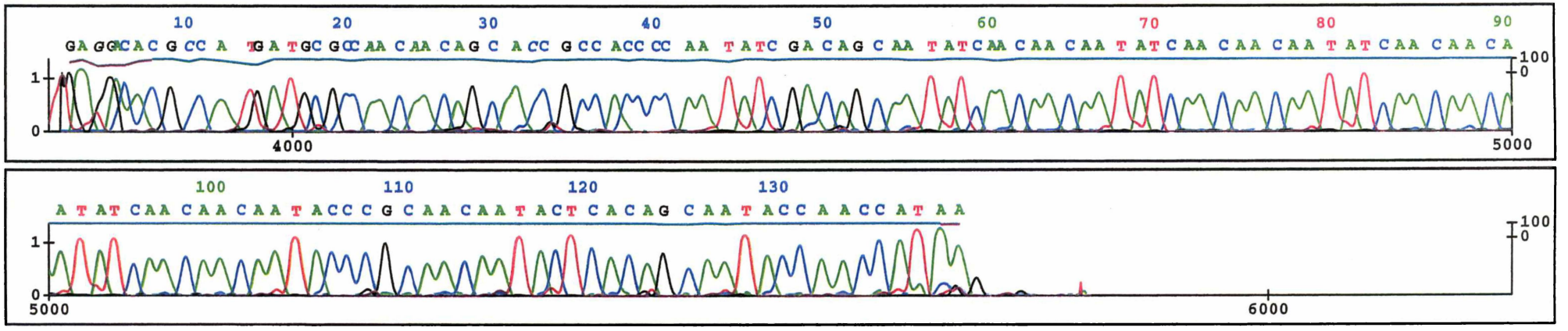
MegaBACE

MegaBACE 1030	020906A	Cimarron 3.12	Injection: 2.0 kV, 50.0 s
ICM ver. 2.4		Read length: 152	Run: 5.5 kV, 180.0 min
1030-13833	1025_53(EWA-F)	Overall quality: 93.0	Started Fri Sep 06 12:23:56 2002
BIO-MEGABACE	C03		ET Terminators



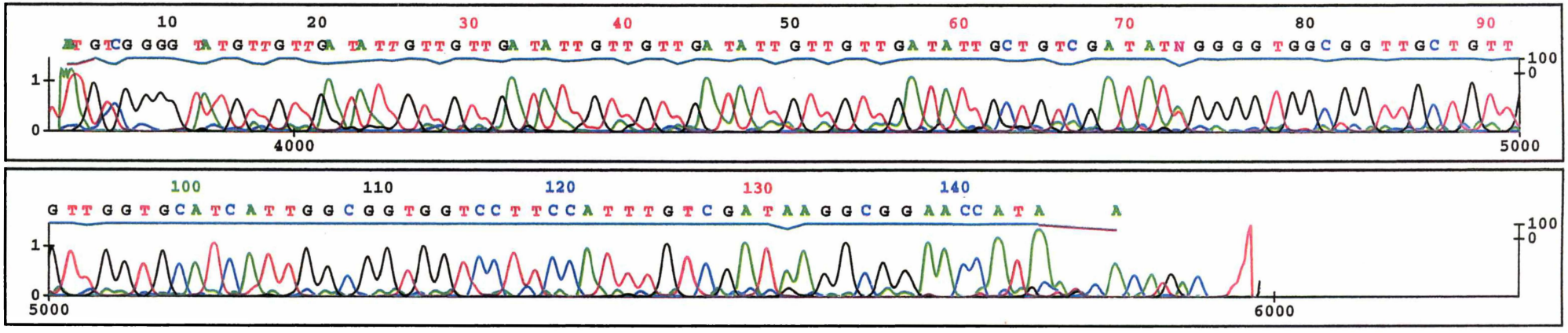
MegaBACE

MegaBACE 1030	020906A	Cimarron 3.12	Injection: 2.0 kV, 50.0 s
ICM ver. 2.4		Read length: 130	Run: 5.5 kV, 180.0 min
1030-13833	1025_70(EWA-R)	Overall quality: 96.0	Started Fri Sep 06 12:23:56 2002
BIO-MEGABACE	F03		ET Terminators



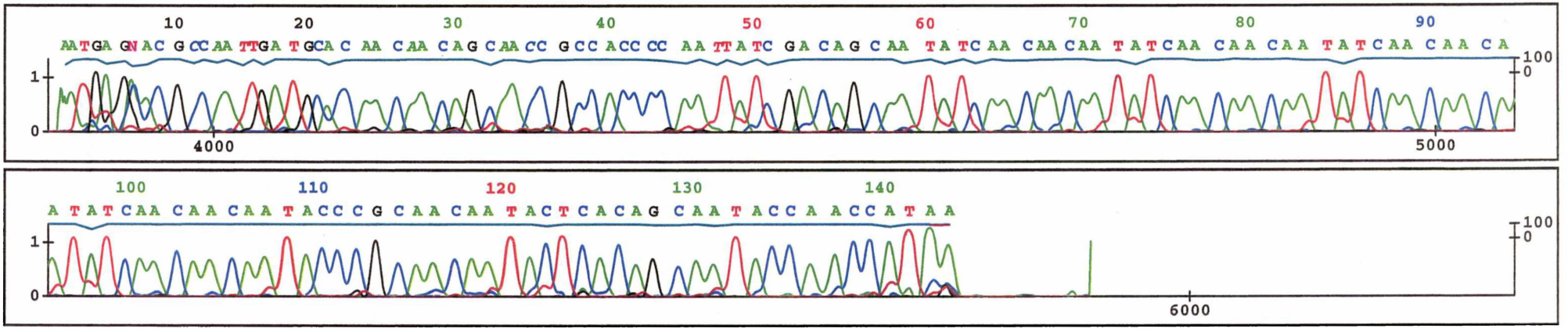
MegaBACE

MegaBACE 1030	020917A	Cimarron 3.12	Injection: 2.0 kV, 50.0 s
ICM ver. 2.4		Read length: 140	Run: 5.5 kV, 180.0 min
1030-13833	1092_70EWA(EWAF)	Overall quality: 91.0	Started Tue Sep 17 15:05:34 2002
BIO-MEGABACE	B03		ET Terminators



MegaBACE

MegaBACE 1030	020917A	Cimarron 3.12	Injection: 2.0 kV, 50.0 s
ICM ver. 2.4		Read length: 141	Run: 5.5 kV, 180.0 min
1030-13833	1092_70EWA(EWAR)	Overall quality: 91.0	Started Tue Sep 17 15:05:34 2002
BIO-MEGABACE	C03		ET Terminators



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