CHAPTER 1.0: INTRODUCTION

1.1 Why study Antarctica?

The Antarctic includes some of the most extreme terrestrial environments by very low temperature, frequent freeze-thaw and wet-dry cycles, low and transient precipitation, reduced humidity, rapid drainage and limited organic nutrients which are considered as unfavorable condition (Yergeau *et al*., 2006).

Located in the Earth's south hemisphere, Antarctica has received considerable amount of attention due to its untapped resources. Renowned for its cold, harsh conditions, there is an abundance of microbial life which has undergone their own adaption and evolutionary processes to survive. Since, Antarctic microbial habitats have remained relatively conserved for many years; there are unique opportunities for studying microbial evolution (Vincent, 2000).

Besides that, presence of psychrophiles and other extermophiles in the environment are interesting specimens of nature's wonder. Psychrophilic enzymes produced have high potential in biotechnology for example the conservation of energy by reducing the need of heat treatment, the application of eurythermal polar cyanobacteria for wastewater treatment in cold climates (Tang *et al*., 1997) and the integration of proteases, lipases and cellulases into detergents to improve its mode of action in cold water.

1.2 Antarctic microorganisms

Although bacterial researches have been conducted since early 1900s by Ekelof (Boyd and Boyd, 1962), little is known about the presence and characteristics of bacteria in the Antarctica.

Microorganisms dominate Antarctic biology compare to other continent (Friedmann, 1993) as well as they are fundamental to the functioning of Antarctic ecosystems. Baseline knowledge of prokaryotic biodiversity in Antarctica is still limited (Bohannan

and Hughes, 2003). Among these microorganisms, bacteria are an important part of the soil. Researchers reported greater density and diversity of microorganisms in younger soils in comparison with older and concluded that the development of microbial ecosystems in terrestrial Antarctica requires a favorable complex of interacting microclimatic and edaphic factors (Cameron *et al.,* 1970). Sometimes chemical and microbiological contamination brought by human activity was also identified by changing in bacterial diversity.

Based on this, it is important to discover the diversity of bacteria and their relationship between bacteria and their environment by studying the structural and functional of soil bacterial communities and their reaction to various natural or artificial disturbances.

1.3 Methods for microbial community analysis

Most of the early studies of Antarctic microbial ecology have been involved culture-dependent and molecular methods. The current understanding of Antarctic soil microbiology is mostly based on culture-based studies (Smith *et al*., 2006). This is because; it reveals the physiological character of isolate. However, it does not show all the diversity such as fastidious or unculturable microbes. Despite the difficulties encountered with the identification, isolation, and quantification of microorganisms, especially in complex environments, recent developments in molecular systematic offer exciting opportunities. Molecular biological techniques such as the PCR amplification, cloning and sequencing of 16S rRNA gene have had a major impact on our understanding of bacterial diversity in soils (Kowalchuk, 1999; Paavolainen *et al,* 2001; Pennanen *et al.,* 2001). However, new molecular approaches relying on 16S rRNA gene sequences allow qualitative and quantitative analysis of microbial diversity, the DNA is often very hard to extract and purify from the organic-rich environmental samples. Therefore, different methods which are available to assess the bacterial communities in

soils can be combined together to complement each other to obtain a more complete picture of bacterial diversity.

Based on earlier studies, culture-dependent and culture-independent methods discover different phylogenetic information about bacterial diversity of Antarctic soil. Phyla Proteobacteria, Cytophagales, Actinobacteria and Firmicutes are well represented by cultivated organisms and these four phyla account for 90% of all cultivated bacteria characterized by 16S rRNA sequences. Some phyla which are revealed by clonal analysis, such as Acidobacteria and Verrucomicrobia, are poorly represented by sequences from cultivated organisms (Zhang and Xu, 2008).

In this study the general approach starts with the isolation and purification of different colony types of culturable bacteria and a screening of the genotypic diversity by sequencing of different ribotype of bacteria and lastly is data analysis, using a fast fingerprinting method such as RFLP, and subsequent comparison with a reference database. Strains of bacteria are selected by 16S rRNA gene sequence analysis to determine their phylogenetic position.

Advances in the study of microbial diversity through molecular methods have shown that there are more than 99% of microorganisms which are not cultivated in the laboratory (Pace, 1997). Development of more effective culturing and isolation techniques of pure cultures has contributed to the understanding of these organisms and consequently, our living world. It is therefore not surprising that extensive research and focus has been given towards the discovery and manipulation of microorganisms for beneficial purpose. In view of an increasing need in scientific discovery and development in the biotechnological industry as well as importance of isolate pure culture, this study aims to isolate and characterize culturable bacteria from the soil samples of Anchorage Island in Antarctica.

1.4 Objectives

- Isolation and purification of culturable bacteria from soils collected of three sites on Anchorage Island, Antarctica.
- Grouping isolates using molecular methods such as RFLP.
- The bacterial isolate were classified based on phenotype (colony morphology, cells structure and gram stain) and genotype (16 S rRNA gene sequence).
- Determination of the 16S rDNA sequences for the identification of the bacterial isolates by using online databases, CHROMAS and GENBANK.
- Determination of biodiversity of culturable bacteria isolated from the soil samples.

CHAPTER 2.0: Literature review

2.1 The importance of biodiversity

According to the definition of International Union for Conservation of Nature and Natural Resources, biodiversity included all life forms, ecosystems and ecological processes and acknowledges the hierarchy at genetic, taxon and ecosystem levels (Kapur and Jain, 2004). Research suggests that level of plant and animal biodiversity directed function in agroecosystems and it performs a variety of ecological services beyond the production of food, such as recycling of nutrients (Altieri, 1999). Although most people are very familiar with the diversity of life in the plant and animal kingdoms, few actually realize the huge amounts of variability present in the bacterial community. Microbial diversity includes the diversity of bacteria, protozoans, fungi, unicellular algae and constitutes the most extraordinary reservoir of life in the biosphere (Kapur and Jain, 2004). In addition, important biological materials which are useful to humans such as antibiotics, drugs, enzymes, herbicides, growth promoters and sources of novel pharmaceuticals are produced by microorganisms. On the other hand, efficient exploitation of microorganisms such as sources of novel pharmaceuticals requires knowledge of the distribution of microbial diversity (Bohannan and Hughes, 2003**)**.

In order to produce novel pharmaceuticals from bacteria is important to solve new and emerging disease problems and to advance biotechnology. In addition, microbial diversity is the key to human survival and sustainability of ecosystem. Contributions of soil bacteria in stabilizing the ecosystem are well-known (Dubey *et al*., 2006). They are able to catalyze the transformation of organic matter from plants and animals into humus (Whitman *et al*., 1998; O'Donnell *et al*., 2001). Thus, they played essential roles in various biogeochemical cycles processes and nitrogen fixation (Weber *et al*., 2001), which are responsible for the recycling of organic compounds that are essential for

sustainable agriculture (Molin and Molin, 1997; Yao *et al*., 2000). Hence, the occurrence of soil and sediment bacteria, and the studies on these bacteria with their ecosystem effects are important.

Unfortunately, evaluating the biodiversity of microbial communities remains an indefinable task because of taxonomic and methodological difficulties (Zak *et al.*, 2002) and their extremely high abundance and diversity (Bohannan and Hughes, 2003) as some studies indicate that the 5,000 identified species of prokaryotes represent only 1 to 10% of all bacterial species to be discovered(Kapur and Jain, 2004). There for, new technologies, particularly in nucleic acid analysis, bioinformatics, analytical chemistry, and methodology and characterization with ecological sound methods to access the diversity, can provide us with a plentiful source of new and valuable products. Therefore, continued research is needed to describe and protect the unexplored resources for the preservation of natural ecosystems and the future benefit of human.

2.2 Antarctica

Gondwanaland was previously known as part of super continent which joined Africa, Australia, India, New Zealand and South America about two hundred million years ago, the continent has since drifted to where it is now; located at most southern part of earth that called Antarctica. Antarctica is the continent of superlative, is constantly referred by many as the coldest, windiest and highest continent and is considered the largest and most pristine wilderness continent on earth (Lohan and Johnston, 2005).

The most southernly among the seven continents and the fifth largest, Antarctica covers 14.2 million square kilometers out of which 98% are ice sheets and remaining 2% are barren rocks. Furthermore, this isolated continent is permanently covered with ice and snow. During the summer period, less than 1% of this land is free of snow and ice, and most of these snow free areas can be found at low heights close to sea (Bokhorst *et al.* 2007a). Average elevation of the landmass is 2160 m. Antarctica containing about 70%

of the world's freshwater and 90% of the world's ice. Antarctica is certainly the least tainted large landmass in the world, with many unique ecosystems, animals and landscapes (Roper-Gee, 2003).

Environmental conditions are generally unfavorable to the majority of terrestrial life-forms in Antarctic regions, such as low thermal capacity of the substratum, frequent freeze-thaw and wet dry cycles, low and transient precipitation, low humidity, rapid drainage, and limited organic nutrients (Wynn Williams, 1990). These generally poor conditions support relatively simple ecosystems with a noted reduction in the complexity of food webs Annelids, mollusks, winged insects and mammals are effectively absent from these systems, and only two vascular plant species (Antarctic hair grass, *Deschampsia antarctica,* and Antarctic pearlwort, *Colobanthus quitensis* (Smith, 2003) have been found to inhabit Antarctic terrestrial environments (Davis 1981). On the other hand, numerous animal species that prosper, in the surrounding waters include fish, krill, penguins, seals, whales and various kinds of sea birds (Davis, 1981). The largest terrestrial organisms and plant cover are soil arthropods and lichens respectively (Convey, 2001).

Extreme climate makes the Antarctica a huge crystal desert. In contrast with general belief, desiccation stress is the most important limiting biological activity factor in Antarctica instead of temperature. Terrestrial biologists admit three distinct biogeographical zones in the Antarctica: the continental, the maritime and the sub-Antarctic (Engelen *et al.,* 2008). The sub-Antarctic including the islands near to the Polar Front (South Georgia), the Maritime Antarctic comprising the western part of the Peninsula and, including the outlying archipelagos of the South Orkney, South Shetland, and South Sandwich Islands (56 - 72ºS), and the continental Antarctic is the third region containing the Great Circle of east and west Antarctica (Chown and Convey, 2007).

Antarctica has attracted many scientists recently and provides unique opportunities for scientific studies. It has been the key sources of novel information that aids human understanding of the Earth system. Ice cores from Antarctica have revealed detailed records of climatic and environmental changes that have prevail over the past million years (Petit *et al.,* 1999). Changes in Antarctic community composition and biodiversity are affecting human life in numerous ways. Moreover, Antarctic researches can give us valuable information from the way such an isolated area may have been colonized (Tindall, 2004).

2.3 Anchorage Island

Among all researches that have been done on Antarctic the continental cold desert soils of the Dry Valleys in Victoria Land and coastal areas of Wilkes Land of the maritime Antarctic have most focused research (Engelen *et al*., 2008) but study on Anchorage Island (Fig. 2.1) associated with biodiversity using culture-dependent work is still unexplored. Most of the research on this Island focused on comparison of soil springtails, soil arthropods, vascular plant and effects of climate change in Antarctica (Hawes *et al.,* 2007; Bokhorst *et al.,* 2007b; Bokhorst *et al.,* 2007c; Bokhorst et al., 2008; Chong *et al.,* 2009; Convey and Smith, 1997; Yergeau *et al.,* 2007a; Yergeau *et al*., 2007b; Yergeau *et al.,* 2008a; Rinnan *et al*., 2009). In this study we tried to determined bacterial diversity in soil samples from three different sites.

Anchorage Island (67º61'S 68º22'W, southern maritime Antarctic), lies in Marguerite Bay south close to the Rothera Research Station of the British Antarctic Survey (BAS). The island is 2.5 km long and 500 m wide and is partly covered by semi-permanent snow and ice fields, although recently these have been decreasing rapidly in extent (Fox and Cooper, 1998). Annual soil temperature is around -3 °C and annual precipitation approximates 500 mmy^{-1} (Turner *et al.,* 2002). Anchorage Island includes the least developed terrestrial ecosystems, in terms of macroscopic vegetation. There is one

beach used by elephant seals for moulting and haul out on the island (Bokhorest *et al.*, 2007d).

The island includes several rocky ridges, with a maximum height of 57 m. On the slopes of these ridges, there are some carpets of the moss *Sanionia uncinata*. The larger size of the island, 3 km in length, allows the development of moss stands dominated by *Drepanocladus uncinatus, Andreaea spp., Brachythecium austrosalebrosum* and *Polytrichum alpinum* on moist east-facing slopes. Small quantities of both Antarctic flowering plants are also present (Convey and Smith, 1997) as Loeske and clumps of the grass *Deschampsia antarctica* Desv. However, the vegetation consists predominantly of lichens (*Pseudephebe minuscula, Umbilicaria decussata, Usnea* spp. and many crustose species, with *Usnea antarctica* being most prominent (Bokhorest *et al.,* 2007c). The former consisted of patches (2–4 mm) of complete moss coverage located between rocks. A layer of dead moss of 0–10 cm underlies this vegetation. The lichen-dominated community consisted of bare rock boulders with a partial coverage of *U. antarctica* and other lichen species. *Cryptopygus antarcticus* is by far the most abundant springtail in vegetated habitats of Anchorage Island (Convey and Smith, 1997).

Anchorage Island1 or AI_1, is located in S67° 36.285', W68° 12.919', with elevation of 10.4 m, which has sandy coarse soil and weathered rock with trace of moss and lichen, air temperature about 2.3°C and soil temperature 3.3°C. AI_2, Anchorage Island 2, is located in S67° 36.285', W68° 12.919', with elevation of 28.6 m which is between scattered rock and cover with moss and it has black and fine soil (wet) by the air temperature of approximately 3.0°C and the soil temperature of 4.1°C. Whereas, AI_3 (S67° 36.896', W68° 13.987') is located between large rocks on rocky outcrop and it is high in nutrient input and it is penguin moult site with elevation of 16m. Air

temperature in this site is 10.5°C and the soil temperature is 8.4°C that are different from other two locations (AI_1, AI_2).

2.4 Antarctic soil

Ice-free terrestrial habitat in Antarctica is limited to less than 0.35% of the entire Antarctic continent whit the remainder permanently covered by ice (British Antarctic Survey 2004) representing cold rock deserts. Precipitation of terrestrial ice-free habitats is very low so they are classified as frigid deserts (Yu *et al.,* 2010). Soils and their properties are vital factors involved in the Antarctic ecosystems.

Scientists categorized Antarctic soil into two groups based on analysis of a range of abiotic and biotic factors: 1) nutrient-poor, dry, bare mineral soils and 2) moist, relatively nutrient-rich, vegetated soils. As compared between mineral soil and temperature soil revealed lower bacterial diversity (Smith *et al.,* 2006) while diversities and community compositions in vegetated soils are rather similar to soils from temperate environments. These results hypothesized that Antarctic soils under plants offer a relatively stable and nutrient-rich environment (Yergeau *et al.* 2008b). Since in non vegetated areas of Antarctica microbiota are subjected to unfavorable environmental conditions such as sub-freezing temperatures, desiccation, hypersalinity, and transient and diurnal freeze-thaw cycles distribution are limited compare to vegetated soil. Besides that, the high velocity of katabolic winds in the Antarctic continent also disrupt the soil surface and restrict the ability of microorganisms to colonize within the region (Kastovska *et al.,* 2005) also reported that polar soils with no vegetation generally support fewer microbes than soils associated with mosses.

2.5 Microorganism in Antarctica

Microorganisms have been developing for nearly 4 billion years and are capable of exploiting a huge range of energy sources and flourishing in almost every habitat. For 2 billion years microbes were the only form of life on Earth. It is estimated that 50% of

the living protoplasm on this planet is microbial (Kapur and Jain, 2004). Considerable amount of researches has been done on microbiota of the polar region especially on the Antarctic, within the past 20 years.

Prokaryotes control many Antarctic ecosystems and manage most of the biological flux of carbon, nutrients and energy (Yu *et al.*, 2010). They also have a crucial role in existence of life by maintaining the dynamic equilibrium and integrity of the biosphere (O'Donnell, 2001)**.** One of the best ways to study about Antarctica is to focus on its microbial life. The Antarctic microbiology studies were started from sixties by a group of Indian expedition for the first time in 1981 (Loka Bharathi *et al.*, 1999). On the other hand, the inhospitable environment makes Antarctica home to only organisms that are adapted to tolerate it. One of such microorganism is psychrophiles or cold-adapted microorganisms that are able to grow below 15°C and cannot grow above 20°C. On the other hand, Psychrotrophs or cold-tolerate microorganisms can survive at 0°C, but around 20-30°C is their optimal growth and their growth stop at 35°C and above. Researchers reported that bacterial processes are particularly sensitive to environmental conditions yet bacteria are also highly adaptable to extreme and changing environments (Eriksson *et al.*, 2001). Several studies revealed Antarctic terrestrial bacteria are mostly cold-tolerant bacteria rather than cold-adapted bacteria. This may not be surprising because cold-tolerant organisms are better adapted than true cold-adapted microorganisms to survive the large and frequent variation in temperatures occurring in Antarctica (Yergeau *et al.*, 2008b). Although, several authors believed that microbial species isolated from terrestrial Antarctica are cold- and desiccation-tolerant species that are also found in more temperate zones yet other authors have indicated that Antarctic isolates of some species are capable of growing at lower temperatures than those of the same species collected in more temperate locations. Hughes (2003) reported that Antarctic microorganisms are the best organisms that can adapt themselves to

biochemical and physiological conditions. Distinctive features in their proteins and membranes and their responses to thermal shifts as a result of their genetic in addition to their survival in low nutrient condition are examples of their special abilities that adopt them to extreme environment (Deming, 2002).

All these reasons make Antarctic microorganisms such valuable issues to concentrate. Soil micro fauna supply important clues into trophic interaction in Antarctic system (Newsham *et al.*, 2004). However, there is still a big gap between our understanding of diversity and its importance in ecosystem processes (O'Donnell 2001) as well as the most researches of this subject have been restricted to measuring macrophyte and animal diversity, and baseline knowledge of prokaryotic biodiversity in Antarctica is still limited (Hughes, 2003). Generally, it has been observed that bacterial activity, numbers and community structure are related to soil type, nitrogen content, water abundance and type of plant cover (Yergeau *et al.* 2007b). On the other hand, due to importance of temperature in polar region such as Antarctica, temperature plays a huge pivotal role in influencing the growth of microorganisms. Commonly temperatures fluctuate widely over a relatively short period of time in the soils of continental Antarctica, and influence the microorganisms directly or indirectly. Since most Antarctic bacterial species are cold-tolerant as opposed to cold-adapted, it can be hypothesized that an increase of a few degrees in temperature will increase bacterial activity (Yergeau *et al.* 2008b). However, increasing temperature alone without combined increase in nutrient input will not improve growth if bacterial communities are nutrient limited (Waldrop and Firestone, 2006). This is because other evidence suggests that bacterial abundance and activity are controlled by the availability of organic matter or the presence of vegetation. So microbial abundance demonstrated a significant positive relationship with vegetation and vegetation-associated soil factors such as water contents, organic C and total N. Between these factors the availability of

water was the major factor affecting bacterial distribution, abundance and activity (Smith *et al.,* 2006) as water in a frozen state cannot be used by organisms. In addition, report showed that microbial community structure was mainly related to latitude or location and latitude-dependent factors such as mean temperature, NO_3, pH (Yergeau *et al*., 2008b). Therefore, water availability, temperature and species diversity are closely related to abundance of bacteria in the Antarctic. In addition to climate and edaptic factors, disturbances by humans and animals like penguin rookeries and camp-scavenging birds can also enhance the microbial population due to accumulation of organic matter from these sources (Zunino *et al.*, 1985).

Studies of the microeukaryote diversity across a range of Antarctic terrestrial sites showed no clear pattern of decreasing diversity with latitude. Nevertheless, this patchy distribution followed the pattern of vegetation, moisture retention or bird activity (Yergeau *et al*., 2008b). Generally, some studies revealed limiting biodiversity of soil bacteria by increasing stressful environmental conditions at higher Antarctic latitudes.

2.6 Antarctic bacteria

Microbiology is one of the most exciting areas of Antarctic science. Bacteria from Antarctica have dramatic potential, for example producing antibiotics. In addition some Antarctic phytoplankton has ability to develop sun screens against the increased radiation caused by the thinning of the ozone layer over Antarctica.

Many species of Antarctic fauna are found nowhere else on the planet.This is because, unlike other large ecosystems, Antarctica is an island continent and is isolated from other parts of the Southern hemisphere by distance, air and water currents (Strugnell and Linse, 2007).

Within the past 20 years extensive diversity of bacterial strains has been isolated from a variety of Antarctic environments. Many of these strains were novel species, which are unique to the Antarctic. Tanner (1985) reported microbiological researches in

Antarctica have been conducted since the 18th century with one of the earliest documented expeditions in which the Swedish Antarctic found a significant number of bacteria in the soil of Snow Hill Island of the Palmer Peninsula. After that, bacteria were isolated from snow, ice and frozen algae at Adelie Land near the South Pole. Then, a wide variety of strains have been isolated from various marine or terrestrial environments of Antarctica. In many cases, the strains isolated were proven to be unknown taxa (Yu *et al.*, 2010).

2.6.1 Antarctic Lake

In glacial valleys the major Antarctic lakes are found. During winter the lakes are frozen but may thaw for a few weeks during summer (Ferris and Burton, 1988).

In Antarctic lakes primarily microbial composition consisted of Cyanobacteria and heterotrophic bacteria (Wharton *et al*., 1983). After that, *Phormidium frigidum* and *Lyngbya martensiana* were found to be the dominant filamentous Cyanobacteria present in microbial mats using culture based studies (Wharton *et al*., 1983).

In Lake Fryxell, morphological and molecular analyses of Cyanobacterial diversity were conducted and results discovered the presence of *Nostoc* sp. and *Schizothrix* sp. as well as morphotypes such as *Hydrocoryn* cf. *spongiosa, Nodularia* cf. *harveyana* and *Phormidium* cf. *autumnale* (Taton *et al.*, 2003). Other autotrophic bacteria include photosynthetic green sulphur bacteria such as *Chlorobium vibrioforme* and *Chlorobium limnicola* (Volkman *et al*., 1988).

2.6.2 Sea ice

The major nutrient source in Maritime Antarctica is sea ice. Although ice may appear to be an unfavorable habitat to support microbial growth, brine inclusions, sheets of floating ice and the ice-water interface support the survival of rich microbial populations (Palmisano and Garrison, 1993).

Various morphological bacteria have been found to be associated with sea ice. Presence of rods, cocci, straight and branching filamentous, fusiform and prosthecate bacteria have been showed using light. Scanning electron microscopy studies found about 70% of the bacteria in a sea-ice community at McMurdo Sound were free-living, whereas 30% were attached to either detritus or active algal cells (Sullivan *et al.,* 1984). In the Antarctic ice microorganisms are non-spore like *Pseudomonas* and spore forming bacteria included a variety of *Bacillus* species. Between non-spore bacteria *P. fluorescens* and *P. alcaligenes* have displayed psychrophilic properties (Stokes, 1963). In addition, two representative genera include *Streptomyces* and *Nocardia* from Actinomycetes have also been found in the ice and soil. Staley *et al* (1989) reported isolation of red to orange filamentous pigmented and gas vacuolated bacteria which is unsure whether the gas vacuoles confer an adaptive advantage for the sea-ice habitat.

2.6.3 Terrestrial bacteria

The terrestrial properties Antarctica provides a hostel habitat for the colonization of microorganisms. Seventy-one percent of soil bacteria were related to coryneform bacteria, within the genera *Arthrobacter, Brevibacterium, Cellulomonas, Corynebacterium* and *Kurthia* using culture based technique (Brambilla *et al*., 2001). *Pseudomonas, Flavobacterium* and other gram negative aerobic rods like *Alcaligenes* and *Arthrobacter* were also identified (Brambilla *et al*., 2001). They also reported by employing molecular analysis the presence of anaerobic, gram positive *Clostridium* sp. Other study on mineral soil reported dominating group was either *Actinobacteria* (Dry Valleys) or *Bacteroidetes* (Victoria Land) (Aislabie *et al*., 2006; Smith *et al*., 2006). *Cyanobacteria* were also reported to be highly diverse and common in several Antarctic mineral soils. Results of all these studies indicate that culture based methods alone remain inadequate for providing accurate information of the microbial diversity in an environment.

2.7 Methods for microbial community analysis

In the studies of soil microbes, Pace and co-workers found that prokaryotic evolution is divided into two major branches, which are bacteria and Archaea (Pace, 1997). Studies have also revealed that 1 g of soil contains more than 2.6×10^{29} prokaryotes and may contain up to 4000 different species of bacteria (Whitman *et al*., 1998; Torsvik *et al*., 1990). Besides that, soil microbial diversity controlled the homeostasis of the entire soil ecosystem as redundancy of soil microorganisms and their enzymes, and the combination effects of their metabolic activities (Torsvik *et al.,* 1990). The paucity of adequate research methods is the cause to unanswered questions about soil microorganisms. For the suitable analysis of soil microbial diversity, all or at least most of these soil compartments should be analyzed as each soil is populated by specific microbial communities. Direct cultivation (culture-dependent) and modern molecular genetics (culture-independent) are two different methods that can be used to reveal prokaryotic populations in environmental samples (Juck *et al.,* 2000, Tindall, 2004).

2.8 Culture dependent methods

In conventional culture-dependent methods, bacterial diversity is studied by the cultivation of bacteria on solid media or agar plate. The development of this method has led to the study of bacteria in pure cultures according to their nutritional criteria and the morphology examinations through microscope (Torsvik *et al*., 1990).

Microorganisms from different environmental samples can be obtained by culture-dependent method to isolate single colony **(**Prosser and Embley, 2002) by, inoculation of soil suspensions on nutrient agar plates. Single colony is a population consisting of bacteria that all derived from a single cell. The purpose of isolation is to achieve pure culture through isolation of single colonies. This kind of culture which is free from contamination with other microorganism is called “axenic.” To prepare axenic growth physical separation of single cells from others and inoculation into sterile medium and

incubation under conditions is necessary. Pure cultures almost never occur in nature (Prosser and Embley, 2002). On solid media, colony morphology and other properties such as swarming over the agar surface can be easily observed (Overmann, 2006).

Culture-dependent methods, involves recovery of viable culturable cells. Bacterial strains are isolated from collected samples and plated using an adequate medium. After incubation single colonies are enumerated so that total CFUs per volume or gram of sample can be calculated (Prosser and Embley, 2002). Single colonies picked can be purified by successive streaking. These culturable microorganisms are then preserved for classification by phenotypic and genotypic characteristics. Another way for determine biodiversity in culture dependent methods is bacteria are isolated from environmental samples with culture medium then nucleic acid is extracted from the bacterial culture (Kapur and Jain, 2004).

Although,there is a big challenge to mimic the growth condition of some bacteria, especially those bacteria living in extreme habitats, such as in the cold and hot regions (Lorenz and Schleper, 2002). The fact is culture-dependent techniques provide an undeniable understanding of previously undescribed environments. Hence, culture-dependent studies can act as a solid backbone to future investigations that apply culture-independent procedures. Our recent knowledge reveals that most Antarctic environments contain a diverse range of prokaryotes. For cultivation based studies large data set of isolates are needed. Although our current knowledge of soil microbiology in Antarctica is contributed by both methods (Tindall, 2004), but it is mostly related to culture-dependent techniques (Smith *et al.,* 2006).

2.8.1 Advantages of culture-dependent technique

Isolation of pure culture is an essential step in microbial ecology which helps to better understand physiology and ecology of bacterial species (Bernard *et al*., 2000). Culture-

based techniques can select organisms without regard to their numerical or functional significance in situ, which is extremely useful for understanding the physiological potential of isolated microorganisms (Pearce *et al*., 2003). Until recently, this method was considered standard and was performed in most microbial ecology studies. The advantages of cultivation method are to provide an excellent understanding of the physiological potential of isolated organisms (Prosser and Embley, 2002). Besides that, recovery of viable culturable cells is very important. In the search for novel natural products that can be developed as a resource for biotechnology we need isolated bacteria in pure culture.

For example, cold-active enzymes have a huge biotechnological potential in so many field such as detergent formulations (e.g. proteinases, lipases, amylases, cellulases), in the dairy industry (e.g. ß-galactosidase), as environmental biosensors (e.g. dehydrogenases), for bioremediation (e.g. oxidases), for cleaning of contact lens (e.g. proteinases) and for biotransformation (many specific enzymes, e.g. methylases, aminotransferases). By isolation these protein from psychrophilic or psychrotrophic bacteria, these enzymes may greatly reduce and the cost for heating/cooling step of production (Karasova-Lipovova, 2003). The Gram-positive Antarctic bacterium *Arthrobacter* sp (Karasova-Lipovova, 2003) and the Antarctic gram-negative bacterium *Pseudoalteromonas haloplanktis* (Hoyoux *et al.*, 2001) have ß-galactosidase enzyme that could be used as a biotechnological tool in the production of lactose-reduced dairy products at refrigeration temperatures. Since, ß-galactosidase catalyzing the hydrolysis of lactose into glucose and galactose, has fascinated the attention of researchers at the technological (crystallization), environmental problems (pollution) (Hoyoux *et al*., 2001) and the mostly it has two main biotechnological uses in the dairy industry because the removal of lactose from milk for lactose-intolerant persons and the

production of galactooligosaccharides for use in probiotic foodstuffs (Karasova-Lipovova, 2003).

Based on these advantages, it is very vital to discover new culturable method to culture the rest of bacteria, due to the medicinal and industrial properties of known bacteria, it is hypothesized that uncultured species have many useful applications to human society.

2.8.2 Disadvantages of culture-dependent technique

Culture-dependent method similar to other methods has some drawbacks and high degree of bias induced is inevitable. The most important drawback is limited number of microorganisms that could be cultivated by traditional plate culture methods. Even if at appropriate culture condition is provided only a small fraction of bacteria (approximately <1 %) could be successfully cultured in the laboratory (Hugenholtz and Pace, 1996; Mclnerney *et al*., 2001; Fuhrman and Ouverney, 1998), possibly due to the limitations of culture-dependent methods (Torsvik *et al*., 1990; Amann *et al*., 1995). This indicates that the huge bacterial diversity in soil remains unexplored (Hammond, 1995). For instance, *Cyanobacteria* do not or hardly grow on media (Antoon *et al*., 1994). Besides, some of important organisms are fastidious or co-culture-dependent. There are also another group of organisms that may be in non-culturable state of their life cycle (Smith *et al.*, 2006). Thus, the community composition of unculturable prokaryotes in situ remains unknown (Orphan *et al.*, 2000). This indicates the conventional culture-dependent methods (media plate) do not reveal the entire bacterial community of a habitat (Handelsman, 2004), but only allow certain bacterial species like aerobic comes to grow on the media plates. For instance, *Cyanobacteria* do not or hardly grow on media (Antoon *et al*., 1994).

There are also difficulties in analyzing the bacterial diversity using conventional culture-dependent methods. As we know, different species of bacteria require different medium conditions to grow. There would be a loss of major portions of bacterial

communities if the handling techniques (agar plate spread as an example) are not properly administered (Amann *et al*., 1995; Ogram and Feng, 1997). Hence, the results given are biased and might not be true to reveal the whole bacterial diversity of a habitat.

As for the observation of bacterial morphology using a microscope in the conventional culture-dependent method, there are also several limitations in describing the taxonomic diversity due to the simple bacterial morphology (Madigan *et al*., 1999) as well as researchers must be very experienced to be able to identify colonies at the genetic level. Usually, bacterial communities are the most difficult to phenotypically analyze as compared to other organisms. This might be due to their tiny sizes and high level of diversity among the most complex assemblages in the biosphere (Zhou *et al*., 2003). Furthermore, the majority of the viable microscopically bacteria could not be cultured on plates (Staley and Konopka, 1985; Roszak and Colwell, 1987), therefore increasing the difficulty of phenotypical analysis of bacterial diversity.

Conventional culture-dependent methods that employ various media, incubation conditions, and preservation of bacterial colonies on plates, are time-consuming and labour-intensive (Atlas and Bartha, 1997). Therefore, culture-independent molecular approaches, which analyze bacteria based on their DNA, have been introduced to overcome the difficulties and limitations in conventional culture-dependent methods to study bacterial diversity in environmental samples (Steffan and Atlas, 1988).

Lastly, based on phenotypic characteristics only some genera, such as *Bacillus*, *Cytophaga*, and *Rhodococcus*, can be identified to the species level and in the majority of cases of analysis to the species level, phenotypic studies should be combined with the molecular genetics and chemotaxonomic methods of analysis of bacterial isolates, such as the determination of the G+C content of their DNA, the DNA–DNA hybridization with reference strains, and the chemical analysis of the cell wall constituents (membrane

lipid) (Dobrovol'skaya *et al.,* 2001). The failure to cultivate many of the ecologically relevant bacteria clearly suggests that there is a need for the development of novel cultivation methods (Overmann, 2006).

2.8.3 Possible reasons for "Non-culturability"

It has been showed that by culture-independent microscopic techniques the number of colony-forming units obtained from a given sample in nearly all cases is significantly lower than the actual number of prokaryotic cells in the sample (Overmann, 2006). This observation has been termed the "great plate count anomaly" and is considered to be due to several factors. In is possible that, cells of not-yet-cultured prokaryotes in natural samples are in a specific physiological state which prevents them from growing in conventional cultivation media and/or the physiology of not-yet-cultured species of prokaryotes is fundamentally different from that of known prokaryotes to such extent so that cultivation methods applied do not meet the requirements for growth (Overmann, 2006). Another research reported that, estimation of bacterial population by direct count and the culture technique may differ by 100–1000 times in the case of soil communities and by 10 times in the case of rhizosphere communities that shows the estimate of a soil bacterial population by the culture technique significantly depends on the state of bacteria (Dobrovol'skaya *et al*., 2001).

To overcome this, first of all, different nutrient media and cultivation conditions may comprise hundreds of variants. Since, only a small fraction of soil microorganisms will grow on any one type of medium under given cultivation conditions, therefore, increasing the number of test media and cultivation conditions such as aerobic and anaerobic and so on which is corresponding to particular soil microhabitats may significantly enlarge the range of detected soil microorganisms (Dobrovol'skaya *et al.*, 2001). Besides that, using the standard test media for the detection of the so-called physiological bacterial groups such as nitrifiers, denitrifiers, nitrogen fixers,

cellulolytics, enhance the bacterial diversity compare with the inoculation of one universal test medium. Dobrovol'skaya *et al*. (2001) also explained that many bacteria occur in soil in a specific state known as viable but non-culturable. In this case bacteria must be incubated for some time in the presence of the key intermediates of the Krebs cycle, such as pyruvate or acetate.

2.9 Culture-independent (molecular based method)

Since the last two decades, culture-independent molecular approaches, which analyze bacteria based on their genetic contents, have been widely used to identify bacterial species without the need of bacterial cultivation on agar plate (Amann *et al*., 1995; Hugenholtz and Pace, 1996).

As in culture-independent molecular approach, a universal and highly conserved and small subunit ribosomal ribonucleic acids (SSU rRNA) gene sequence in prokaryotes: 5S rDNA or 16S rDNA, which act as a culture-independent tool, has been studied to determine the bacterial diversity in environmental samples (Stahl *et al*., 1984; Olsen *et al*., 1986; Ward *et al*., 1992; Amann *et al*., 1995).

This method involved the extraction and analyzing of total environmental nucleic acids from the sample (Kapur and Jain, 2004; Tian *et al.,* 2009). It often involves the amplification of DNA or cDNA from RNA extracted from environmental samples by PCR and the following analysis of the diversity of the amplified PCR products. It should be noted that, some researchers prefer to extract rRNA gene, which is analyzed similarly to DNA and allows actively growing soil microbial populations to be detected. Nevertheless, others believe that the application of reverse transcriptase gives less accurate results than the application of Taq polymerase (Kennedy and Gewin, 1997).

After that, the amplified products may be cloned and sequenced to identify and enumerate bacterial species present in the sample. Each single bacterial species can identify base on ribosomal sequence which acts as a phylogenetic markers (Marsh *et al.,*

2000). The ribosomal sequences exist in all organisms, contain variable and highly conserved regions. These conserved regions can compare with a lot of data in databases to identify the phylogenetic position of the corresponding bacteria (Pinzari *et al*., 2010). Based on the studies of this specific rRNA gene sequence, phylogenetic trees can be constructed to study the bacterial diversity of environmental samples (Pace, 1997; Hackl *et al*., 2004).

2.9.1 The use of 16S rRNA gene fragment in bacterial diversity studies

16S rRNA gene forms the basis for analyses of microbial diversity. The first attempts to analyze environmental samples using culture-independent molecular approach was made by studying the rRNA gene fragment, which began in the mid-1980s (Xu, 2006). The rRNA gene fragment is amplified from genomic DNA of environmental samples using PCR, cloned into a vector and then sequenced in order to identify the bacteria species (Stahl *et al*., 1984).

In the past, c-type cytochromes, globins and other proteins were also used for mapping phylogenetic relationships but these molecules are limited to the 'higher' eukaryotic systems (Goodman, 1982). On the other hand, rRNA gene studies are much less complicated. In addition several features of 16S rRNA gene make it the most practical genetic marker used to discover bacterial phylogeny and taxonomy (Olsen *et al*,. 1986). These reasons include, as rRNA`s are basic elements for synthesizing proteins therefore functionally and evolutionary homologous in all organisms, rRNA`s are prehistoric molecules and their overall structure and nucleotide sequences are conserved. The conserved regions are essential as they supply primer directed sites for PCR as well as convenient hybridization targets for the cloning of rRNA genes. Finally, with the proper primer design to target the rRNA gene conserved regions; sufficient sequence information can be obtained to facilitate significant comparisons between organisms (Stackebrandt and Woese, 1981).

By the late 1960`s, the 5S rRNA with the length of 120 bp was the most popular used to characterize microorganisms in environmental samples (Stahl *et al*., 1984; Amann *et al*., 1995). However, the small size of 5S rRNA gene sequence limited bacterial gene information (Madigan *et al*., 1999).

Later, a larger rRNA molecule, 16S rRNA gene fragment with a fragment length of 1500 bp was then used to analyze bacterial diversity. Highly conserved sequences and sufficient sequence variability in 16S rRNA gene fragment have made these molecules an excellent indicator of relatedness in the studies of bacterial diversity (Madigan *et al*., 2000).

Since 1990s, 16S rRNA gene fragments have result in many uncultivable bacteria. A phylogenetic tree of bacteria that contains 36 phyla, 13 of which do not have culture representative was discovered (Hugenholtz *et al*., 1998). Besides that, studies have shown that a bacterium which 16S rRNA gene fragment differs by more than 3% from similar sequences from other organisms, should be considered as a new species (Madigan *et al*., 1999). To date, the rRNA gene fragment has been established as a unique identity molecular marker for microbial studies (Janssen, 2006).

2.9.2 Polymerase chain reaction (PCR) and RFLP

The Polymerase Chain Reaction (PCR) technique, invented by Kary Mullis in 1985 (Innis and Gelfand, 1990) is an *in vitro* procedure that can exponentially replicate either single or multiple DNA up to a million fold (Arnheim *et al.,* 1990). The reaction mixture consists of DNA template, thermostable DNA polymerase, two primers consisting of short oligonucleotides 10 to 30 bps long, dinucleotides, $MgC1_2$ and other additives in a buffer. This reaction mixture is then subject to repeated temperature cycles in a thermocyler (Peakall, 1998).

With the advancement of molecular techniques, the development of PCR has overcome the limitations of culture-dependent methods. It is a time saving modified gene cloning

method that is able to clone genes in a test tube by mixing DNA with a set of reagents and carry out the reaction in a thermal cycler (Brown, 2001). Hence, it is a useful and important tool in studying bacterial diversity based on culture-independent molecular approaches.

PCR of the 16S rRNA gene using universal primers has been one of the most widely used techniques for bacterial determination in the environment. Proper selection of primers is most effective in successful PCR (Dobrovol'skaya *et al.*, 2001). Universal primers do not guarantee the amplification of all types of the bacterial DNA extracted from soil, whereas the use of specific primers may lead to mistaken results (Dobrovol'skaya *et al.*, 2001). Sometimes detection of some microorganisms requires the use of highly specific primers and particularly careful work of researchers due to their small amounts (less than 1%) in soil.

Digestion of PCR amplicons with restriction enzymes followed by electrophoretic separation allows determination of characteristic restriction fragment length polymorphism (RFLP) patterns. Variations in the resulting 16S rDNA-RFLP are able to represent differences in microbial community structure along with proportions of individual populations. Although PCR-RFLP technique can be biased by several factors e.g. distinct gene copy number, different GC content, the annealing temperature, and primer homologies, the number of cycles of replication and the interspecies operon heterogeneities. This approach has been successfully applied in several investigations. Thus, in the present study similar method was used to assess microbial diversity (Ramıirez-Moreno *et al.*, 2004).

RFLP analysis of the 16S rRNA gene is a rapid and inexpensive method that can be useful in studies of the identity and diversity of microbial communities (Zhang, 2006) and referred to as amplified ribosomal DNA restriction analysis (ARDRA) (Kapur and Jain, 2004). In this method amplified rRNA gene products are digested by restriction

enzyme producing different fragment lengths that can be detected using agarose or non-denaturing polyacrylamide gel electrophoresis for community analysis (Ranjard *et al*., 2000; Kirk *et al*., 2004).

This technology depends on comparison of band profiles produced after digestion of target DNA with restriction enzymes (Ramirez-Moreno *et al*., 2004). The key advantage of RFLP analysis over PCR-based protocols is that no prior sequence information, nor oligonucleotide synthesis, is required. Choice of restriction enzymes to use for RFLP detection is the main step of this technique (Yang *et al*., 2007; Caleb, 2010). This technique has been used to distinguish changes in microbial community structure as a result of changes in environmental conditions and diverse soil types (Soares *et al*., 2006; Matsuyama *et al*., 2007), as well as, contact of soil to toxic compounds (Smit *et al*., 1997). The number of rRNA gene and the amount of complementarity between primers and target sequences are biases of RFLP analyses. Furthermore, from the ecological point of view both features must be considered (Ramirez-Moreno *et al*., 2004).

2.9.3 Advantages of molecular methods

To overcome culture-dependent deficiencies, several molecular approaches have been developed to accomplish and facilitate detection the analysis of bacterial populations and communities the last 30 years (Orphan *et al.*, 2000) to provide a better understanding of entire microbial population in various types of environments. Despite the difficulties encountered with the identification, isolation and quantification of microorganisms especially in complex environments recent developments in molecular systematic offer exciting opportunities (Tian *et al.*, 2009). Evaluation and enhancement of soil microorganism's diversity without using the tedious and time-consuming procedures of enrichment, cultivation, and isolation of microorganisms in pure cultures, allow determine so called non-culturable microbial form to be easily detected and

revealed phylogenetic tree of prokaryotes are considerable advantages of molecular based methods (Dobrovol'skaya, 2000).

2.9.4 Disadvantages of molecular methods

Although these methods are less difficult, faster and cheaper to perform (Fuhrman and Ouverney, 1998), but still have some limitations. Mostly, it is very difficult to extract and purify the DNA from the organic-rich environmental samples (Zhang and Xu 2008). Soil samples require a harsh extraction method because of the tight bonds that exist between inhabiting microorganisms and soil particles (Priemé *et al*., 1996). Gene clone library construction also needs to be carried out which is an expensive and time consuming procedure. In addition, many soil clones are recognized as new phylogenetic groups because of the low degree of DNA homology with the known bacterial taxa, and molecular genetics methods often fail to detect common soil inhabitants, such as actinomycetes, which are easily detected by the culture techniques. By this method, the detection of particular bacterial groups in soil provides no further information on their physiological peculiarities and ecological functions as it mentioned before. To obtain such information, soil bacteria must be isolated in pure cultures. Furthermore, obtained results from molecular method often ignore the ecological direction of the problem, for example, particular bacteria can live or just be preserved in particular habitats. Thus, the data on the microbial diversity of soils achieved by molecular genetics methods are much more defined than those obtained by the culture technique. On the other hand, the reason of higher diversity of the 16S rRNA genes of the total DNA extracted compare to the diversity of these genes in the DNA extracted from soil isolates may due to the higher genetic in homogeneity of natural microbial populations as compared with the populations of collection cultures which belong to non-culturable bacteria or other microorganisms except bacteria (Dobrovol'skaya *et al.,* 2001). In addition, the PCR bias, which is usually caused by lysis efficiency of bacterial cells, could affect the

results of bacterial diversity studies. Hence, the lysis efficiency of bacterial cells that varies between and within microbial groups should be concerned, to avoid PCR bias (Prosser, 2002). Besides, the purity (humic acid content) of extracted DNA should be taken care of. This is because humic acid contaminated DNA sample interfere with the reaction of *Taq* DNA polymerase in PCR, hence resulting in a poor PCR products (Tebbe and Vahjen, 1993; Neef *et al*., 1998).

According to the previous studies, culture-dependent and culture-independent methods reveal different phylogenetic information about bacterial diversity of Antarctic soil. Phyla Proteobacteria, Cytophagales, Actinobacteria and Firmicutes are well represented by cultivated organisms and these four phyla account for 90% of all cultivated bacteria characterized by 16S rRNA sequences. Some phyla which are revealed by clonal analysis, such as Acidobacteria and Verrucomicrobia, are poorly represented by sequences from cultivated organisms (Zhang and Xu, 2008).

Apart from these problems arise from methodological limitations in both methods; there is also lack of taxonomic knowledge which influences both techniques. It is difficult to study the diversity of microbial groups when it is not understood how to categorize or identify present species (Malik *et al.,* 2008). Comparing these two available methods in assessing the bacterial communities in soils, each method has its limitation and only provides a partial picture of one aspect of soil microbial diversity that can combine with other method to obtain a more complete picture of bacterial diversity (Zhang and Xu, 2008). Therefore, the use of a polyphasic approach involving a combination of molecular biology techniques, microbiological methods and geochemical techniques or microsensors is necessary to obtain a better understanding of the interaction between the microorganisms and their natural environment (Dobrovol'skaya *et al.,* 2001; Kapur and Jain, 2004).

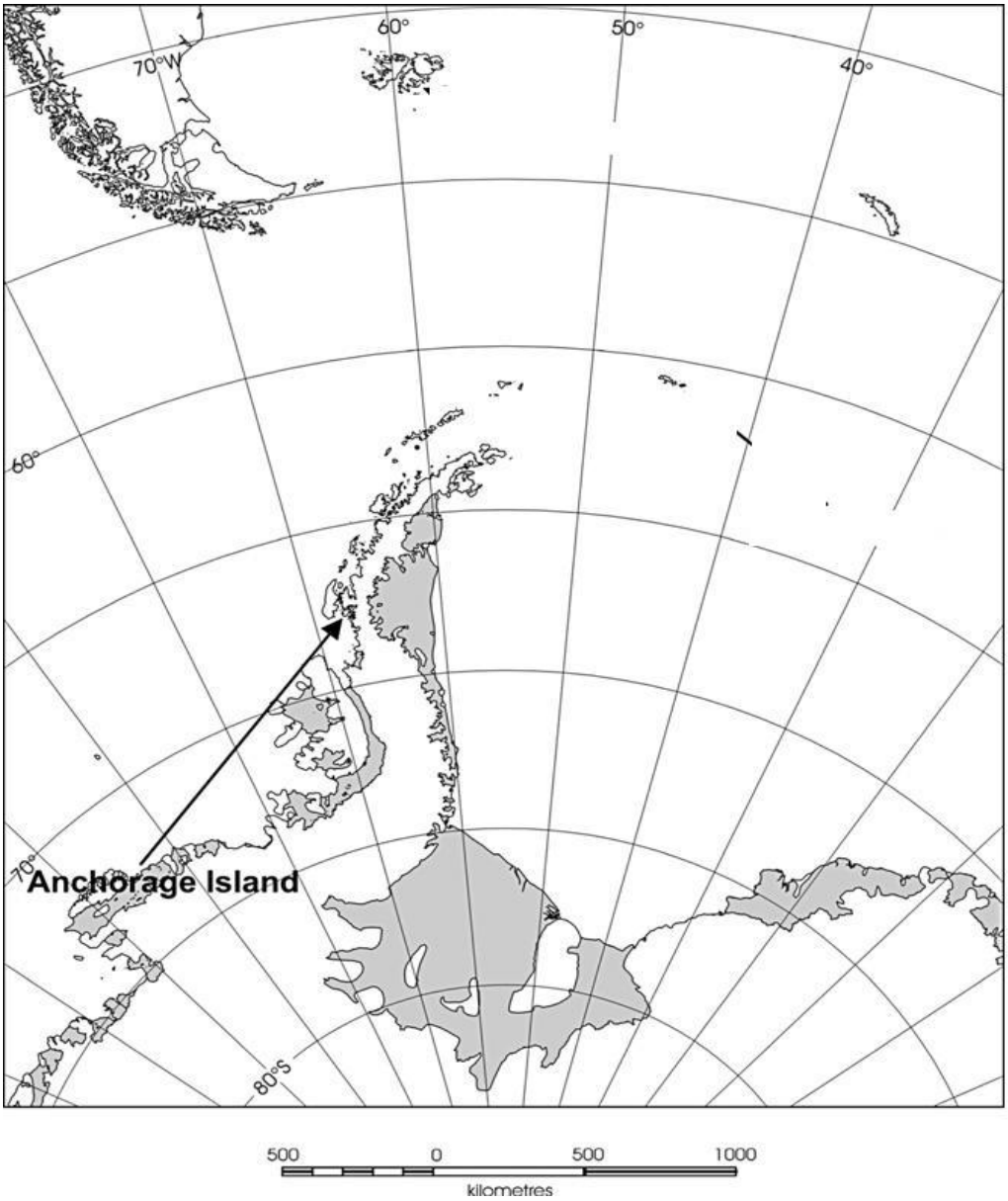


Fig.2.1: Map indicating the location of Anchorage Island (adapted from Bokhorst *et al.*, 2008).

CHAPTER 3.0: MATERIALS AND METHODS

3.1 Sample collection

Surface soil samples (top 4-6 cm depth) were collected by Ms. Goh Yuh Shan and Mr. Chong Chun Wie (PhD students) from three different locations in Anchorage Island, off Rothera Research Station in Peninsular Antarctica (67°34'S 68°08'W). Soil samples were collected during the austral summer between 10 December 2006 and 18 February 2007. At each location, six replicate samples of approximately 50 g were collected using sterile falcon tubes. Samples were stored at -20°C until use. To prevent contamination of the soil samples in the laboratory, each soil sample was transferred to a clean sterile 50 ml centrifuge tube to be used as a stock sample.

Table 3.1: Description of location and characteristics of soil sample from three different sites on Anchorage Island

Sample	Location GPS	Description & Date of collection	Elevation (m)	Air Temperature (°C)	Soil Temperature (°C)
AI_1	S67° 36.178' W68° 12.550'	Sandy coarse soil weathered rock with trace of moss &lichen (16/12/2008)	10.4	2.3	3.3
AI_2	S67° 36.285' W68° 12.919'	Between scattered rock cover with moss black and fine (16/12/2008)	28.6	3	4.1
AI_3	S67° 36.896' W68° 13.987'	Between large rocks on rocky outcrop high in nutrient (30/12/2008)	16	10.5	8.4

As shown in Table 3.1, AI_1 or Anchorage Island1, is located in S67° 36.178', W68° 12.550', with elevation of 10.4 m which has sandy coarse soil and weathered rock with trace of moss and lichen, air temperature about 2.3°C and soil temperature 3.3°C. AI_2,

Anchorage Island 2, is located in S67° 36.285', W68° 12.919', with elevation of 28.6 m which is between scattered rocks and covered with moss and it has black and fine soil (wet) by the air temperature of approximately 3.0°C and the soil temperature of 4.1°C. Whereas, AI_3 (S67° 36.896', W68° 13.987') is located between large rocks on rocky outcrop and it is high in nutrient input and it is penguin moult site with elevation of 16m. Air temperature in this site is 10.5°C and the soil temperature is 8.4°C that are different from other two locations (AI_1, AI_2).

3.2 Culture medium

Nutrient agar was used as medium to culture microbes from each soil sample. Nutrient Agar is a complex medium; advantage of using a complex media in bacterial isolation is that they support the growth of a wide range of microbes. Generally, nutrient Agar is used for the cultivation of bacteria and for the enumeration of organisms which are not fastidious. This medium contains beef extract, peptone and agar in water. The beef extract contains water soluble carbohydrates, vitamins, organic nitrogen compounds and salts. Peptone is casein (milk protein) that has been digested with the enzyme pepsin and it is the sources of organic nitrogen, particularly amino acids and long chained peptides. Agar is purified from the cell wall of the red algae and it is the solidifying agent and it has no nutrient value. It is an excellent solidification agent because it dissolves at near boiling but solidifies at 45°C. This relatively simple formulation provides the nutrients necessary for the replication of a large number of microorganisms that are not excessively fastidious (Downes and Ito, 2001).

3.3 Medium preparation

Amount of culture medium composition based on protocol were weighted and dissolved in distilled water into universal bottle. For example, 0.8 g of nutrient medium containing beef extract, peptone and 12 g of agar were weighed and added to 1 L of

distilled water. This was then sterilized at 121°C for 20 minutes. As Antarctic soil have the least developed soils in the world and are generally nutrient poor (Beyer and Bolter, 2000) for culturing the soil bacteria from Antarctica samples nutrient strength of medium was prepared as 1/10.

After autoclave, 2 ml of Cycloheximide stock solution was added into the agar medium to a concentration of 0.02 mg/ml to eliminate fungal growth. The Cycloheximide stock solution (10 mg/ml) was prepared by dissolving 0.5 mg of Cycloheximide powder in 1 ml of water and then the solution was filter-sterilized before use. The Cycloheximide stock solution and the prepared agar plates were stored in 4°C fridges until use.

3.4 Dilution method

From six replicate of each location, three replicates were chosen to isolate culturable bacteria. Around 1 g soil sample was weighed and suspended into 9 ml sterile water in 15 ml sterile centrifuge tube. The tube was mixed thoroughly and this was the 10^{-1} dilution of the soil. From here, 1 ml of suspension was pipetted into another15 ml sterile centrifuge tube contained 9 ml sterile water and this was mixed thoroughly to get homogenous suspension. The second tube was the 10^{-2} dilution of the soil suspension. The following dilutions (10^{-3} and 10^{-4}) were prepared in a similar manner. Each tube was labeled by its dilution factor and the name of the sample. A serial dilution plating method was used to plate each soil sample on the nutrient agar medium which contained Cyclohexamide (0.02 mg/ml).

3.5 Plating sample

Spread plate method was used to cultivate the bacteria from the Anchorage Island soil samples. 50 µl of the diluted suspension mentioned in session 3.4 was pipetted onto the nutrient agar plate contain Cyclohexamide (0.02 mg/ml) and distributed over the surface of the agar using a sterile hockey steak. Duplicate plating was prepared from each

dilution factor except 10^{-1}. Plates were labeled according to the name of sample, dilution factor and date of plating. Plates were sealed with Parafilm®, inverted and incubated at 4ºC for 7 days.

3.6 Plate counting and colony forming unit determination

Colony forming units (CFU) per gram fresh soil was counted after incubation times of 7days. Counting for a second time was carried out to determine if additional colonies would grow after one day of incubation. Plate counts were recorded along with a description of all visible colonies. CFUs/g fresh soil was calculated from the countable dilution plate (30 to 300 colonies) by multiplying the number of colonies by the dilution factor and 10,000 to account for the 50 µl aliquot.

3.7 First purification

As mentioned in Session 3.3 nutrient agar contains Cyclohexamide (0.02 mg/ml) as antifungal antibiotic which is vital for bacterial isolation was used for plating and isolation. After 8 day counts were recorded, from the countable plates the first isolation of pure cultures was transferred to the same nutrient medium by dilution-streak method to isolate individual colonies. Uncountable plates were not useful due to in this kind of plates colonies were stick to each other and it was impossible to pick single colony. Colonies was chosen based on their shape, color and appearance. If there were multiple colonies with similar morphological characteristics on a dilution plate, two or three were selected for isolation. In try to isolate sufficient representatives of the culturable bacteria, from all sample sites, all unique colonies from each dilution plate were transferred. Colonies were selected from all plates of the duplicate set. Each plate divided into two sections, in each section on picked colony was grown. Plates was sealed, inverted and incubated at 4ºC until pure cell growth is observed.

3.8 Second purification

After incubation time around 7 to 10 days, when the first isolate colonies were observed the second purification was done on nutrient agar medium from the isolated colonies of first isolation to make sure to have pure colonies. Each plate was sealed, inverted and incubated at 4ºC until pure cell growth was monitored. Each plate was labeled by the given name and date. These pure isolate were used as template for polymerase chain reaction.

3.9 Naming of isolate samples

Records for each isolate included a name of site, sample and colony description like color. So, the order of given name for every single isolate were, site name, sample, number of pick and the first letter of isolate color. For example name of first isolate from AI_1 called AI_1a-1p (p, means pink color). In total, 196 colonies were picked from the three sites.

3.10 Polymerase Chain Reaction (PCR)

Pure bacterial isolates of interest were identified by PCR amplification and sequencing of the 16S rRNA gene. The primer set used were uni-for (5'-TGCCAGCAGCCGCGGTA-3'; *E. coli* positions 516 to 532) and uni-rev (5'-GACGGGCGGTGTGTACAA-3'; *E. coli* positions 1390 to 1407). The PCR amplification was performed in a 25 µl reaction mixture containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Biotools, Spain), 12.5 pM of each oligonucleotide primer, 1× PCR buffer [200 mM Tris–HCl (pH 8.4), 500 mM KCl] and 0.1 U *Taq* DNA polymerase (Invitrogen, USA) (Table 3.2). A single colony from a freshly-prepared bacterial culture was picked and transferred into the PCR reaction mixture as DNA template. Amplification was managed using a thermal cycler (BioRad, U.S.A.). The following program was used: initial denaturation at 95°C for 5 min followed by 30 cycles of

denaturation at 94°C for 11 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 10 min.

Table 3.2: The reaction mixture for PCR

PCR stock solution	Volume (µl)	Final concentration
10x PCR Buffer	2.5	1x
50 mM $MgCl_2$	0.75	1.5mM
10 mM dNTP	0.5	0.2mM
Forward primer (5'-TGCCAGCAGCCGCGGTA-3')	2.5	12.5pmol
Reverse primer (5'GACGGGCGGTGTGTACAA-3')	2.5	12.5pmol
Taq DNA Polymerase(5U/µl)	0.5	0.1U/ µl
sdH_2O	14	-
DNA template	1.75	-
Total	25	-

3.11 Template preparation

3.11.1 Colony PCR

A single colony from pure culture on nutrient agar was picked using a micropipette tip and was suspended in 50 µl sterile distilled water. This was used as template for amplification of 16S rRNA gene PCR in session 3.10 (1.8 µl for each 25 µl PCR reaction).

During the PCR, faced difficulties amplifying some bacteria via colony PCR, hence other methods were utilized to obtain the PCR template (3.11.2 and 3.11.3).

3.11.2 DNAzol Reagent

DNAzol (Invitrogen, U.S.A.) is a reagent for the isolation of genomic DNA from samples. One ml of DNAzol reagent was added to plenty of colonies by gently pipetting to lyse the cells. 0.5 ml 100% ethanol was added to precipitate DNA by inversion mixing. Solution centrifuged after 1-3 minutes at 6000 rpm for 1-2 minutes. For DNA solubilization pellet was washed by 1ml 75%ethanol twice. After 5-15 second, 50µl autoclaved distilled water was added and mixed using pipette tip. This product use as template and has ability to keep in fridge for long time.

3.11.3 Genomic DNA extraction kit

G-spin ™ Genomic DNA extraction kit by Intron Biotechnology (Korea) was also used to prepare template for PCR. According to the company protocol, solution of 50 µl pre-buffer and 3 µl Lysozyme mixed with appropriate amount of colonies were incubate at 37°C for 15 to 30 minutes followed by incubation at 65°C for 15 minutes. 250 µl of binding buffer was added and mixed well by pipetting. Cell lysates were loaded on sterile column and centrifuged at 13,000 rpm for 1 minute. In the next step, 500 µl of washing buffer A was added to the column and centrifuged at 13,000 rpm for 1 minute. To complete washing, 500 µl of washing buffer B was added and centrifuged 1 minute at 13,000 rpm. After removing the solution, empty column were centrifuged at same rpm to dry. G-spin ™ column were placed in clean 1.5 ml microcentrifuge tube and between 50-200 µl of Elution buffer was added directly onto the membrane. After incubation for 1 minute in room temperature, tubes were centrifuged for 1 minute at 13,000 rpm. The solution contain extracted DNA can be used as template for several times.

3.12 Detection of PCR product

Amplified DNA fragments were visualized using agarose gel (1.0% w/v) electrophoresis (40 minutes run at 100V, 1xTAE buffer). Gel electrophoresis separates

DNA molecules according to their size. In practice the composition of gel determines the size of DNA molecules that can be separated.

3.12.1 Gel preparation

10 x TAE stock solution was diluted to 1 x using dH_2O for routine DNA analysis by gel electrophoresis.

Agarose powder (Molecular Biology Grade, Vivantis)	0.7 g
1 x TAE buffer (Tris Acetate ETDA) (First base, Malaysia)	70 ml

The suspension in beaker was heated in microwave oven for 1-2 minutes, till the agarose powder had completely dissolved. The melted agarose was poured into Perspex gel tray inserted with gel combs. When the agarose has solidified, the combs were removed and the gel together with the tray was submerged in horizontally electrophoresis tank (Scie-plas, U.K.), filled with 1 x TAE until the gel was covered by buffer.

5 µl of PCR product were mixed with 2 µl, 6 x loading dye and loaded into each well. The 100 bp DNA Ladder (Invitrogen, US) was used as a molecular size marker.

3.12.2 Gel staining

As mentioned above, after 40 minutes run at 100 V gel was stained in ethidium bromide 2.5 µg/ml (Promega, Madison, U.S.A) for 5 minutes and distained in water for 10 minutes. Gel images were viewed and photographed using UV transilluminator (Syngene, Bio Imaging).

3.13 Restriction Fragment Length Polymorphism (RFLP)

The amplified PCR products were subjected to restriction digestion by *Hae*III enzyme (Promega, Madison, U.S.A.). Master mix for each reaction tube was prepared using, 2 µl buffer C, 0.5 µl *Hae*III enzyme, 0.2 µl BSA (Bovine Serum Albumin) (Promega, Madison U.S.A.) and 2.3 µl sterile distilled water(table3-4). Digestion was conducted using thermocycler (BioRad, U.S.A.) for about 4 ½ hours.

Final products of the digestion were examined by agarose gel electrophoresis as described in session 3.12.1 except difference of preparing 1.5% gel instead of 1%. For example, adding 70 ml 1X TAE buffer to 1.05 g of agarose.

Table3.3: The reaction mixture for RFLP

Digestion reagent	Stock concentration	Volume (µl per reaction)	Final concentration
Buffer C	10 x	2	1 x
*Hae*III	10U/µl	0.5	0.25U/µl
BSA	150µl	0.2	1.5µl
dH_20		2.3	-
PCR product		15	-
Final volume		20	-

3.14 Grouping

RFLP analysis of 16S rRNA genes was employed to differentiate between various species. Due to cost of sequencing for 196 isolate were high, RFLP analysis effectively reduce the number of isolates needing to be sequenced. Resulting 16S rRNA gene fragments were grouped by different pattern obtained by restriction fragment length polymorphism using restriction enzymes *Hae*III. Different groups were obtained by comparing the fragments respective to marker ladder (Invitrogen, US). The ladder is designed for sizing DNA fragments on agarose gel.

3.15 Gram stain

From each group of RFLP patterns different colonies was picked for gram staining. Gram stain is the most commonly used staining procedure in microbiology. It is extremely useful in identifying bacteria as well as their morphological shape. Gram stain divides bacteria into two groups, gram positive and gram negative based on their chemical composition of cell wall.

3.15.1 Smear preparation

Clean microscope slides were labeled by name of isolate, the colony of isolate was picked using sterile loop by flame, mixed gently in drop of distilled water which placed before to emulsify. Smears were allowed to air dry. Air dried smear were passed through a flame for two or three times. Fixed smear left for few minutes to be cool before staining.

3.15.2 Staining procedures

The fixed smears were flooded into Crystal violet for 1 minute, crystal violet was decanted and slides were washed by water gently. Smear were cover with iodine for 1.5 to 2 minutes to make strong complex between cell wall peptidoglycon of gram positive bacteria and crystal violet. To decolorize, Iodine was rinsed with water and smear washed by ethanol until run off become clear. For the last step of staining, smear were flooded by Safranin for less than 1 minute, rinsed with water gently and left to become dry. Slides was observed using microscope (Leica Microsystem, Germany).

3.16 DNA preparation for sequencing

PCR products from unique RFLP patterns based on different isolate morphology were chosen and purified by gel electrophoresis prior sequencing. Purified PCR product should be single band and free from salt, protein and primer dimmers before sequencing to obtain good result from sequencing. MEGA quick-spin^{TM} PCR and Agarose DNA Extraction kit by Intron Biotechnology (Korean) was used. 5 volume of BNL buffer was added to the PCR product in 1.5 ml microcentrifuge tube, mixed well and centrifuged briefly. Solution was loaded into the MEGA quick spin^{TM} column assembled to the collection tube and centrifuged at 13,000 rpm for 1 minute. Flow- through was discarded and 700µl of washing buffer was added into the column, centrifuged at 13,000 rpm for 1 minute. This step was repeated with 500 µl of washing buffer. After second wash solution was removed and column was centrifuged for 1 minute at 13,000

rpm to dry the spin membrane since residual ethanol from washing buffer may interfere with other reaction. After that, the column was placed into the clean and sterile 1.5µl centrifuge tube. About 30-100 µl Elution buffer was applied directly to the center of the column without touching the membrane by pipette tips. After 1 minute incubation in room temperature, centrifuged at 13,000 rpm for 1 minute. DNA is eluted from the column membrane. The amount of purified DNA was determined spectrophotometrically at OD_{260} nm as well as gel electrophoresis to check purity. 10µl of purified sample were allocated to the 0.5 ml PCR tubes, sealed by Parafilm and stored in -20° C. Forward primer was 10 times diluted and send for sequencing together with samples.

3.17 Sequencing

The PCR products were screened by restriction fragment length polymorphism (RFLP) analysis using *Hae*III digestion. Fingerprint profiles were compared for similarity using the program NEB Cutter version 2.0 software was adopted to interpret the RFLP pattern results. This program has been designed to search for the recognition site of endonuclease to locate the position of these sites in the sequences and to calculate the size of fragments after digestion. Representatives of each RFLP group were sequenced. As described in session 3.14 the PCR products were purified using PCR quick-spin PCR Product Purification Kit (INTRON Biotechnology, Korea) and sent to a 1st Base commercial laboratory (Selangor, Malaysia) for sequencing the samples. 16S rRNA sequences were a BLAST search using Chromas version 1.45 (Conor McCarthy, School of Health Science, Griffith University, Southport, QLD, and Australia, 1996–1998,). National Center for Biotechnology Information (NCBI) was performed to identify the nearest species to the amplified sequence. Phylogenetic trees were created using MEGA4 (Tamura *et al*., 2007). The tree topologies were evaluated by bootstrap value of

5000 replicate with PHYLIP (Felsenstein, 1993) and Phylogenetic trees were inferred using the neighbor-joining method (Saitou and Nei, 1987).

Sequences were obtained using the sequence match search tool in the Ribosomal Database by RDP classifier tool (Cole *et al*., 2005) to make reclassified sequences with 80% similarity. Nucleotide distance of the sequences was analyzed by MEGA 4 (Tamura *et al*., 2007). The data were then used to compare the variation between locations, with higher values depicting larger taxonomic diversity. Besides that, MEGA 4 (Tamura *et al*., 2007) was used to obtain nucleotide distance between different samples. Some sequences were checked for possible chimeras using the Pintail-Chimera (Ashelford *et al.*, 2005). NEB Cutter version 2.0 software was used to check the RFLP patterns.

3.18 Storage of representative isolates

From unique RFLP patterns and based on different isolate morphology in each different sites samples, two isolates was stored in 20% glycerol and kept in -80°C for future work.

CHAPTER 4.0: RESULTS

4.1 Colony Forming Units

Three replicates of soil surface samples from each site within the Anchorage Island region were studied. Using the dilution plating technique on nutrient agar, different numbers of colonies were retained from samples. The same dilution from 10^{-1} to 10^{-4} was done for all samples. Plate counts were recorded along with a description of all visible colonies after one week. Counting for a second time was done to determine if additional colonies would grow after one day of incubation. Number of colonies in Anchorage Island 1 (AI_1) and Anchorage Island 3 (AI_3) samples increased after one day compared to Anchorage Island 2 (AI_2) samples which did not show any difference between two counts. Number of colonies observed in AI_1 and AI_3 samples was higher than AI_2 samples. Highest number of colonies was determined in AI_1 samples. More than 300 colonies were detected in 10^{-1} and 10^{-2} of AI_1 as well as 10^{-1} of AI_3 samples. The least number of colonies growing were detected in AI_2 samples. In 10^{-4} dilution of AI_2 colonies did not grow after incubation time (Figure 4.2). CFUs/g of soil was calculated from the countable dilution plate (30 to 300 colonies) by multiplying the number of colonies by the dilution factor and 10.000 (final volume in µl) to account for the 50 µl aliquot (Table 4.1).

The number of colony decreased with increasing dilution. In addition, range of CFU/g fresh soil decrease along with increasing the dilution. According to the Table 4.1 the CFU data of samples were calculated from range 1.4×10^5 to 4.9×10^6 CFUs/g fresh soil. Highest CFU value (4.9×10^6) was observed in 10^{-3} dilution of AI_1 sample. Lowest number of samples with no detectable CFUs/g were in 10^{-3} and 10^{-4} of AI_2.

Table 4.1: Values of CFU/g fresh soil and number of colonies recovered on nutrient agar medium for the four dilutions of three Anchorage Island site samples.

Sample	Dilution	First count 27.07.2009	Second count 28.07.2009	Cfu/g fresh soil
AI_1	10^{-3} 10^{-4}	249 63	263 71	4.9×10^6 1.2×10^6
AI_2	10^{-1}	134	134	2.6×10^5
AI_3	10^{-2} 10^{-3}	259 74	263 74	5.1×10^5 1.4×10^5

The number of colony-forming units obtained from a given sample in almost all cases is considerably lower than the actual titer of prokaryotic cells in the soil.

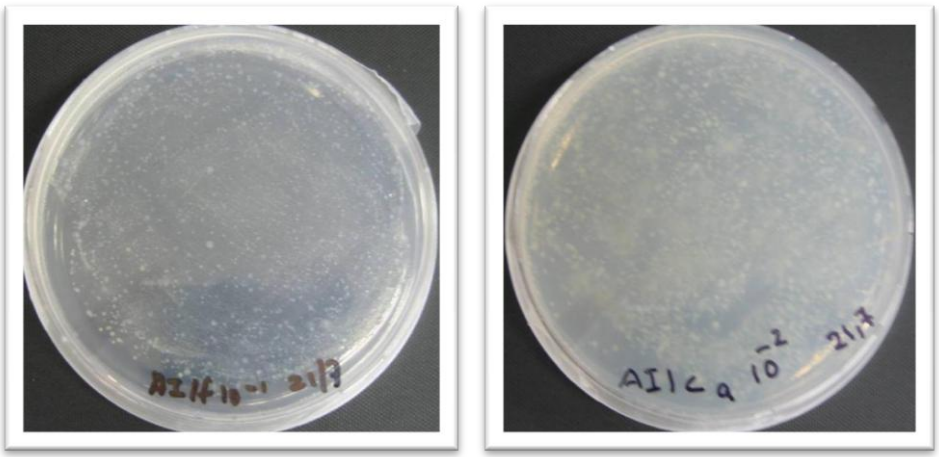


Figure 4.1: Uncountable plate (more than 300 colonies) in 10^{-1} and 10^{-2} dilutions of Anchorage Island 1 (AI_1) sample**.**

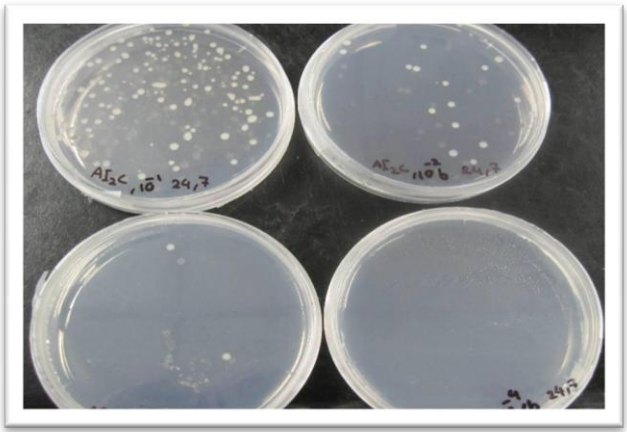
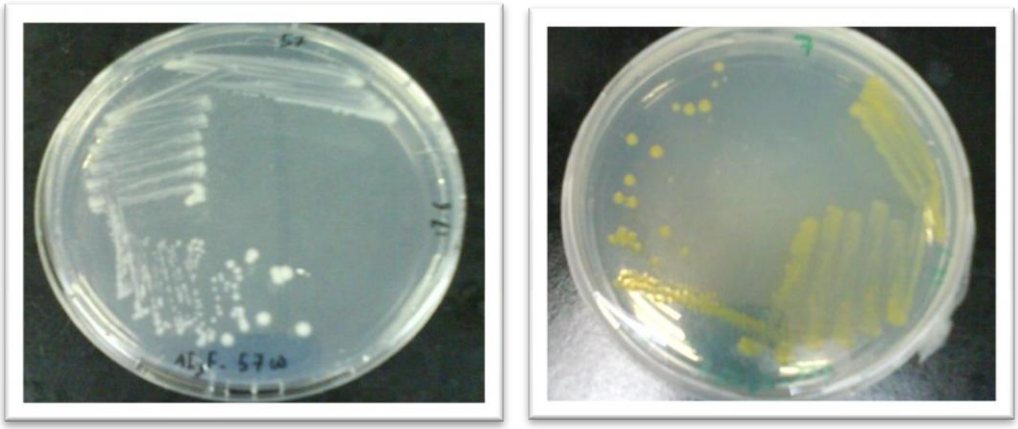


Figure 4.2: Plates of Anchorage Island 2 (AI_2) samples from 10^{-1} to 10^{-4} dilution.

4.2 Purification

All the isolates so far characterized have been isolated under aerobic growth conditions. Initially each isolate is characterized phenotypically for colony morphology.

From countable plates as much as possibly different colonies based on their shape and color were isolated on nutrient agar plate to obtain pure culture using dilution streaking method (Fig.4.3). 80 pure colonies from AI_1, 34 colonies from AI_2 and 82 colonies from AI_3 were picked and isolated.



(A) (B)

Figure 4.3: Typical appearance of pure colony (A) *Pseudomonas fragi,* and (B) *Sejongia antarctica.*

4.3 PCR amplification and biases

PCR amplification has become well accepted method for obtaining rRNA gene sequence from microbial communities or pure cultures prior to fingerprinting studies (Drancourt *et al.* 2000). In this study, 196 pure isolate colonies (80 isolate from AI_1, 34 isolate from AI_2 and 82 isolate from AI_3) on nutrient agar from all three site samples were subjected to amplification of 16S rRNA gene by PCR using uni-for (5'-TGCCAGCAGCCGCGGTA-3'; *E. coli* positions 516 to 532) and uni-rev(5'-GACGGGCGGTGTGTACAA-3'; *E. coli* positions 1390 to 1407) primers.

The 16S rRNA gene sequences determined for all isolates. In some isolates, amplified 16S rRNA gene was not in high quality, no bands were seen on agarose gel after

electrophoresis and staining with ethidium bromide (Fig. 4.4). This issue happened due to using colony PCR to amplify the 16S rRNA gene. In this case, bacterial cell wall was not broken by subjected to 95° C in thermal cycler. To overcome this issue, DNAzol reaction (Ligozzi and Fontana, 2003) and genomic DNA extraction kit was used. Treating microbial cells with specific cell-wall degrading enzymes greatly facilitates the susceptibility of these cells to DNAzol and genomic DNA extraction kit procedures (Fig. 4.5).

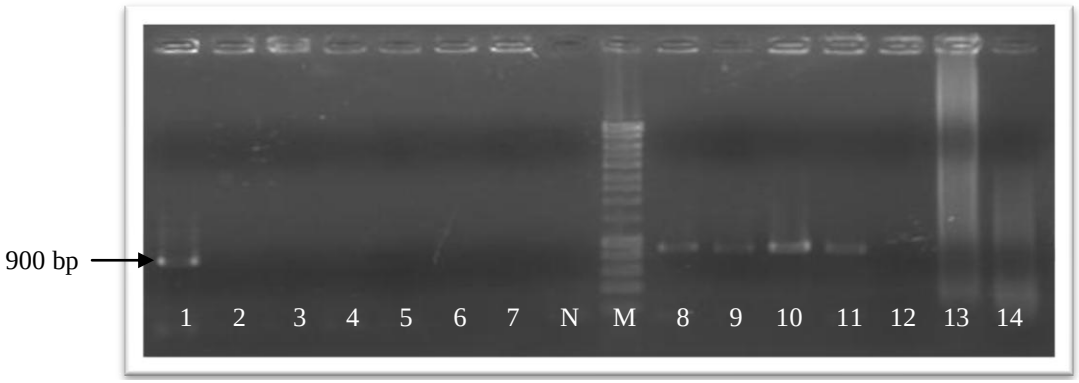


Figure 4.4: Agarose gel electrophoresis (1%) of the genomic DNA isolated by simple colony PCR. M shows marker and N is negative control without template. 1,2,….are DNA extracted from colonies by colony PCR.

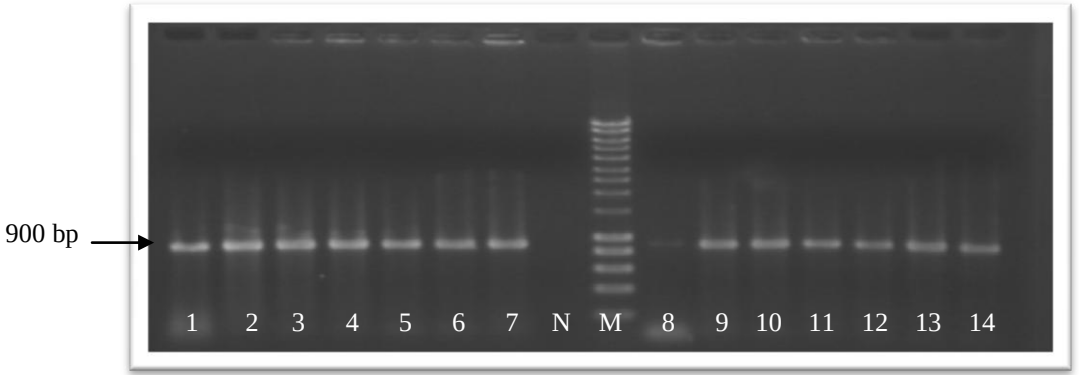


Figure 4.5: Agarose gel electrophoresis (1%) of the genomic DNA isolated by the modified DNAzol and genomic DNA purification kit methods. M shows marker and N is negative control without template. 1,2,….are DNA extracted from colonies by using DNAzol reaction and DNA extraction kit.

DNA extraction kit was costly to obtain huge amount of isolate thus DNAzol reaction which has same features as genomic DNA extraction kit used to extract DNA from the

rest of colonies. Besides that, DNAzol and genomic DNA extraction kit were complete and ready to use reagent. The extracted DNA from these two methods was reproducible, kept in -20°C before used as template. In addition, there is no need to sub culture old isolate since fresh DNA sample is not required.

4.4 RFLP fingerprinting

The PCR products of 16S rRNA gene from isolates were each about 900 bp long. RFLP analysis of the 16S rRNA gene is useful to obtain band patterns, studies of the identity and diversity of microbial communities as well as rapid and economical method (Zhang *et al.* 2006).

The key step of the technique is selection of suitable endonucleases. Each amplified 16S rRNA gene was digested using two different restriction enzymes, *Hha*I and *Hae*III for RFLP analysis on agarose electrophoresis. As show in Fig. 4.6 employing these two enzymes caused over digestion of PCR amplicons. Although, to produce differentiating band patterns, it is better to use minimum number of enzymes based on their cost, availability and steps as well as avoiding enzymes that producing too many small fragments (<50 bp) (Yang *et al.* 2007). Since, the size of gain amplicons were 900 bp and it was short for being subjected to digest with two different restriction enzymes with different restriction sites that cause occurrence of many fragments. Therefore, the restriction endonuclease *Hae*III which specifically recognizes and cuts the site ‘‘GC/GC’’ was selected for the digestion of the PCR products of the16S rRNA gene for RFLP fingerprinting analysis (Fig. 4.7).

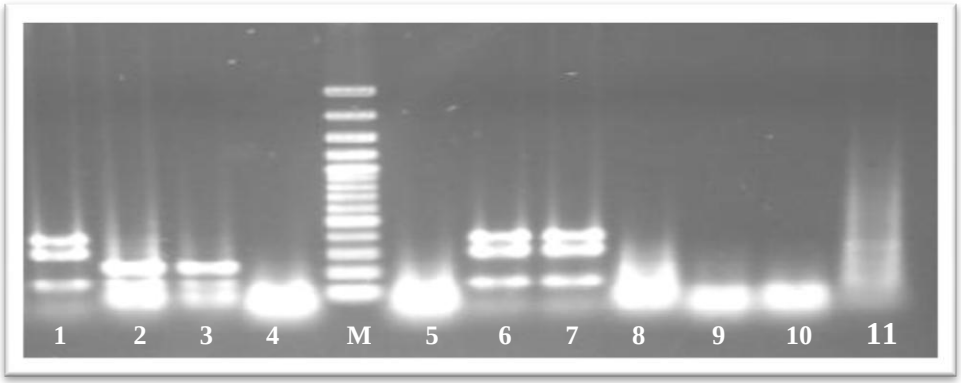


Figure 4.6: Agarose gel electrophoresis of restriction fragments of 16S rDNA amplification product of isolate associated with Antarctic soil with restriction enzymes *Hha*I and *Hae*III. M used as marker ladder. 1,2,3,….are amplified 16S rRNA gene digested by two RE enzymes.

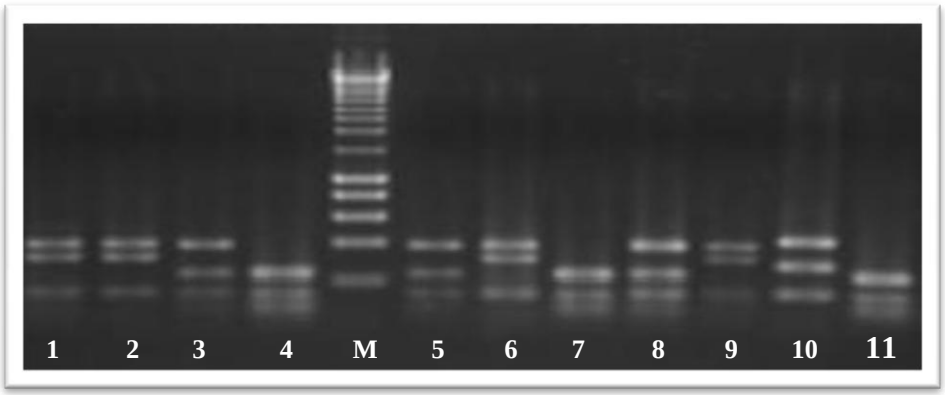


Figure 4.7: Agarose gel electrophoresis of restriction fragments of 16S rDNA amplification product of isolate associated with Antarctic soil with restriction enzyme *Hae*III. M used as marker ladder. 1,2,3,….are amplified 16S rRNA gene digested by one RE enzyme.

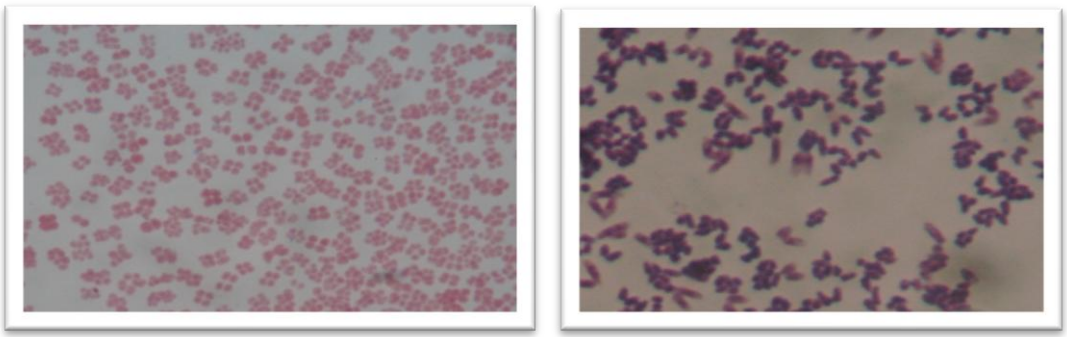
In total among 196 isolates, fourteen different patterns were delineated with have overlap between three different site samples (Table 4.4). The main digestion products were 900 bp in size.

The total number of isolates recovered from pattern A among the fourteen different patterns, were observed the highest number of isolates in AI_1 and AI_3 samples. While in AL_2 samples, the most number of isolate belonged to pattern B. On the other hand, patterns C and F in AI_3 samples, E and F among AI_2 samples and pattern J in AI_1 samples indicated the lowest isolate between all patterns. Based on this, pattern F has

lowest number of isolate in all samples. Among 80 isolates from AI_1 eight different RFLP patterns were visually delineated, I and J patterns were specific and A, B, C, D, E and G patterns had overlap with others. From AI_2 that contain 34 isolates, five different patterns (A, B, E, F, H) were described which had no specific pattern for this sites samples. In AI_3 site samples, which contained 82 isolate, among twelve different RFLP patterns eight patterns (A, B, C, D, E, F, G, H) were common with others and rest patterns (K, L, M, N) were detailed to this site samples.

4.5 Gram stain

Each representative strain was subjected to gram stain (Fig. 4.7) as different colony color showed same RFLP pattern like pattern A and B. on the other hand colonies with same morphology and color illustrate different RFLP pattern like patterns K, L and M.



(A) (B)

Figure 4.8: (A) Photographs showing Gram negative cocci from samples observed under oil emulsion (x 1000 magnification) and (B) indicates Gram positive rod from samples observed under oil emulsion (x 1000 magnification).

Table 4.2: Restriction fragment length polymorphism (RFLP) fingerprinting patterns and representative strain of each site.

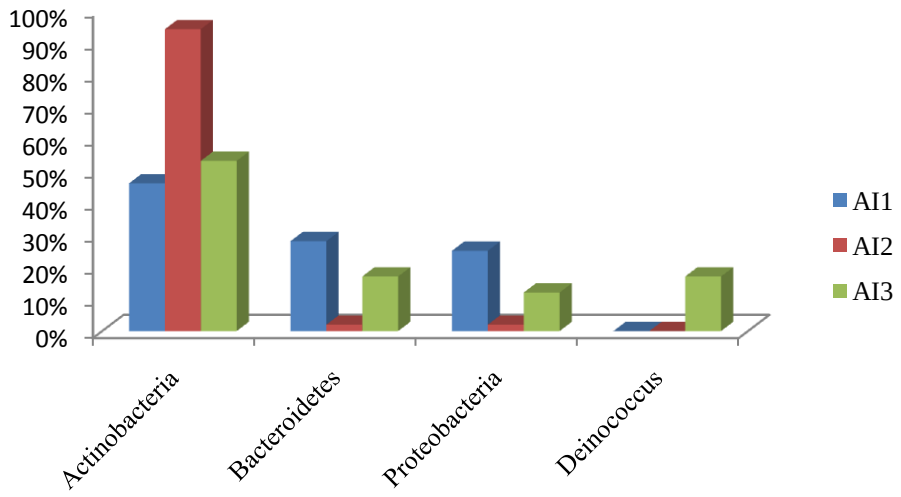
RFLP Pattern	Sites	Number of Isolate	Representative Strain	Colony Color	Gram Stain
A	AI_1	30	AI_1-23C	Cream	+
			AI_1-32W	White	+
	AI_2	12	AI_2-9C	Cream	+
			AI_2-29W	White	+
	AI_3	28	AI_3-18C	Cream	+
B	AI_1	7	AI_1-58C	Cream	+
	AI_2	15	AI_2-8C	Cream	+
	AI_3	3	AI_3-58C	Cream	+
C	AI_1	11	AI_1-29W	White	_
	AI_2	0	-	-	
	AI_3	1	AI_3-57W	White	_
D	AI_1	16	AI_1-56Y	Yellow	_
	AI_2	0	-	-	
	AI_3	3	AI_3-11Y	Yellow	_
E	AI_1	7	AI_1-18Y	Yellow	_
	AI_2	1	AI_2-13Y	Yellow	_
	AI_3	11	AI_3-30Y	Yellow	_
F	AI_1	0	-	-	
	AI_2	1	AI_2-27O	Orange	+
	AI_3	1	AI_3-69O	Orange	+
G	AI_1	3	AI_1-3W	White	+
	AI_2	0	-	-	
	AI_3	5	AI_3-49W	White	+
H	AI_1	0	-	-	
	AI_2	5	AI_2-7W	White	+
	AI_3	5	AI_3-12W	White	+
			AI_3-19W	White	_
I	AI_1	4	AI_1-1O	Orange	_
	AI_2	0	-	-	
	AI_3	0	-	-	
J	AI_1	2	AI_1-49W	White	_
	AI_2	0	-	-	
	AI_3	0	-	-	
K	AI_1	0	-	-	
	AI_2	0	-	-	
	AI_3	11	AI_3-40P	Pink	+
			AI_3-78R	Red	+
L	AI_1	0	-	-	
	AI_2	0	-	-	
	AI_3	2	AI_3-37R	Red	+
M		0	-	-	
	AI_1	0	-	-	
	AI_2	2	AI_3-52P	Pink	+
	AI_3				
N	AI_1	0	-	-	
	AI_2	0	-	-	+
	AI_3	10	AI_3-41O	Orange	

4.6 Phylogenetic analysis of 16S rRNA gene sequences of isolates

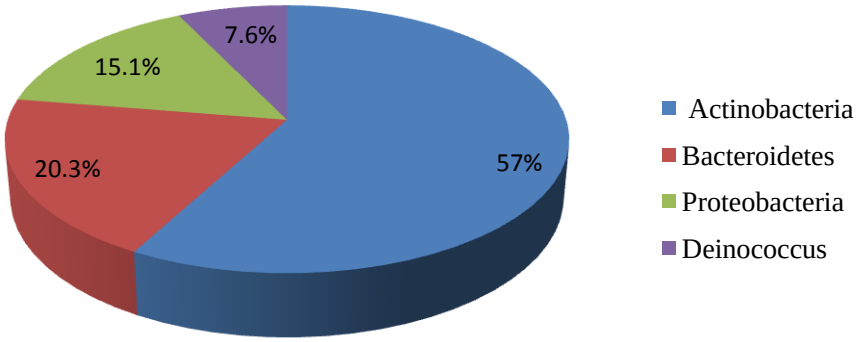
RFLP analysis can successfully decrease the number of isolates needing to be sequenced (Zhang *et al.* 2006). There for among 196 isolates samples of all three sites, one isolate from diverse RFLP patterns belong to each site samples as well as colonies with visibly different morphologies such as color and shape, were selected for subsequent 16S rRNA gene sequencing and phylogenetic analysis (Table 4.5).

Regards to Table 4.5 patterns A and B are represented by strains AI_1-23c, AI_1-32w, AI_2-9c, AI_2-29w, AI_3-18c and AI_1-58c, AI_2-8c, AI_3-58c belong to genus of *Arthrobacter*. Strains yielding patterns C is represented by strains AI_1-29w and AI_3-57w belonged to *Pseudomonas*. Pattern D is represented by strains AI_1-56y and AI_3-11y belong to *Flavobacterium*; Pattern E is represented by AI_1-18y, AI_2-13y and AI_3-30y belonged to *Sejongia* and *Chryseobacterium.* Pattern F is represented by strains AI_2-27o and AI_3-69o belong to *Sphingomonas*. Strains yielding pattern G is belong to *psycobacter* that represented by AI_1-3w and AI_3-19w. Pattern H is represented by AI_2-7W and AI_3-12W are belonging to *Agreia* and *polaromonas.* Pattern I belongs *Naxibacter* represented by AI_1-1O. AI_1-49W represented pattern J belongs to *Janthonobacterium.* Patterns K, L, and M are belong to *Deinococcu*s characterized by AI_3-40P, AI_3-78R, AI_3-37R and AI_3-52-P. Lastly, pattern P represented by AI_3-41O is belong to *Rhodococcus.*

According to table 4.5 sequencing result indicated that the isolates belonged to four major groups: the high-G + C Gram-positives Actinobacteria, the Bacteroidetes, Proteobacteria, and the Deinococcus phylum which is just represented in AI_3 site samples (Fig. 4.9A). The most abundant and diverse isolates belonged to the Actinobacteria phylum, the *Arthrobacter* in particular. After that is Bacteroidetes phylum followed by Proteobacterium and Deinococcus (Fig. 4.9B).



(A)



(B)

Figure 4.9: Relative abundance of the principal phylum groups found in the three distinct soil sample sites in the Anchorage Island, Antarctica (A). Relative abundances of the principal phylum groups found in all soil sample sites in the Anchorage Island, Antarctica (B).

Table 4.3: Restriction fragment length polymorphism (RFLP) fingerprinting patterns and sequence relationships of 16S rRNA gene in all soil sample sites in the Anchorage Island, Antarctica.

RFLP pattern	Representative strain	Nearest Species	Similarity	Accession No.	Division
A	AI_1-32 white	*Arthrobacter polychromogenes*	99%	AB167181	Actinobacteria
	AI_1-23 cream	*Arthrobacter sulfonivorans*	99%	FM955888	Actinobacteria
	AI_2-9 cream	*Arthrobacter citreus*	98%	AM237346	Actinobacteria
	AI_2-29 white	*Arthrobacter psychrolactophilus*	98%	AF134181	Actinobacteria
	AI_3-18 cream	*Arthrobacter oxydans*	99%	GU391465	Actinobacteria
B	AI_1-58 cream	*Arthrobacter luteolus*	99%	AJ640198	Actinobacteria
	AI_2-8 cream	*Arthrobacter livingstonensis*	99%	GQ406811	Actinobacteria
	AI_3-58 cream	*Arthrobacter stackebrandtii*	99%	AJ640198	Actinobacteria
C	AI_1-29 white	*Pseudomonas migulae*	100%	EU111739	Gammaproteobacteria
	AI_3-57 white	*Pseudomonas fragi*	99%	AM933514	Gammaproteobacteria
D	AI_1-56yellow	*Flavobacterium hydatis*	99%	AM230487	Bacteroidetes
	AI_3-11 yellow	*Flavobacterium saccharophilum*	99%	AB473208	Bacteroidetes
E	AI_1-18 yellow	*Sejongia antarctica*	98%	NR_025809	Bacteroidetes
	AI_2-13 yellow	*Sejongia marina*	98%	EF554366	Bacteroidetes
	AI_3-30 yellow	*Chryseobacterium hominis*	96%	AM261868	Bacteroidetes
F	AI_2-27 orange	*Sphingomonas sp. Ens34*	100%	DQ339629	Alphaproteobacteria
	AI_3-69 orange	*Sphingomonas sp.Tianshan*	98%	FJ005007	Alphaproteobacteria
G	AI_1-3 white	*Psychrobacter namhaensis*	99%	AY722805	Gammaproteobacteria
	AI_3-49 white	*Psychrobacter urativorans*	97%	AJ609555	
H	AI_2-7 white	*Agreia pratensis*	99%	NR_025460	Actinobacteria,
	AI_3-12 white	*Agreia pratensis*	99%	NR_025460	Actinobacteria,
	AI_3-19 white	*Polaromonas naphthalenivorans*	99%	CP000529	Betaproteobacteria
I	AI_1-1 orange	*Naxibacter indica*	97%	FJ812371	Betaproteobacteria
J	AI_1-49 white	*Janthinobacterium agaricidamnosum*	99%	AY167838	Betaproteobacteria
K	AI_3-40 pink	*Deinococcus radiopugnans*	96%	NR_026403	Deinococcus-Thermus
	AI_3-78 red	*Deinococcus aquaticus*	96%	EU834252	Deinococcus-Thermus
L	AI_3-37 red	*Deinococcus saxicola*	96%	AJ585984	Deinococcus-Thermus
M	AI_3-52- pink	*Deinococcus radiopugnans*	97%	NR_026403	Deinococcus-Thermus
N	AI_3-41 orange	*Rhodococcus fascians*	100%	AJ576249	Actinobacteria

16S rRNA gene sequence phylogenetic analysis of Actinobacteria phylum isolates from three sites mostly related (>98%) to the genus *Arthrobacter* showing that these isolates presented a more scattered relationship with several species, namely, *Arthrobacter polychromogenes, Arthrobacter sulfonivorans, Arthrobacter psychrolactophilus, Arthrobacter luteolus, Arthrobacter livingstonensis, Arthrobacter stackebrandtii, Arthrobacter oxydans, Arthrobacter citreus*. Other isolates were affiliated with different genera in the linage of the Microbacteriaceae, namely, *Agreia* and *Rhodococcus*, with >99% sequence similarity. *Agreia pratensis* was isolated from both AI_2 and AI_3 samples. AI_3-41o, isolated from AI_3 site samples, was most closely related to Nocardiaceae of the suborder Corynebacterineae, with 100% sequence similarity to *Rhodococcus fascians* (Fig. 4.11).

The isolates belonging to the CFB phylum (Bacteroidetes) were less diverse. AI_1-56y and AI_3-11y showed highest sequence similarity (99%) with *Flavobacterium hydatis* and *Flavobacterium saccharophilu* respectively. AI_1-18y and

AI_2-13y was closely related to *Sejongia antarctica* and *Sejongia marina* with 98% sequence similarity. The remaining AI_3-30y had the least similarity by 96% with *Chryseobacterium hominis.*

Next relatively diverse of the organisms belonging to Proteobacteria fell into three major lineages: alpha, beta and gamma subdivisions.

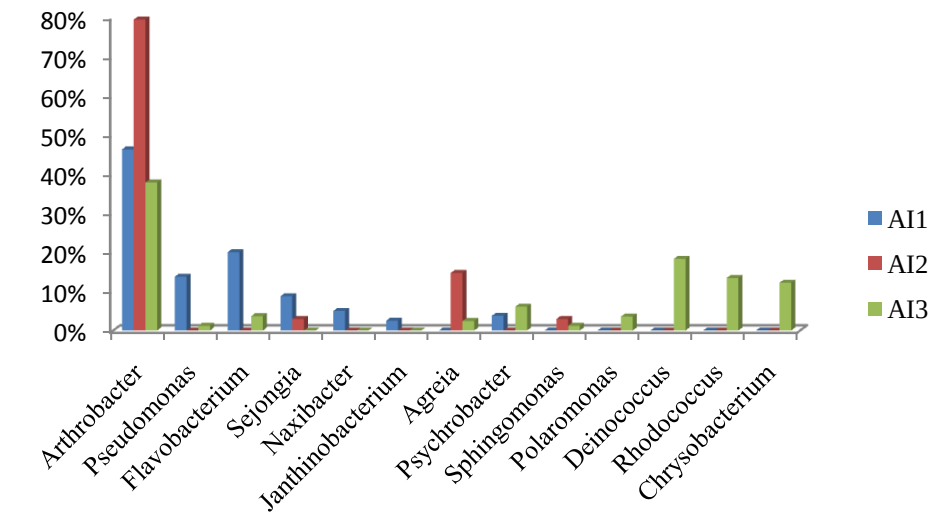
All isolates belonging to the alpha subdivision of Proteobacteria were from AI_2 and AI_3 (Fig. 4.11, 4.13 and 4.14) samples affiliated with the genus *Sphingomonas*. *Sphingomonas sp.Ens 34* with 100% sequence similarity was belonged to AI_2-27o samples and *Sphingomonas sp.Tianshan with 98%* sequence similarity was belonged to AI_3-69o.

The isolates affiliated with the beta-subdivision of Proteobacteria belonged to AI_1 and AI_3 samples (Fig. 4.11, 4.12 and 4.14). AI_1-49w and AI_1-19w were most closely related

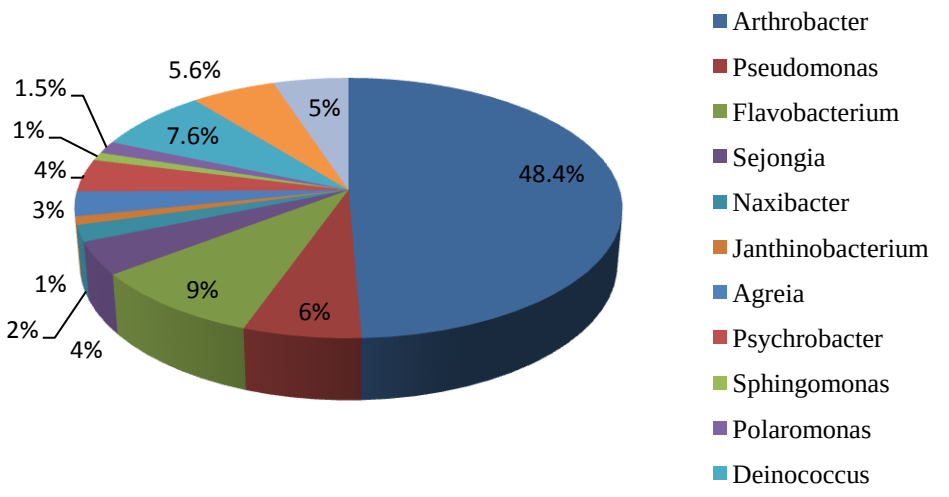
to *Polaromonas naphthalenivorans* and *Janthinobacterium agaricidamnosum*, with 98% sequence similarity. The remaining one, AI_1-1o, showed 97% sequence similarity to *Naxibacter indica.*

Isolates related to the gamma subdivision of Proteobacteria formed two subgroups supported by more than 97% sequence similarity. One subgroup was affiliated with two species of the genus *Psychrobacter*. AI_1-3w and AI_3-49w had 97% and 99% sequence similarity to *Psychrobacter namhaensis* and *Psychrobacter urativorans* respectively. The other subgroup, AI_1-29w and AI_3-57w were within the genus *Pseudomonas.* AI_1-29w and AI_3-57w showed 100% sequence similarity to *Pseudomonas migulae* and 99% similarity to *Pseudomonas fragi* respectively.

The last determined phylum was Deinococcus which only presented in AI_3 samples (Figs. 4.11, 4.14). The highest sequence similarity to *Deinococcus radiopugnan* with 97% was AI_3-52p showed. AI_3-40p, AI_3-78R and AI_3-37R showed 96% sequence similarity to *Deinococcus radiopugnans*, *Deinococcus aquatics* and *Deinococcus saxicola*, respectively. According to Fig. 4.10, AI_3 showed the highest diversity in genus and phylum meanwhile AI_2 the least diversity in this study.



(A)



(B)

Figure 4.10: Relative abundance of the various genuses found in three distinct soil sample sites in the Anchorage Island, Antarctica (A). Relative abundances of the various genus found in all soil sample sites in the Anchorage Island, Antarctica (B).

The isolates sequenced were compared with the data available in chromas using the sequence match tool, to determine the relative phylogenetic positions. In order to determine the relationship between four different bacterial phyla that were isolated, from all three soil samples, phylogenetic tree was constructed using Mega 4.software with bootstrap confidence estimates using the neighbor joining method.

From the tree constructed AI_2-8, AI_3-58, AI_1-32, AI_2-29, AI_1-23, AI_1-58 represented different genus of *Arthrobacter livingstonesis, Arthrobacter psycrolactophilus, Arthrobacter stackebradentii, Arthrobacter oxydanse, Arthrobacter sulfonivorans* and *Arthrobacter luteous* and AI2-7, AI_3-12 was represented by *Agreia prentesis* and AI_3-41, *Rhodococcus fascians,* derived from the same branch but different genus. All species are related to each other and are in the same family belong to Actinobacteria. Bacteroidets is closest phyla to Actinobacteria compare to other phyla represented by two different genus, *Sejongia antarctica, Sejongia marina* and *Flavobacterium hydatis, Flavobacterium saccharophillum* by AI_1-18, AI_3-30, AI_1-56 and AI_3-11 respectively. AI_2-27 and AI_3-69 *Sphingomonas* sp. Ens34, *Sphingomonas* sp.Tianshan, respectively showed by 5000 bootstrap values related to α-proteobacteria. ß-proteobacteria contains isolates, AI_3-19 *Polaromonas naphthalenivorans,* AI_1-1 *Naxibacter indica* and AI_1-49 *Janthinobacterium agaricidamnosum* constructed by bootstrap values of 5000. AI_1-29 *Pseudomonas migulae,* AI_3-57 *Pseudomonas fragi* AI1-3 *Psychrobacter namhaensis* AI3-49 *Psychrobacter urativorans* are represent by γ- Proteobacteria. This tree confirms the close connection between Actinobacteria, Bacterioidetes and Proteobacteria compare to Deinococcus-Thermus. This phyla that represented by AI_3-40, AI_3-78, AI_3-78 and AI_3-52 belong to diferrent species *Deinococcus radiopugnans, Deinococcus aquaticus, Deinococcus saxicola* and *Deinococcus radiopugnans,* respectively.

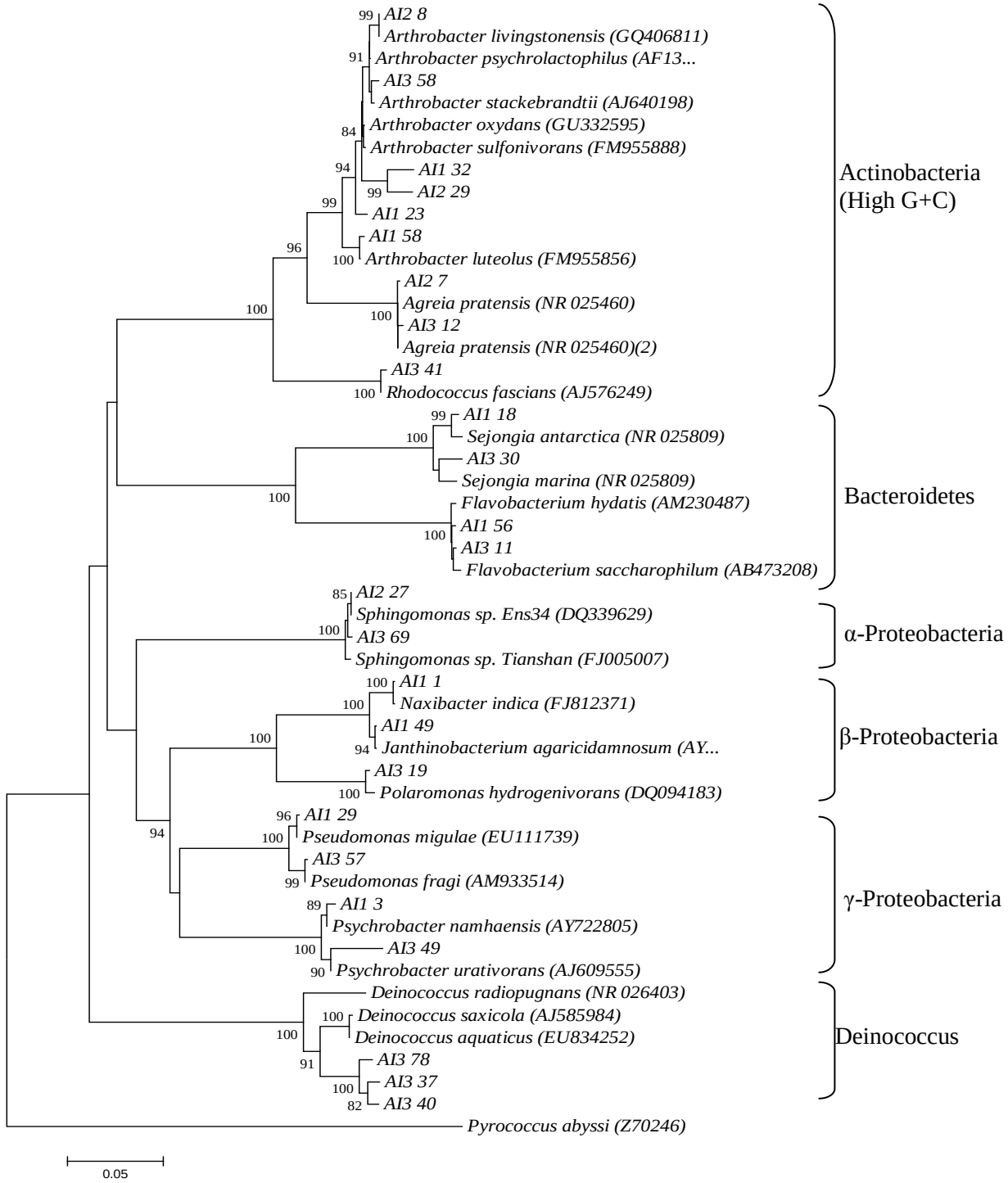


Figure 4.11: Phylogenetic relationship of the Antarctic isolates based on 16S rRNA gene homology. The tree was constructed using the neighbor- joining method with Clustal Wand pair wise deletion model analyses implemented in the program MEGA version 4.0.2. The resultant tree topologies were evaluated by bootstrap analysis based on 5,000 replicates. Numbers at nodes represent percentage levels of bootstrap support (%). Gen Bank accession numbers of 16S rRNA sequences are given in parentheses. *P.abyssi* (Z70246) was used as out group**.**

To confirm the obtained result of the sequences and high-throughput determination of accepted taxonomic affiliation, the recovered sequences were greatly facilitated by the RDP classifier tool (Cole *et al*., 2005) with 80% confidence threshold. RDP Classifier tool was greatly facilitated taxonomic affiliation of the recovered sequences and detailed results up to the family level. Besides that, randomly 50% of isolates check for chimeric using Pintail program. As we expected there was no chimeric in those sequences.

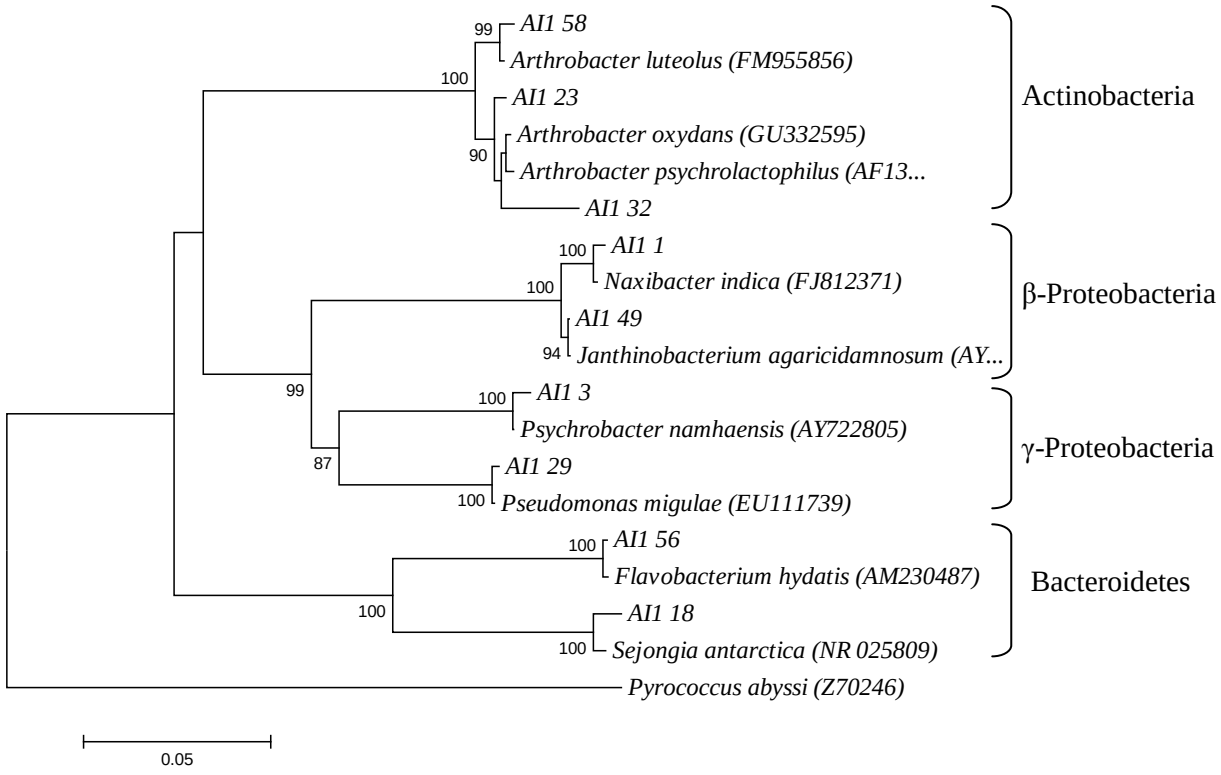


Figure 4.12: Neighbor-joining phylogenetic tree of partial 16S rDNA sequences of representative isolates affiliated with AI_1 isolates and their closest Phylogenetic relatives. Bootstrap values (n = 5000 replications) were indicated at nodes. *Pyrococcus abyssi* (Z70246) was used as out group.

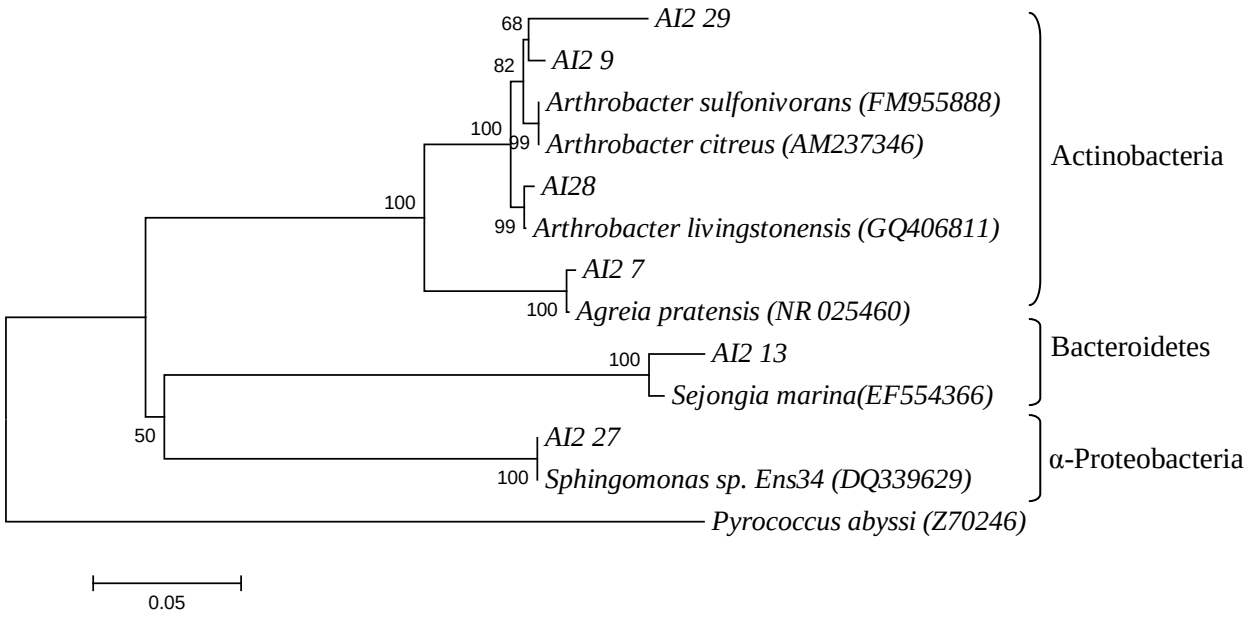


Figure 4.13: Neighbor-joining phylogenetic tree of partial 16S rDNA sequences of representative isolates affiliated with AI_2 isolates and their closest Phylogenetic relatives. Bootstrap values (n = 5000 replications) were indicated at nodes. *Pyrococcus abyssi* (Z70246) was used as out group.

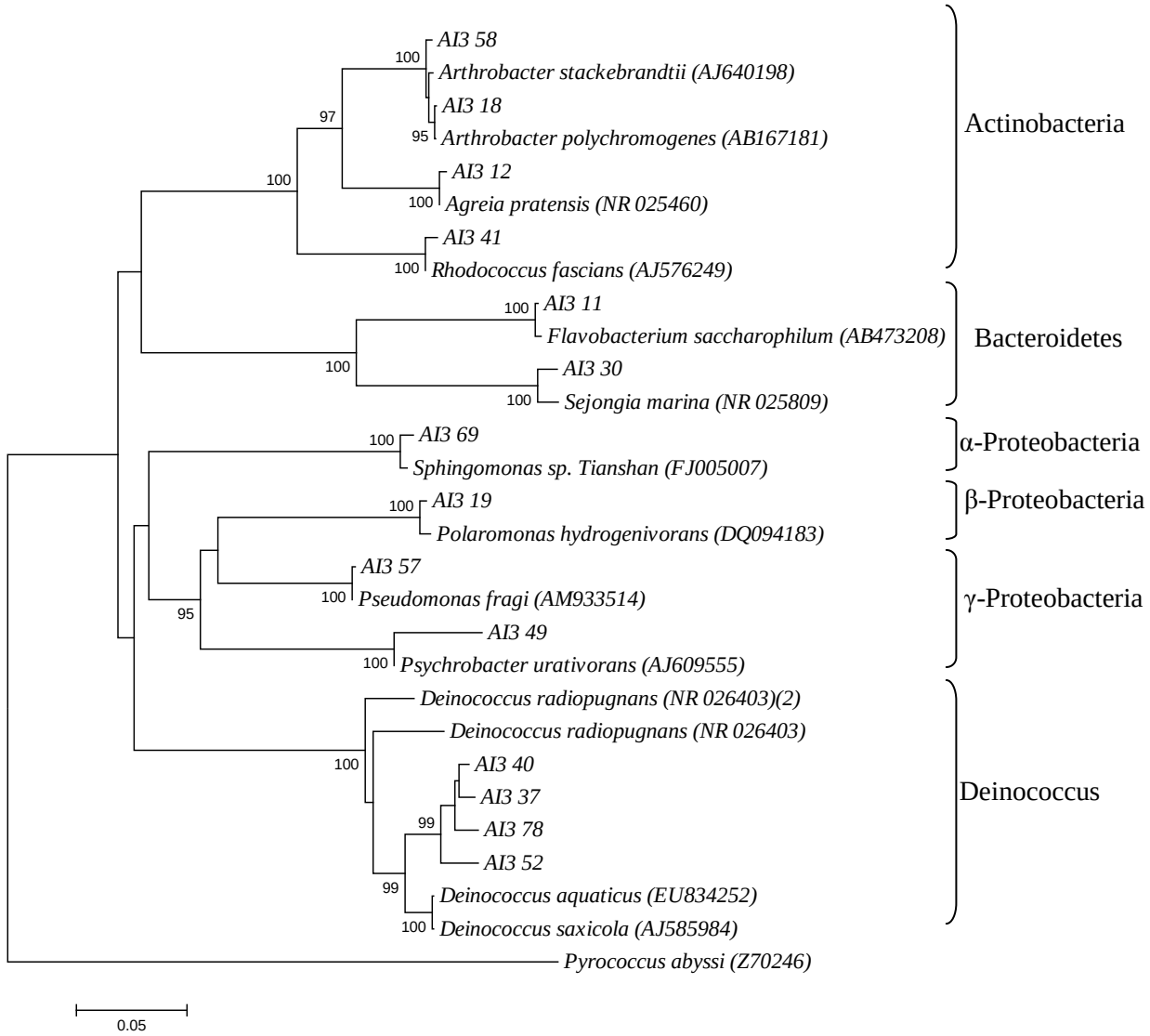


Figure 4.14: Neighbor-joining phylogenetic tree of partial 16S rDNA sequences of representative isolates affiliated with AI_3 isolates and their closest Phylogenetic relatives. Bootstrap values (n = 5000 replications) were indicated at nodes. *Pyrococcus abyssi* (Z70246) was used as out group.

CHAPTER 5.0: DISCUSSION

5.1 Methods used for assessment of bacterial diversity

Anchorage Island in Antarctica is underexplored niche for microbes and microbiological analyses of this Island are very scarce. Previous reports on this Island had mainly focused on the comparison of this island with other places in Antarctica.

It should be noted that, like all other methods, the method of analysis of bacterial communities by plating with the subsequent differential count of grown colonies and identification of dominant bacteria to the level of groups or genera has some shortcomings and limitations. For instance, all or at least most of soil compartments should be measured or/ and a variety of media need to be utilized to gain suitable analysis of soil microbial diversity.

Generally, it is difficult to compare the result observed in this study with those observed in other studies on similar environments because of the scarcity of reports on culturable microbial communities in Anchorage Island. Besides, our sampling was in summer only. Since, growing season in Antarctic is limited to summer month only (Peck *et al.*, 2006), thus, our data refer to the period when the vegetation is most active. The reason why we were unable to isolate more than these phyla is, one type of media under aerobic condition was used, and no single technique can be expected to cultivate majority of the physiological diversity within the samples. In addition, specific ecological knowledge for most species is absent, including their specific functional roles among the extremely diverse bacterial community in penguin guano in Antarctica.

Soils contain a great diversity of bacteria, while many of them belong to groups known as un-culturable. It has been estimated that less than 0.1% of the total microbial population can be successfully isolated in pure culture (Hugenholtz and Pace, 1996; Mclnerney *et al*., 2001; Fuhrman and Ouverney, 1998). Furthermore, study of

prokaryotic biodiversity has been delayed for many years due to the difficulty of assessing true diversity by culture dependent methods. It has been reported that plate count methodologies are not suitable for cultivation of soil bacteria. Identification of a suitable isolation method that optimizes the recovery of genetically diverse bacterial lineages can helpful to better understand the culturable microbial community in soil. It has been proved that use of serial dilution techniques can be effective for isolating soil bacteria (Janssen *et al.,* 2002).

5.2 Type of culture medium

Successful enrichment, isolation and cultivation of prokaryotes mostly depend on the choice of appropriate growth media and incubation conditions. Culture dependent methods such as cell count were most used in the early studies of Antarctic microbial ecology to determine the presence and abundance of species. Although, difference by 100–1000 times in bacterial population between direct count and population in soil communities can be expected frequently, species reported in these studies were found to be viable only under laboratory conditions.

The aim of this study was to use media and conditions of incubation which give the maximum possible counts of soil bacteria capable of growth under aerobic conditions.

To cultivate soil bacteria on plates a variety of media have been developed. Some media were chosen for nutritional requirement of major groups of specific bacteria whereas others were intended to satisfy the requirements of major groups of soil bacteria. According to many pre-experiments and the principal characteristics of Antarctic soil, we chose low concentrations of nutrient agar medium for isolation and it supported the growth of different species of bacteria from Antarctic soils. Since our seniors could grow many type of Antarctic soil bacteria on 1/10 strength of nutrient agar medium, this low strength of nutrient agar was used. Previous studies (Overmann, 2006; Bai *et al.*, 2006) showed, nutrient agar diluted by 100-fold generated higher number of colonies

compared with full strength nutrient medium. Besides, low strength nutrient agar media had been established for the isolation of fastidious bacteria as well as enhanced Acidobacteria, Actinobacteria, pseudomonads, Proteobacteria, Low G+C Grampositive bacteria, and Verrucomicrobia from soil samples (Overmann, 2006; Bai *et al.,* 2006).

On the other hand, previous reports indicated that no single medium will allow growth of all types of prokaryotes and employing different types of media could significantly increase the efficiency of plate counting instead of a single medium (Dobrovol'skaya *et al*., 2001). As mentioned in chapter four from AI_1 samples 80 colonies, $AI_{2,}$ 34 colonies almost all colonies on all sample plates and $AI_{3,}$ 82 colonies were picked and purified. Bacteria are highly selective in their growth requirements; nutrient agar may not have all requirements for bacteria. On the other hand, water availability is one of the important factors to bacterial growth, in this situation; excess water allows a high level of connectivity and plenty of opportunity for the few species that adapted to local condition and most bacterial species are eliminated by competitive exclusion (Aislabie *et al.,* 2009).

5.3 PCR and RFLP

Many procedures in molecular biology require the isolation of high quality genomic DNA. In diversity studies, differential amplification of 16S rRNA gene can affect the PCR results. For instance, by decreasing G+C content in DNA sequences sequence the efficiency of separation in the denaturing step of PCR enhance, therefore these sequence amplified more (Malik *et al*., 2008). A few problems may lead to PCR biases are: small volume of template and implementing the proper annealing temperature (Ishii and Fukui, 2001). Tusnady and colleagues (2005) suggested that implementing elevated annealing temperature during the PCR should result in more specific and efficient amplification. However, according to other research (Qiu *et al*., 2001), PCR biases can be minimized by careful planning and controlling experimental condition.

Isolates recovered from the soil samples were identified by 16S rRNA gene sequencing and RFLP in order to examine the genetic diversity of culturable bacteria isolated from these soils. If only distinct bacterial phenotypes from each sample were studied it was possible a larger number of genetically distinct strains, which are morphologically similar, may be missed. That's why we classified isolates based on different RFLP patterns and their distinct morphologies.

Based on such patterns, each isolate is identified in terms of its nearest, previously-characterized relative. Although most phenotypically and distinct RFLP patterns have different sequences result, some with different pigments from colonies with different morphologies have been found to have identical sequences. For example, one RFLP pattern was represented two different phyla of bacteria, e.g. pattern H associated with *Actinobacteri*a and *proteobacteria* (Table 4.5) which is one of the shortcomings of RFLP analysis.

Digestion of PCR amplicons with restriction enzymes followed by electrophoretic separation allows determination of characteristic restriction fragment length polymorphism (RFLP) patterns. Variations in the resulting 16S rDNA-RFLP are able to represent differences in microbial community structure along with proportions of individual populations. Although this PCR-RFLP technique, can be biased by several factors like, distinct gene copy number, different GC content, the annealing temperature, and primer homologies, the number of cycles of replication and the interspecies operon heterogeneities. Thus, in the present study similar method was used to assess microbial diversity (Ramirez-Moreno *et al.,* 2004). Despite the fact that using this method, large amount of PCR product is required and there are low levels of polymorphism between some species. This method is useful for detecting structural changes in microbial communities but not as a measure of diversity or detection of specific phylogenetic groups by banding patterns in diverse communities become too

complex to analyze using RFLP since a single species could have four to six restriction fragments. The best to use in the search for RFLPs can be chosen by mainly considering the rate at which RFLPs can be detected based on the enzyme chosen. Another consideration is whether the enzyme is optimally active with genomic DNA obtained from the bacteria and cost is also an important consideration. Perhaps by using a six-base cutting enzyme, the number of restriction fragments per species could be reduced, thereby increasing the resolution of this method. In addition the choice of restriction enzymes is crucial for obtaining optimal resolution of rRNA gene fragment lengths. Preliminary test on enzyme(s) of choice must be conducted to ensure that optimal resolution is obtained in detecting shifts in microbial communities (Ranjard *et al*., 2000).Different RFLP pattern can be viewed after application of restriction enzymes. Since the scoring and the degree of cleavage site of restriction enzymes are vital to settle these enzyme (Yang *et al*., 2007), candidate restriction enzyme may not sufficient. Besides, by using one enzyme all species could not be distinguished. In this case, NEB Cutter version 2.0 software was used to compare the RFLP experimental design. This program has been designed to search for the recognition site of endonuclease, to locate the position of these sites in the sequences and to calculate the size of fragments after digestion. In some cases results were different from visualized RFLP pattern as shown in gel electrophoresis. Generally, the unequal densities of DNA in the bands reproduce the different sizes of the fragments after digestion. Brightness and size of each fragment has direct impact on each other (Yang *et al*., 2007). It means smaller fragment appears fainter band under UV illumination after ethidium bromide staining. In addition, voltage fluctuation in running gel may cause shift of distance between bands which occur false different band. In gel picture, the lowest band sizes visualized were about at 200 bp, NEB cutter software shows all band sizes even less than 100 bp. Although, NEB cutter version 2.0 as existing design tools for the PCR-RFLP assay, has been used to search

for cleavage sites and to calculate the sizes of restriction fragments, the selection of restriction endonucleases by manual comparison of restriction fragment profiles of different sequences with various restriction enzymes is still very time consuming. Furthermore, by comparing the experimental results, the software analysis demonstrated high reliability of the RFLP fingerprints except few object.

With respect to the proximity of one site to another there was not obvious difference in microbial community at three different sites in Anchorage Island. In addition, substantial overlap was observed across sites visibly affected by collection was from the same Island.

5.4 Phylogenetic analysis of 16s rRNA gene sequencing of isolates

According to our results, the isolated bacteria fell in four phylogenetic groups: Actinobacteria, Bacteroidetes, Proteobacteria (α, ß and γ-proteobacteria) and Deinococcus. Some of them have previously been found while Firmicutes seems to be common cultarable bacteria in Antarctic soil (Aislabie *et al.,* 2006; Aislabie *et al.,* 2008; Aislabie *et al.,* 2009; Selbmann *et al.,* 2010; Pearce *et al.,* 2003) but was not detected in this study. Deinococcus phyla exclusively found in AI_3 isolates. The finding of the genus *Deinococcus*, exclusively in AI_3 samples was remarkable. Deinococcus were previously obtained from Antarctic lichens, McMurdo Dry Valley's soil and Antarctic samples of snow (Selbmann *et al.,* 2010).

Our recent knowledge reveals that most Antarctic environments contain a diverse range of prokaryotes. So, large data sets of isolates are needed for cultivation based studies. In cold environments isolates related to the alpha, beta and gamma of *Proteobacteria* are typical and dominant group by cultivation methods (Bai *et al.,* 2006). Although in our study the Gram-positives were diverse and predominant in culturable populations. A high proportion of members of the Actinobacteria were found at all sample sites. This

phylum is one of the largest phyla among gram-positive bacteria with high G+C content in their DNA. Actinobacteria members can adapt themselves to different conditions. They can have different morphologies, from cocci to fragmenting hyphal forms, and highly variable physiological and metabolic characteristic (Ventura *et al*., 2007).

Within the Gram-positives group, the genus *Arhtrobacter* formed the largest group in terms of diversity and high abundance and extremely numerous in soils of all three sites isolates especially in AI_2. Many studies have shown that *Arthrobacter* are numerically predominant native bacterial flora of soils as well as many low temperature environments, such as alpine ice caves, Antarctica ice shells, Siberian permafrost and alpine permafrost environment of Tianshan (Hagedorn and Holt, 1975; Bai *et al.*, 2006), suggests that their extreme resistance to drying factors and nutritional versatility play an important role to survive these microorganisms in soil (Jones and Keddie, 2006). Members of the genus *Arthrobacter* were originally described as being highly aerobic, nutritionally nonexacting and capable of liquefying gelatin slowly and may be important agents of mineralization in soil. This cultivable population show as Gram-negative rods in fresh cultures and Gram-positive cocci in older cultures (Jones and Keddie, 2006). *Rhodococcu*s and *Agreia* are other genera related to Actinobacteria phyla which were absent in AI_1 isolates. *Agreia* was isolated from AI_3 and AI_2 samples and *Rhodococcus* was found in AI_3 exclusively. *Rhodococcus* is psychrotolerant, gram-positive bacteria with hydrocarbon-degrading ability and massive catabolic versatility that seems to be an interesting target for developing bioremediation in cold environments (Giudice *et al.*, 2005). *Sejongia* and *Flavobacteria* from Bacteroidetes phyla were common in these three site isolates. *Flavobacterium* and *Sejongia* are yellow-pigmented, gram-negative and aerobic bacterial strains that are widely distributed in soil and freshwater habitats and isolated from temperate to polar low salinity ecosystems. Isolated *Flavobacterium*

from cold environment have biotechnological potential to produce cold-active enzymes (Bernardet and Bowman, 2006; Yi *et al.,* 2006).

All three sites also contained a number of isolates belonging to Proteobacteria. This phylum represents the largest and phenotypically most diverse phylogenetic lineage which scattered over five major Phylogenetic classes such as α-proteobacteria, ß-proteobacteria, γ-proteobacteria, Deltaproteobacteria and Epsilonproteobacteria. Between these sub phyla, some genera of α-proteobacteria, ß-proteobacteria and γ-proteobacteria were isolated among all samples. Sphingomonas genus of Alphaproteobacteria was isolated from the AI_2 and AI_3 sites capable of producing aromatic compounds important in biotechnological application (Kersters *et al.,* 2006). Another group that has biodegradation properties is members of the "Betaproteobacteria". In this phylum gram-negative isolates of *Janthinobacterium* and *Naxibacter* genus were found in AI_1 soil and *Polaromonas* genus was isolated in AI_3 samples. Like Beta-subclass of Proteobacteria, *Pseudomonas* and *Psycobacter* genera from Gammaproteobacteria were originated in AI_1 and AI_3 isolates. *Psychrobacter* species were prevalent, indicating their fitness for guano decomposition which is present in AI3 site due to exhibit of penguins (Aislabie *et al.,* 2009).

The fourth and exclusive found phylum among all three sites is Deinococcus. *Deinococcus* is gram-positive bacteria found to form pink or reddish colored colonies. Members of the *Deinococcaceae* are famously known for their ability to survive and limit the DNA damage due to exposition to extremely large amounts of ionizing radiations and extremes cold temperature and desiccation (Selbmann *et al.,* 2010). Such property to withstand to radiation is an advantage to *Deinococcus* to live through such intense blasts of radiation. From a Phylogenetic point of view, our *Deinococcus* isolates are distantly related with the others already reported from the Antarctic. In a separate comparison, the sequences of these strains appeared to be very distinct from all the

others, including ours. This is hypothesized that, soil moisture of AI_3 soil is high in summer; soil moisture levels were conducive for bacterial activity (Aislabie *et al.*, 2006). According to Figure 5.2 AI_3 showed most diverse site in genus and phylum meanwhile AI_2 illustrated the least diversity in this study as well as Isolates from AI_1 and AI_3 are most similar compare to AI_2. It is proved by nucleotide distance obtained from MEGA (version4) in different sites which is 0.299 in AI_1, 0.144 AI_2 and 0.246 in AI_3.As it mentioned before AI_3 soil has high nutrient and highest soil and air temperature as well as presence of penguin compare with other two sites. According to other research highest water content, carbon and nitrogen content were recorded from penguin rookeries (Chong *et al*., 2010).

It is obvious that, the presence of suitable substrate may be of greater significance than climatic conditions in favor of the presence and/or diversity of soil microorganisms in soil as well as combined effects of greater nutrient availability and more favorable physical conditions, these microhabitats had higher diversity. As a broad generalization, low temperatures and severe water deficiency are two of the main limiting factors for life in the ice-free areas of the Antarctic continent (Selbmann *et al.,* 2010), and increases in temperature and water availability have led to positive responses in native Antarctic biota Convey, 2003). Therefore, water availability, temperature, rapid thermal fluctuations and species diversity are closely related in the Antarctic but it should be mentioned that our estimates of air and soil temperature were necessarily limited at the sampling date.

Although, water content in AI_1 samples are significantly lower than AI_2 and AI_3 samples, this parameter was not found to be well correlated with abundance, distribution, or the types of microorganisms found in these area. In addition, most mineral and nutrient input in the Antarctic is thought to come from the marine ecosystem via wind blast that dispersed mineral in soils equally.

CHAPTER 6.0: CONCLUSION

The results of the present study provide an examination of bacterial isolation and diversity across three sites in Anchorage Island of Antarctic. Bacterial diversity was shown to be almost the same between different locations. A total of 196 bacteria were isolated from all 3 samples using nutrient agar medium. 16S rRNA gene of all isolates obtained by colony PCR, DNAzol reagent and purification kit were subjected to RFLP analysis.

80 isolates of AI_1 samples fell into Actinobacteria, Bacteroidetes and Proteobacteria (ß and γ-Proteobacteria). These isolate affiliated with the genera of *Pseudomonas, Psychrobacter, Naxibacter, Janthinobacterium, Arthrobacter, Sejongia* and *Flavobacterium.* Approximately 50% of isolates are belonging to Actinobacteria and another 50% are divided between other phyla.

34 isolates of AI_2 like AI_I fell into Actinobacteria that contain 98% of isolate, Bacteroidetes and Proteobacteria (α-Proteobacteria) phyla which affiliated with the genera of *Arthrobacter, Agreia, Sejongia* and *Sphingomonas.*

82 isolates of AI_3 samples fell into Actinobacteria, Bacteroidetes, Proteobacteria (α, ß, and γ-Proteobacteria) and Deinococcus phyla. These isolates affiliated with genera of *Arthrobacter, Rhodococcus, Sejongia, Flavobacterium, Polaromonas, Pseudomonas, Psychrobacter, Sphingomonas* and *Deinococcus.* Near 60% of isolate belong to Actinobacteria while amount of Bacteroidetes and Deinococcus were equal (15%) and least amount belong to Proteobacteria.

In summary, based on the result diversity of culturable bacteria in three different site samples from Anchorage Island were the same.

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