Isolation, identification and characterization of novel actinomycetes from Antarctic soil samples

By

Natasha R Mavengere

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Supervisor: Prof. D.A. Cowan

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Abstract

Actinomycetes are Gram-positive bacteria that form filamentous branching hyphae with asexual spores. They occur in a variety of environments and are important industrially and pharmaceutically. The aims of this study were to isolate and identify novel actinomycetes from Antarctic soil samples and screen these isolates for the production of secondary metabolites, especially antibiotics. Actinomycetes were successfully isolated from Antarctic Dry Valley soils and optimum growth conditions were determined by investigating the effects of different temperatures and growth media on growth rates. Using phylogenetic (16S rRNA gene sequence), physiological, morphological and biochemical analyses, seven different actinomycete strains were identified, one of which was potentially novel. Isolates were screened for the production of antimicrobial compounds using the disc diffusion and stab culture methods. Antibiotic screening showed that one isolate had antimicrobial activity against eight of the ten bacterial strains tested. Another isolate was observed to inhibit the growth of some contaminating fungi. This observation implied the need to screen isolates for antifungal activity in further studies. Thin layer and high performance liquid chromatography were used in an attempt to characterize the antibiotics.

DECLARATION

I declare that **Isolation, identification and characterization of novel actinomycetes from Antarctic soil samples** is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Natasha R Mavengere

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List of abbreviations

ATCC	American Type Culture Collection
bp	Base pair
BLAST	Basic Local Alignment Search Tool
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide tri-phosphates
°C	Degrees Celsius
EDTA	Ethylendiaminetetraacetatic acid
× g	Centrifugal force
g	Gram
GC	Guanosine-cytosine
GeneBank	Nucleotide sequence database
IPTG	Isopropyl- D-thiogalactoside
kb	Kilobase
LB	Luria Bertani
mg	Microgram
ml	Milliliter
µl	Microliter

NCBI	National Center for Biotechnology Information
ng	Nanogram
O.D	Optical Density
Ω	Ohm
PCR	Polymerase Chain Reaction
PVPP	Polyvinylpolypyrrolidone
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium Dodecyl Sulfate
sp.	Species (single)
spp.	Species (plural)
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
U	Units
v/w	Volume per weight
w/w	Weight per weight
X-Gal	5-Bromo-4-Chloro-3-indolyl- -D-galactopyranoside

Chapter 1: Introduction

1.1 Antarctica

Antarctica is the southern-most continent and overlies the South Pole. It is situated in the southern hemisphere and is surrounded by the southern waters of the world oceans. Antarctica covers a region of approximately 14 million km^2, most of which is covered by ice and snow (Russell, 2006). It is the coldest, driest and windiest continent. Approximately 98% of Antarctica is covered by the Antarctic ice sheet, which is a minimum of 1.6km thick. During the coldest winter month (September) the ice sheet may cover an area of more than 20 million km^2, but by midsummer (February) the ice sheet is usually reduced to less than 4 million km^2 (Vishniac, 1993).

The main factors that influence the Antarctic climate are the waters of the Southern Ocean, the seasonal variations of the sea ice, the ice sheet itself and the high latitude and altitude of the continent (Schlatter, 1972). In winter, during periods of continuous darkness, the temperatures may drop as low as -60°C and the wind speeds of up to 100km/h have been recorded. During summer, the average daily temperatures range from -35°C to +3°C (de la Torre *et al.*, 2003). Although the summer daily totals of incident solar radiation are the greatest observed anywhere on earth, Antarctica stills remains the coldest continent (Schlatter, 1972). Similar to all other environments, temperatures are not uniform over the continent and there are distinct environmental niches which have varying temperatures, soil types, sediments, rocks and melt waters. The unique and diverse terrestrial and aquatic habitats found in Antarctica each with

varying climate conditions, impact directly on the diversity of life forms that exist. Antarctica is physically and physiologically demanding but is not devoid of life (Vishniac, 1993).

1.1.1 The Dry Valley Terrestrial environment

The McMurdo Dry Valleys of Eastern Antarctica are located in South Victoria Land and are areas of exposed soil and considered to be the coldest driest deserts on Earth (Cowan *et al.*, 2007). They are one of the largest ice free regions of the Antarctic continent (Fritsen *et al.*, 1998). The climate of the Dry Valley is characterized by extreme cold and dryness. The poorly developed soils have a coarse texture (Virginia and Wall, 1999). These soils are unique amongst desert soils in having large amounts of soluble salts, a high pH and permafrost at 10-30cm depth (de la Torre *et al.*, 2003). The atmospheric water content of the Dry Valleys is low which results from low precipitation and atmospheric humidity. The soil water activity is therefore low and further reduced by very high levels of salinity (Cowan *et al.*, 2002). Organic accumulation of carbon and nitrogen is low and is a limiting factor in the development of microbial populations in the Dry Valley soils (Cowan *et al.*, 2007)

1.1.2 Antarctic Life Forms

Organisms that thrive in and may even require extreme conditions that are detrimental to the majority of life on Earth are known as extremophiles. There are many different classes of extremophiles, each corresponding to the way their environmental niche differs from mesophilic conditions (Niehaus *et al.*, 1999).

Psychrophiles are extremophilic organisms which are capable of growth and reproduction in cold temperatures. There are two groups of psychrophiles; obligate and facultative psychrophiles. Obligate psychrophiles were once defined as the true psychrophiles. They have a growth temperature optimum of 15°C or lower and are unable to grow at temperatures greater than 20°C. They are largely found in cold regions (such as in Antarctica) and on the ocean floor. Facultative psychrophiles can grow at temperatures ranging from 0°C to 40°C and are therefore more common in isolation experiments than obligate psychrophiles (Stolp, 1988). Although facultative psychrophiles are generally not able to grow at temperatures below 0°C, they can maintain basic cellular functioning at these temperatures. Facultative psychrophiles have evolved to tolerate cold, but they are not as physiologically specialized as obligate psychrophiles and as such are usually not found in the coldest habitats. Such organisms are also known as psychrotolerant (Morita, 1975).

The survival of psychrophilic microorganisms in cold environments is the result of unique features of their proteins and membranes and specialized responses to the extreme temperatures (Deming, 2002). Their membranes have altered fatty acid constituents and distribution in order to maintain fluidity. This is critical for the transport of substrates and nutrients at conditions which are otherwise rigidifying (Russell, 1997). Psychrophiles induce the production of cold-shock proteins which enable the continuation of metabolic activities as the environmental temperature drops (Deming, 2002).

The extreme environmental conditions characteristic of the Antarctic greatly lower the diversity of organisms that are capable of surviving there. The greatest diversity of species is found along the western side of the Antarctic Peninsula where the climate is generally warmer and wetter than elsewhere in Antarctica (Kennedy, 1993). These life forms have evolved through natural selection to be able to occupy habitats that offer protection from the desiccating conditions (de la Torre *et al.,* 2003). Soil communities in Antarctica are very different from those of other terrestrial ecosystems because they lack higher plants, have simple soil communities with low species diversity, and are almost completely isolated from human influence (Virginia and Wall, 1999).

The Antarctic flora is composed of approximately 380 lichen species, 130 species of bryophytes and some fungi (Alberdi *et al.,* 2002). Only two native vascular plants, the Antarctic hair grass *Deschampsia Antarctica,* and the cushion-forming pearlwort *Colobanthus quitensis*, have been recorded. They occur in isolated clumps near the shore of the west coast of the Antarctic peninsula (van de Wouw *et al.,* 2007). Land fauna is dominated by invertebrates which live in the soil and vegetation. They range from protozoa, rotifers, tardigrades and nematodes to arthropods (mainly mites and springtails). The largest terrestrial invertebrate is the wingless midge, *Belgica antarctica*, which grows to 12 mm in length (Freckman and Virginia, 1998).

Microorganisms are the most abundant life forms in Antarctica and a wide diversity of cold adapted microorganisms including bacteria, archaea, yeast and microalgae have been isolated (Russell, 2006). Prokaryotes dominate many Antarctic ecosystems where they play a major role in the food chains, biogeochemical cycling of nutrients, and the mineralization of pollutants

(Nichols *et al.,* 1999). Light and scanning electron microscopy has revealed that a variety of bacterial morphological types including rods, cocci, straight and branching filamentous, fusiform, and prostecate bacteria can be found (Vincent *et al.,* 1993). The use of effective culture dependent isolation techniques has been shown to yield relatively high microbial numbers (Cowan *et al.,* 2005). More recent studies have used culture independent techniques such as 16S rRNA gene amplification by PCR to provide a more complete record of the microbial community structure of the Antarctic Dry Valleys (Cowan et al., 2007).

1.2 Actinomycetes

Actinomycetes are filamentous, Gram positive bacteria that form branching filaments with hyphae and asexual spores. They were originally incorrectly classified as fungi because they possess true aerial hyphae and form spores, both of which are considered to be fungal characteristics (Lechevalier and Lechevalier, 1967). Actinomycetes are defined as bacteria that have the ability to form branching hyphae at some stage of their development (Goodfellow and Williams, 1983). They are characterized by having a high G+C content in their DNA (Stackebrandt and Goebel, 1994).

1.2.1 Occurrence of Actinomycetes.

Actinomycetes are primarily found in terrestrial habitats but they are widely distributed in a variety of other habitats including compost, river mud, and lake bottoms (Alexander, 1977). They are among the most widely distributed microorganisms in nature and constitute a significant

component of the microbial populations in most soils (Barakate *et al.*, 2002). Actinomycetes are found in cultivated and uncultivated soils (Goodfellow and Simpson, 1987). They play a major role in the carbon cycle due to their ability to grow at low concentrations of carbon and to degrade recalcitrant organic compounds (Terkina *et al.*, 2002).

Actinomycetes are also found in aquatic environments. Some indigenous marine actinomycetes like *Rhodococcus marinonascens* and *Salinispora* spp. have been identified (Helmke & Weyland, 1984, Mincer *et al.*, 2002, Maldonado *et al.*, 2005, Pathom-aree *et al.*, 2006). It has been argued that the actinomycetes isolated from water are of terrestrial origin as these bacteria can produce resistant spores that may be transported from land to sea and remain as dormant but viable spores for many years (Mincer *et al.*, 2002). *Salinispora* represent the first taxon to be reported exlusively from the ocean. The studies also suggested a world wide distribution of these bacteria in the oceans (Mincer *et al.*, 2002, Maldonado *et al.*, 2005).

Actinomycetes are also widespread in the lacustrine environment. Some studies showed that very low numbers of actinomycetes occurred in the water column but high numbers could be recovered from lake sediments (Johnson and Cross, 1976). Other studies have concluded that actinomycetes make up a small proportion of the bacterial flora in marine habitats and their numbers are much lower than in terrestrial environments (Takizawa *et al.*, 1993).

Most actinomycetes are free living but a few are opportunistic pathogens to humans, animals, and plants. Pathogenic species include *Actinomyces israelii,* a primary cause of tooth decay in humans (Zaitlin and Watson, 2006). Nocardiosis is a serious infectious disease affecting either

the lungs (pulmonary nocardiosis) or the whole body (systemic nocardiosis). It is commonly caused by *Nocardia asteroides* or *Nocardia brasiliensis*. Fermentative actinomycetes of the genera *Actinomyces, Propionibacterium* and *Bifidobacterium* have also been shown to cause inflammations refered to as actinomycoses (Zaitlin and Watson, 2006).

1.2.2 Antarctic Actinomycetes

Actinomycetes have been isolated from a number of Antarctic environments (Cowan *et al.,* 2007). Novel actinomycete strains which include *Friedmanniella antarctica* (Schumann *et al.,* 1997) and *Micromonospora endolithica* (Hirsch *et al.,* 2004), both isolated from sandstone rock samples, as well as *Pseudonocardia antarctica* sp. nov. (Prabahar *et al.,* 2004) have been characterized from Antarctica.

The actinomycetes *Streptomyces flavis* and *Microbispora aerata* were isolated from Antarctica and were interestingly shown to grow on sheep wool. This was the first report of Antarctic actinomycetes that produced keratinolytic enzymes (Gushterova *et al.,* 2005). *Streptomyces cyaneus, Streptomyces tendae* and *Streptomyces caelestis*, which have been identified as potent xylanase producers, have also been isolated from Antarctic soil samples (Ninawe *et al.,* 2006).

Actinomycetes that produce antibiotics have also been isolated from Antarctica. In one study, 47 actinomyces strains were isolated from Antarctic soils and 19 were shown to have antagonistic activity against Gram-positive and Gram-negative bacteria. Six strains possessed a broad spectrum of antibacterial activities (Moncheva e*t al.,* 2002). A study of the antibacterial activity

of actinomycete isolates from Antarctic soils by Nedialkova and Naidenova (2004) showed that out of the forty actinomycete strains tested, ten were found to have a broad spectrum of antibacterial activity and could potentially be used in the development of new compounds for pharmaceutical or agricultural purposes.

1.2.3 Actinomycete taxonomy

Taxonomy is the science of the classification of living organisms. There are three important reasons for classifying organisms. Firstly, classification is a form of database or information retrieval system containing a large amount of information about an organism. Secondly, classification is important because organisms must be categorized into groups before identification systems can be created for new isolates. Thirdly, classification systems may provide an insight into the origins and evolutionary pathways of organisms (Priest and Austin, 1995). A number of different methods have been used to classify actinomycetes. These include morphological, biochemical and genomic methods. The taxonomy of actinomycetes is, however, still evolving and the taxonomic status of many taxa is currently being re-evaluated (Chiba *et al.*, 1999).

1.2.3.1 Morphological and physiological methods

Actinomycetes have a wide range of morphologies, many of which can be used in classification. Actinomycete taxonomy was traditionally based on morphology and some of the characteristics most considered included the size, shape and colour of colonies on specific media. Gram's stain,

acid fastness, odor and pigment production are also used when classifying using morphology. Other morphological features that are taxonomically important include the colour, morphology and surface arrangement of conidiospores (Shirling & Gottlieb, 1966). These techniques are more accurate on samples that have been freshly isolated. Physiological attributes such as nutritional requirements, fermentation products and growth conditions (oxygen, temperature and inhibitory products) are also important when classifying actinomycetes (Ciantar *et al.*, 2005).

1.2.3.2 Chemotaxonomy

Chemotaxonomy refers to grouping organisms according to their cell chemistry and includes analysis of cell wall constituents, membranes and quinones (Zaitlin and Watson, 2006). Cell wall constituents are a major characteristic used in chemotaxonomy. The composition of cell walls varies greatly among different groups of actinomycetes. The presence of diaminopimelic acid (DAP) isomers is one of the most important cell wall properties of the Gram positive bacteria including the actinomycetes. 2, 6-DAP is widely distributed as a key amino acid and it has optical isomers (Sasaki *et al.*, 1998). Bacteria generally contain either the LL isomer or the meso isomer, mostly located in the peptidoglycan. Four cell wall types based on three major features of peptidoglycan composition and structure can be distinguished in actinomycetes: i) the amino acid in tetrapeptide side chain position 3 ii) the presence of glycine in interpeptide bridges and iii) the peptidoglycan sugar content (Lechevalier and Lechevalier, 1970). Table 1 summarises the four cell wall types.

Table 1: Major constituents of the four cell wall types of actinomycetes

Cell wall type	DAP		Glycine	Arabinose	Galactose
	meso	LL			
I	–	+	+	–	–
II	+	–	+	–	–
III	+	–	–	–	–
IV	+	–	–	+	+

(Lechevalier and Lechevalier, 1970)

Chemotaxonomy also involves the analysis of other macromolecules such as the isoprenoid quinones (menaquinones and ubiquinones), lipids (lipopolysaccharides and fatty acids including mycolic acids), polysaccharides and related polymers (methanochondruitin and wall sugars) and proteins (bacteriochlorophylls, whole organism protein patterns and enzymes) (Ward and Goodfellow, 2004). Although chemotaxonomy is still considered useful in actinomycete taxonomy, it is not always reliable as several genera may exhibit similar chemical properties. For example, members of *Nonomuria, Microbispora, Microtetraspora* and *Actinomadura* species were found to be very difficult to classify using chemotaxonomy (Wang *et al.,* 1999). In addition, the techniques are cumbersome and time consuming.

1.2.3.3 Nucleic acid analysis

The comparison of the DNA nucleotide sequences of two strains provides a rapid and accurate method of establishing relatedness. Techniques for carrying out the comparisons include DNA-DNA hybridization and PCR based gene analysis. The analysis of RNA for taxonomic purposes focuses on three different molecules of ribosomal RNA, 5S (~ 120 nucleotides), 16S (~1600 nucleotides) and 23S (~3000 nucleotides). These molecules are important indicators of relatedness of organisms because the rRNAs are essential elements in protein synthesis and are therefore present in all living organisms (Priest and Austin, 1995). Other factors that make these molecules ideal for the analysis of evolutionary relationships are that i) the lateral transfer of rRNAs between different organisms is extremely rare ii) the longer rRNAs (16S, 18S and 23S) contain regions of highly conserved, moderately variable and highly variable sequences. The conserved regions are essential as they provide primer directed sites for PCR as well as convenient hybridization targets for the cloning of rRNA genes (Gentry *et al.*, 2006, Letowski *et al.*, 2004).

Although the 16S rRNA gene method has served as a powerful tool for finding phylogenetic relationships among bacteria, the molecule is too conserved to provide good resolution at the species and subspecies levels (Cho and Tiedje, 2001). DNA-DNA hybridization is one method that provides more resolution than 16S rRNA gene sequencing. It measures the degree of similarity between the genomes of different species, and is therefore useful for defining proposed new species as well as the definitive assignment of a strain with ambiguous properties to the

correct taxonomic unit. Its disadvantages are, however, that it is time-consuming, labour-intensive, and expensive to perform because of the need of pairwise cross-hybridizations, the requirement for isotope use, and the impossibility of establishing a central database (Cho and Tiedje, 2001).

1.2.3.3.1 16S rRNA molecules and applications of 16S rRNA analysis

16S rRNA is a major component of the small (30S) ribosomal subunit. It is important for subunit association and translational accuracy. The 16S rRNA gene, consisting of 1542 bases, is highly conserved among microorganisms and is therefore an excellent tool for studying phylogenetic relationships (Sacchi *et al.,* 2002). The 16S rRNA genes of many phylogenetic groups have characteristic nucleotide sequences called oligonucleotide signatures. Oligonucleotide signatures are sequences which occur in most or all members of a particular phylogenetic group (Woese *et al.,* 1985) and can be used when designing primers which are genus or species-specific (Bavykin *et al.,* 2004).

PCR-based methods are considered to be a rapid and accurate way of identifying bacteria (Cook and Meyers, 2003). In sequence based techniques, primers to the extremities of the gene are used to amplify the DNA. The amplified DNA can either be sequenced directly or cloned into a phage or plasmid vector prior to sequencing. After the sequences have been generated they are compared by aligning the corresponding nucleotide sites. These type of simple comparisons of sequence positions will provide an estimate of how closely related the organisms are (Priest and

Austin, 1995) Analysis of the 16S rRNA gene offers a time saving alternative to the classical methods of identification summarised above (Alfaresi and Elkosh, 2006).

16S rRNA sequencing is very valuable in clinical settings such as for the accurate identification of *Nocardia* species. Identification of *Nocardia* isolates to the species level is very important for the estimation of pathogenicity, virulence and in predicting how susceptible a strain will be to antimicrobial agents (Roth *et al.,* 2003). Other medically important *Actinomycetes* that can be identified using 16S rRNA sequencing include *Actinomadura, Gordonia, Rhodococcus, Saccharomonospora, Saccharopolyspora, Streptomyces* and *Tsukamurella* (Cook and Meyers, 2003).

16S rRNA gene analysis has been used to reclassify actinomycete species that were incorrectly classified using classical identification methods. An example is the reclassification of the actinomycete strain ATCC 39727 which produces the glycopeptide antibiotic A40926. This actinomycete was originally classified on the basis of morphology and cell wall composition into the genus *Actinomadura.* However, phylogenetic analysis revealed that the strain ATCC 39727 belongs to the genus *Nonomuraea* (Monciardin and Sosio, 2004).

Although any one approach used to assess diversity cannot claim to be more efficient than another, 16S rRNA gene sequence analysis allows for the assessment of a broader range of diversity than that obtained by physiological studies (Brambilla *et al.,* 2001). The 16S rRNA gene can also be analyzed by a number of non-sequence based methods which include amplified rDNA restriction analysis (ARDRA), restriction fragment length polymorphisms (RFLP),

random amplified polymorphic DNA analysis (RAPD), amplified fragment length polymorphisms (AFLP) and rep-DNA (Gütler and Mayall, 2001).

1.2.3.4 Numerical taxonomy

In 1957, Sneath introduced the concept of numerical taxonomy, defined as the grouping of taxonomic units by numerical methods (Priest and Austin, 1995). One of the most interesting features of numerical taxonomy is that all characteristics have equal importance or weight in the classification scheme. This was seen as an advantage as many classical taxonomists insisted that some tests were more important that others when defining taxa and that these should be used to establish relationships.

The genus *Streptomyces* was classified in the family *Streptomyceae* on the basis of morphology and subsequently by the constituents of the cell wall. The development of numerical taxonomy systems, which utilized phenotypic traits, helped to resolve the intergeneric relationship within the family *Strteptomycetaceae* and resulted in the reclassification of the genera *Atinopycnidium*, *Actinosporangium, Chainia, Elytrosporangia* and *Microellobosporia*, to the *Streptomyces* genus (Anderson and Wellington, 2001).

1.2.4 Importance of Actinomycetes

Actinomycetes produce secondary metabolites with diverse chemical structures and biological activities. Many of thes secondary metabolites are of industrial and pharmaceutical interest. The

secondary metabolites are produced during one part of the complex life cycle. The life cycle of actinomycetes has been well studied in streptomycetes. It is a complex cycle that is neither unicellular nor multicellular (Maguelez *et al.,* 2000). The life cycle (as seen in Figure 1) begins with the germination of spores resulting in spreading of filaments into the solid medium to form a vegetative mycelium (steps 0-2). The developed vegetative mycelium forms sporophores which extend vertically toward the surface above the colony ("aerial mycelium", step 3-4). The aerial mycelium spirals (step 5), and then the polynucleated aerial filaments are partitioned (step 6). The resulting sheaths become spores (step 7) and then another cycle begins.

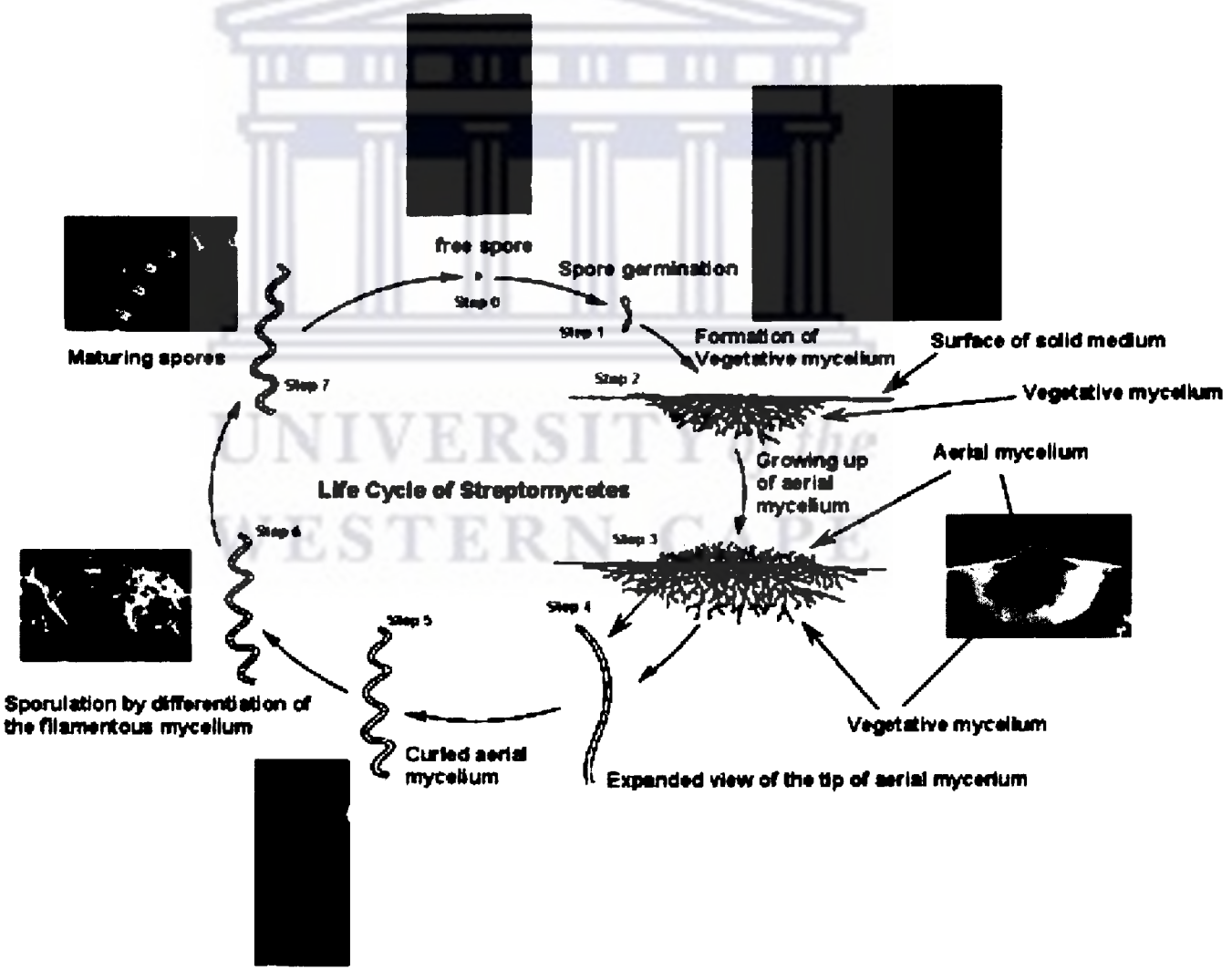

Figure 1: Streptomycete life cycle (Maguelez *et al.,* 2000).

Secondary metabolites are organic compounds that are not directly involved in the normal growth, development or reproduction of the producing organism (Martin *et al.*, 2005). Microbial secondary metabolites include antibiotics, pigments, toxins, enzymes and antitumor agents. The secondary metabolites are secreted during the generation of aerial hyphae from the vegetative mycelium (Maguelez *et al.*, 2000). Studies on γ-butyrolactones have shown that the onset of secondary metabolism is linked to morphological differentiation in streptomycetes. The most characterized γ-butyrolactone, the A-factor (2-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone) of *Streptomyces griseus*, is required for both secondary metabolism (streptomycin and grixazone production) and morphological differentiation (Bibb, 2005).

Organisms produce secondary metabolites usually as defense against predators, parasites and disease or in interspecies competition and in reproductive processes (Demain and Fang, 2000). However, some of these secondary metabolites (e.g., antibiotics and mycotoxins) may be toxic to the microorganisms that produce them. In order to avoid suicide, the genes which code for these metabolites are clustered with genes that confer resistance to that particular compound (Martin *et al.*, 2005).

Microorganisms produce secondary metabolites during the stationary phase of the growth cycle. The process is believed to be triggered by fermentation conditions such as the depletion of nutrients, the biosynthesis of an inducer or a decrease in growth rate. In response to these conditions the microorganism generates signals which trigger a cascade of regulatory events

resulting in chemical differentiation (secondary metabolism) and morphological differentiation (morphogenesis) (Demain, 1998).

Microbes adapt to changes in their surroundings which ultimately affects the type of secondary metabolites they produce. Antarctic microorganisms have had to adapt to harsh environmental conditions within unique ecological niches and therefore Antarctica might be an exciting pool of novel biodiversity. Many of the industrially important antibiotics and secondary metabolites produced by *Streptomyces* species have been well studied, therefore by isolating actinomycetes from an extreme environment such as Antarctica, the potential of discovering novel secondary metabolites of industrial significance is increased.

1.2.4.1 In industry

In addition to antibiotics, actinomycetes also produce other economically important compounds including vitamins, enzymes and immunomodulators (Moncheva *et al.*, 2002). Other secondary metabolites produced by actinomyctes such as aliphatic alcohols, lactones, biogenic sulphides, ketones, esters, thioesters, lactones, furanones and isoprenoids are important to the chemical and pharmaceutical industries (Zaitlin and Watson, 2006).

1.2.4.2 In the environment

Actinomycetes have many roles in the environment. Streptomycetes are saprophytic bacteria and are able to decompose organic matter, especially complex polymers such as lignocelluloses,

starch, chitin, hemicelluloses, pectin, keratin, natural rubber and even some man-made compounds that enter the soil as contaminants (Goodfellow and Williams, 1983, Crawford *et al.*, 1993). Actinomycetes are also important in the rhizosphere where they influence plant growth and protect plant roots against invasion by pathogenic fungi (Goodfellow and Williams, 1983). The potential of actinomycetes as biological control agents of soil-borne root diseases in crop plants has been investigated and some *Streptomyces* species, as well as a few other actinomycete genera, have been shown to protect several different plant species against soil borne fungal pathogens especially in glass house experiments. Some genera have also been shown to produce herbicidal and insecticidal compounds (Crawford *et al.*, 1993). Members of the actinomycete genus *Frankia* can fix nitrogen. They have a broad host range and can form root nodule symbioses with more than 200 species of flowering plants (Mincer *et al.*, 2002)

1.2.4.3 In medicine

Natural products are the single most important source of new medicines (Jensen *et al.*, 2005). Among the potential sources of natural products, bacteria are very important. However, the ability to produce anti-infective agents is limited to five of the 53 known phyla of bacteria. The most productive are members of the class *Actinobacteria* (order *Actinomycetales).* Approximately 7000 compounds reported in the dictionary of natural products are of actinobacterial origin (Jensen *et al.*, 2005). Actinomycetes are therefore of great interest to industry because of their ability to produce important secondary metabolites. The genus *Streptomyces* is the largest antibiotic-producing genus, accounting for approximately 80% of the actinomycete derived natural products reported (Jensen *et al.* 2005). Although thousands of

antibiotics have been described, these are thought to represent only a small fraction of the repertoire of bioactive compounds that members of the genus *Streptomyces* are able to produce (Watve *et al.,* 2001). The discovery of gentamycin, (an aminoglycoside produced by *Micromonospora purpurea* and *M. echinospora* that inhibits bacterial protein synthesis) greatly increased the interest in the screening of non-*Streptomycete* actinomycete genera for novel antibiotics (Abou-Zeid *et al.,* 1978). *Amycolatopsis* and *Actinomadura* species produce vancomycin type glycopeptides. A promising antitumor agent, enediyne, is produced by a non-*Streptomyces* actinomycete. Macrolactam and napthacene-quinone antibiotics have been isolated from *Actinomadura,* whilst *Micromonospora* and *Saccharopolyspora* strains have been found to produce a number of macrolide type antibiotics (Moncheva *et al.,* 2002).

1.3 Metagenomics

Analyses of 16S rRNA gene sequences amplified directly from the environment has shown that uncultured microorganisms represent the vast majority of organisms in most environments. It is widely believed that 0.1 to 1% of bacteria are readily culturable on common media under standard conditions, and hence have been discovered (Handelsman, 2004). Before the advent of molecular techniques, these uncultured organisms remained uncharacterized because a microorganism can only be cultivated after its physiological niche has been characterized and duplicated experimentally. Metagenomics is the culture-independent genomic analysis of microbial communities (Schloss and Handelsman, 2003) and it provides the means to identify novel and industrially useful genes in the environment as well as understanding microbial diversity (Rodriguez-Valera, 2004).

Metagenomic analysis involves isolating genomic DNA from an environmental sample, creating genomic libraries and subsequently screening the clones in the libraries (Cowan *et al.,* 2005). Identification and classification of both well known and novel organisms is made easier by phylogenetic marker genes. The most commonly used phylogenetic markers are genes for the RNA subunits, most frequently 16S rRNA (Deutschbaure *et al.,* 2006). Alternatively, clones may also be screened for conserved genes such as *recA* by hybridization or multiplex PCR, or for expression of specific traits, such as enzyme activity or antibiotic production (Handelsman, 2004).

Two types of analysis (a sequence based approach and a functional approach), have been used to obtain information from metagenomic libraries. In the sequenced-based approach the libraries are initially screened for particular DNA sequences. This can involve the complete sequencing of clones containing phylogenetic markers that indicate the taxonomic group from which the DNA fragment originated. This method relies on conserved regions in the DNA sequences which can be used to design hybridization probes or PCR primers to screen the metagenomic libraries for clones that contain the relevant sequences. Alternatively, the libraries can be screened randomly until the desired genes are obtained. This technique can be used to collect and sequence many genomic fragments from one taxon (Handelsman, 2004; Schloss and Handelsman, 2003). The major disadvantage of the sequence based approach is that it can not be applied to poorly characterized or novel sequences.

The function based analysis of metagenomic libraries is initiated by the identification of clones that express a desired trait, followed by characterization of the active clones by sequence and biochemical analysis (Schloss and Handelsman, 2003). This approach quickly identifies clones that have potential applications in medicine, agriculture and industry. The biggest problem with the function based approach is that it requires the gene to be functionally expressed and many genes, perhaps most, are not expressed in the bacterial vectors routinely used for cloning. Another limitation is that it is dependant on the availability of an assay for the required function (Handelsman, 2004).

1.4 Project Rationale

Research on novel actinomycetes constitutes an essential component in natural product based drug discovery. Thousands of *Streptomyces* strains are screened annually by pharmaceutical companies as a potential source of novel chemical compounds but it is becoming increasingly difficult to obtain novel compounds, and screening usually results in the re-isolation of known compounds (Watve *et al.,* 2001). Metagenomic and mathematical studies have, however, shown that the antibiotic producing organisms have a large potential to produce more compounds (Kennedy *et al.,* 2007; Watve *et al.,* 2001). The study of actinomycete strains from Antarctica is important because it is an extreme environment which has not been extensively studied but is believed to have a large diversity of organisms that have not yet been discovered (Tindall, 2004). On this basis it is presumed that the chances of isolating novel actinomycetes from these soils is high.

1.5 Aims of the study

The aim of this project is to characterise novel psychrotrophic actinomycetes isolated from Antarctic Dry Valley soils and to isolate and characterize secondary metabolites produced by these actinomycetes.

The specific objectives are:

- To isolate and identify actinomycete strains from Antarctic soil samples using 16S rDNA sequence analysis as well as morphological and physiological characterization techniques.
- To screen for the presence of secondary metabolites produced by these strains.
- To investigate the distribution of the novel strain HA8 (isolated during the course of this study) in soils from other regions.

Chapter 2: Materials and Methods

2.1 Chemicals and reagents

Chemicals were supplied by Merck Chemicals and Laboratory Supplies, Sigma-Aldrich Chemical Company and Kimix Chemical and Laboratory Supplies. All chemicals used were of analytical grade. Oxoid Ltd and Biolabs supplied culture media. DNA size markers, and all DNA modifying enzymes (polymerases and restriction endonucleases) were purchased from Fermentas Life Sciences Ltd. Oligonucleotide primers for polymerase chain reaction (PCR) were synthesized by Whitehead Scientific.

2.2 Sampling site and sample collection

Antarctic soil samples were collected by Professor D.A. Cowan in the Miers Valley, Eastern Antarctica, during December 2006 and January 2007. Samples were collected in sterile 50 ml Falcon tubes, mixed thoroughly and resampled before storage at below 0°C for transportation to the laboratory. Samples were stored at -80°C until required. The distribution of the novel strain HA8 was investigated in soil samples from the Miers Valleys, Marion Island and the grounds of the University of the Western Cape in South Africa.

2.2 Isolation of actinomycetes from soil samples.

2.2.1 Isolation method

Actinomycetes were isolated from soil samples using the serial dilution method on differential media. The media used were Humic-acid Vitamin agar (Chi *et al.*, 2001), Middlebrook 7H9 agar (Cook and Meyers, 2003) and Streptomyces General Defined agar (GM (800 ml): 0.17 g Na2HPO4.2H2O, 0.14 g KH2PO4, 0.05 gMgSO4.7H2O, 0.01 g FeSO4.7H2O; pH 7.4; autoclaved at 15 psi (103.5 kPa) for 15 min; after cooling, 100 ml of 100 mM glucose, 50 ml of 50 mM (NH4)2SO4 and 50 ml of 50 mM L-glutamic acid, sodium salt, were added) (Cook and Meyers, 2003). All media was autoclaved at 121°C at >15 psi for 15 minutes prior to use and contained cycloheximide at 50 $\mu g\ ml^{-1}$.1g of soil from each sample was suspended in 9.0 ml of sterile distilled water and diluted 1000 fold and 0.1 ml of last dilution was spread on the agar plates. The plates were incubated at 4°C, 16°C, 30°C or 37°C for 2 weeks. Actinomycete colonies were picked from each plate and were streaked onto fresh plates until pure cultures were obtained.

2.2.3 Determining optimum growth conditions

All isolates were grown in Czapek's solution (CZ) (Cook and Meyers, 2003), 7H9, and HA media at 4°C, 16°C, 30°C or 37°C in order to observe the effect that temperature had on the growth of the different isolates. For each isolate, seven 5 ml cultures were grown in 50 ml specimen tubes at one temperature. Growth was observed by measuring the dry weight of one of the 5ml cultures

over 21 days at 3 day intervals. A total of 168 cultures were grown for each isolate (incuding 1 duplicate).

2.3 Identification of isolates.

2.3.1 DNA Extraction

Pure cultures were inoculated into 50 ml volumes of 7H9, humic acid and Czapek media in 250 ml Erlenmeyer flasks. The flasks were incubated at 29°C and shaken at 120rpm for 14 days. Purity of cultures was determined by Gram staining. DNA was isolated from the cells using a modification of the method described by Wang *et al.* (1996). Cells were harvested by centrifuging the cultures at 13000xg for 2 minutes. Approximately 200µl of cells were resuspended in 500µl of lysozyme buffer (25mM Tris-HCl (pH 8), 50mM glucose, 10mM EDTA, 25mg lysozyme). The cells were incubated in the buffer overnight after which SDS was added to a final concentration of 1% and the tubes were incubated at 65°C for 30 minutes. An equal volume of phenol was added to the cell lysate and the tubes were gently inverted and spun at 10000xg for 30 seconds to separate the phases. The phenol extraction was repeated twice. The top layer was collected into a clean tube. The extraction procedure was repeated with an equal volume of chloroform isoamyl alcohol (24:1). DNA was precipitated with one volume of isopropanol at room temperature and centrifuged at 15000xg for 2 min. The supernatant was discarded and the pellet was air dried and then resuspended in 100µl 1X TE buffer. DNA was stored at -20°C.

2.3.2 DNA Purification

Genomic DNA isolated from colonies grown on humic acid vitamin agar was further purified using polyvinylpolypyrrolidone (PVPP) minicolumns (Berthelet *et al.*, 1996). The columns were prepared by cutting the caps from 1.5-ml and 0.6-ml tubes as well as the lower part of the 0.6 ml tubes, and cutting 20-µl filter tips ca. 2 mm beneath the filter. Both the filter tip and the 0.6 ml tubes were placed inside the 1.5 ml tube. The column was loaded with 200 µl of the PVPP suspension and centrifuged (150x*g* for 2 min). The flow-through was discarded and the step was repeated. The column was washed twice with 150 µl of TE and centrifuged as above and dried by centrifugation (600x*g* for 10 min). The column was placed in a new 1.5-ml tube. 100 µl of a DNA sample (section 2.3.1) was applied to the column and incubated for 1 min at RT. The DNA was eluted by centrifugation (600x*g* for 5 min followed by 1700x*g* for 10 min). The purified genomic DNA was quantified using the Nanodrop ND-1000 spectrophotometer at 260 nm (Nanodrop, Delaware-USA).

2.3.3 DNA amplification using 16S rRNA primers

2.3.3.1 16S rRNA PCR

Universal primers E9F 5'GAGTTTGATCCTGGCTCAG3' (Hansen *et al.*, 1998) and U1510R 5'GGTTACCTTGTTGTTACACTT3' (Baker *et al.*, 2003) designed to target the conserved regions of the 16S rRNA gene were used to amplify an approximately 1.4kb long DNA fragment. The PCR mix contained 5µl of 10X PCR buffer, 4µl of 25mM $MgCl_2$, 5µl of 5µm E9F, 5µl of

5µm U1510R, 5µl of 1mM DNTP's, 0.5µl of *Taq* polymerase (Fermentas) and 2µl of genomic DNA. Each reaction was adjusted to a final volume of 50µl with sterile double-distilled water and amplified in an automated thermal cycler (Thermo Hybaid system). The PCR conditions were an initial denaturation stage at 94 °C for 2 mins, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 45 s, extension at 72°C for 60 s and a final extension step at 72°C for 10 mins. Negative controls with no DNA template were included in all PCR experiments.

2.3.3.2 Agarose gel electrophoresis

Analysis of DNA was performed using agarose gel electrophoresis (Sambrook *et al.*, 1982). Horizontal 1% (w/v) Tris-acetate-EDTA (TAE) agarose gels were cast and run at 100 V in 0.5 × TAE buffer. To allow visualization of the DNA on a UV transilluminator, the gels were supplemented with 0.5 $\mu g.ml^{-1}$ ethidium bromide. The DNA fragments were sized according to their migration in the gel as compared to that of the DNA molecular marker, Lamda DNA restricted with *Pst*I endonuclease.

2.3.3.3 PCR product cleanup

Purification of PCR products was achieved by running the total volume of the PCR reaction on 1% agarose and excising out the appropriate band from the gel. Cleaning the excised DNA bands from agarose gels was performed using the GFX™ PCR and Gel Band Purification Kit (Amersham Biosciences), according to the product specifications.

2.3.4 Cloning of the 16SrRNA gene

2.3.4.1 Preparation of electro-competent cells

Electrocompetent GenHog *Escherichia coli* cells were prepared by inoculating a single freshly streaked colony into 20 ml SOB medium and culturing overnight at 37°C with agitation at 250 rpm. 2 ml of the overnight culture was inoculated into a 250 ml sterile flask containing 25ml LB broth and the cells were grown at 37°C with shaking (250 rpm) for 3.5-4 h to an OD_{600nm} of 0.6. Cells were kept on ice and harvested at 4000xg for 10 min at 4°C. The cells were resuspended in a volume of ice-cold sterile water equal to the original culture volume and harvested as before. The supernatant was discarded and the cells were resuspended in ice-cold sterile 10% glycerol and centrifuged for 15 min at 4000xg.

After decanting the supernatant, cells were re-suspended in ice-cold sterile 15% glycerol, 2% sorbitol using a volume of 2ml per L initial culture. Cell were harvested by centrifugation at 4000xg for 10 min at 4°C and then re-suspended in a volume of ice-cold sterile 15% glycerol, 2% sorbitol equal to that of the cell pellet. 50µl volumes of cells were then aliquoted into sterile 0.5ml Eppendorf tubes. Ethanol at -80°C was used to snap freeze the cells. Cells were stored at -80°C (Inoue *et al.* 1990).

2.3.4.2 Ligation of the 16SrRNA gene into a vector

Ligation reactions were performed using the InsT/Aclone Kit (Fermentas). Ligation reactions were prepared in total volumes of 5µl and consisted of the plasmid vector pGem-T Easy and

insert DNA in a 1: 3 molar ratio, 1X ligation buffer, 1U T4 DNA ligase and dH_2O according to the manufacturer's instructions. Reactions were incubated overnight at 16°C.

2.3.4.3 Transformation of electro-competent cells.

The electro-competent Gen Hog *E.coli* cells were transformed with the recombinant DNA from section 2.3.4.2. An Eppendorf tube containing 50 µl of electrocompetent cells was removed from -80^0C storage and allowed to thaw on ice. For each ligation reaction, 2µl of the ligation mixture was added to the thawed cells and gently mixed. The mixture was returned to ice for ~ 1 min and pipetted into a pre-cooled 0.1 cm sterile electroporation cuvette (Bio-Rad Laboratories, Hercules, CA, USA). Electroporation was performed using the following conditions: 1.25 – 1.8 kV, 25 µF, 200 Ω on the BioRad Gene Pulser machine. Immediately following electroporation, 1ml SOB broth was added to the cuvette. The cells were transferred to a 15 ml Falcon tube and incubated at 37°C for 1 h with agitation. 100µl of the cells were plated onto LB-agar plates supplemented with ampicillin (100 µg/ml), IPTG (20 µg/ml), and X-Gal (30 µg/ml). Recombinant transformants were selected by blue/white colour selection based on insertional inactivation of the *lac*Z gene Messing *et al.,* 1977).

2.3.5 Colony PCR

White colonies were picked from the overnight culture plates using sterile toothpicks and swirled in 50ul TE buffer. 2ul was used directly as a template for PCR. The PCR reaction consisted of 5µl of 10X buffer, 4µl of 25mM $MgCl_2$, 5µl of 5µm T7 promoter, 5µl of 5µm T7 terminater, 5µl

known species in the GenBank database using software from the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov/). Sequences were aligned using Clustal W multiple alignments featured in Bioedit Sequence Alignment Editor version 6.0.5 (Copyright 1997-2001 Tom Hall Isis Pharmaceuticals Inc. Department of Microbiology, North Carolina State University). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura *et al.*, 2007) and were used in the construction of phylogenetic trees based on maximum-parsimony analysis.

2.4 Storage of isolates and DNA

All isolates were stored in two forms: (i) as colonies on agar plates and (ii) as glycerol stocks at -80°C. The plates were stored at room temperature and were maintained by sub-culturing once a month. The glycerol stocks were prepared by adding 300ul of 50% glycerol to 700ul of cell culture. DNA was stored in TE buffer at 4°C for short term storage and at -20°C for long term storage.

2.5 Morphological and physiological characterization of isolates.

Morphological and physiological characterization was based on methods of the International *Streptomyces* Project (ISP) (Shirling & Gottlieb, 1966). Accurate morphological characterisation of actinomyctes is dependent on culture media that promote good sporulation. ISP 2, 3, 4, and 5 media are considered as standard media for morphological observations. ISP 6 and 7 were used

as standard media for observation of melanin production and ISP 9 media was used to test for the utilization of sole carbon sources (Shirling and Gottlieb, 1966). Plates were inoculated by making crosshatch streaks with the exception of the ISP9 plates where the single stroke streak was used.

Washed inoculum was prepared for use in the carbon sources determining tests. The inoculum was prepared by vigorously agitating a cell suspension on a vortex. Ten ml of the fragmented broth culture was transferred into a 15ml Falcon tube and centrifuged. The supernatant was decanted and sterile distilled water was added to restore the volume to 10ml. The cells were resuspended and the washing was repeated twice. The cells obtained from the final wash step were resuspended in 5ml sterile distilled water and were used to inoculate ISP9 plates (Shirling and Gottlieb, 1966).

Each culture was inoculated onto 6 plates of each medium. The plates were incubated at room temperature (approximately 22°C). For each culture, two plates were observed after 7 days, two after 14 and two after 21 days. The colour of the substrate mycelium growing on ISP4 and ISP5 plates was observed on the underside of mass growth. Spore chain morphology was observed by direct examination of the culture surface on opened dishes of the crosshatch cultures under the light microscope at 40x and 100x magnifications. Production of melanin was determined using ISP6 and ISP7 plates. Uninoculated plates were used as a negative control. The ability to utilize sole carbon sources was observed as zones of clearance around the colonies.

2.6.2 Nested PCR

Nested PCR was used to amplify the 16S rRNA gene from genomic DNA extracted from soil samples. HA8 species specific primers were designed from a unique region using the Primer 3 software (Rozen and Skaletsky, 2000). The primers were synthesised by Integrated DNA Technologies Inc. PCR reactions were done in two stages, the first reaction with universal primers as described in section 2.3.3.2. The PCR product was extracted from the agarose gel and the purified DNA was used as a template for the nested PCR. In the nested PCR an HA8 species specific primer 5'CGACTGGGTTTCCCGGACG3' was used as the forward primer whilst the streptomyces specific primer Strep F 5'ACGTGTGCAGCCCAAGACA3' was used as the reverse primer. The PCR reaction using these primers was optimized by varying the $MgCl_2$ concentration. The PCR reaction was carried out as described in section 2.3.3.1, but using a $MgCl_2$ concentration of 2mM. The PCR products were cloned into *E. coli* cells, and the vector inserts were sequenced.

2.7 Screening isolates for antimicrobial activity.

2.7.2 Test organisms

The organisms that were used to test extracts from actinomycete culture for antimicrobial activity were *Micrococcus luteus, Staphylococcus aureus, Streptococcus* sp, *Bacillus megaterium, Citrobacter braaki* strain 90, *Enterococcus faecium, E. coli* ATCC 25922, *Klebsiella pneumoniae* K11, *Proteus mirabilis* strain 87 and *Pseudomonas aeruginosa* ATCC 27853.

Test bacteria were grown by inoculating a loopful of the bacteria from an agar plate culture into 5ml Luria Broth. The culture was vortexed vigorously to disperse the bacteria and produce a turbid suspension. The cultures were incubated at 37°C overnight. Gram stains of the overnight cultures were performed to check for contamination before cultures were used in antibiotic testing. Cell density was estimated by measuring the optical density of the cell suspension at 600nm.

2.7.3 Growth of isolates for solvent extraction and antimicrobial testing

Pure cultures of the actinomycete isolates were grown in humic acid vitamin, 7H9 and the *Streptosporangium* medium broth. Cultures were grown in total volumes of 50ml in 500ml conical flasks at 30°C for 14 days in an orbital incubator with shaking at 100rpm. The cultures were Gram stained in order to check for contamination under the light microscope.

2.7.4 Small scale solvent extraction compounds

Each culture was filtered through a semi-permeable filter paper to separate the cell mass from the medium. The cells were washed with 100ml sterile distilled water. Compounds were extracted from the cell mass using methanol and from the culture filtrate using ethyl acetate, chloroform and hexane (Hace□ne and Lefebvre, 1995, Pfefferle *et al* 2000). The culture filtrate (including the water from the wash) was divided equally into 3 parts (approximately 50ml each). 17ml of each solvent was added to each 50ml culture filtrate. The liquids were mixed by vigorous shaking

for 10 minutes. The phases were allowed to seperate for 15 minutes after which the solvent layer was carefully removed using a Pasteur pippette and placed in a clean universal bottle. The solvent was evaporated in a fume hood overnight and where necessary, the residue was re-dissolved in 400µl of the solvent used for extraction. To extract from the cell mass, the filter paper containing the cells was dried on a paper towel for approximately 10 minutes. The cells were scraped off the filter paper and suspending in 10ml of methanol. The cell suspension was vortexed vigorously for 2 minutes and allowed to settle. The supernatant was removed and placed in a clean universal and allowed to evaporate in the fume hood. All solvent extracts were stored at -20°C.

2.7.5 Disc diffusion antimicrobial testing

100µl aliquots of overnight cultures of each test organism (section 2.7.2) were spread on an LB plate and allowed to dry for 30 minutes. Sterile discs of Whatman filter paper were soaked in 200µl of the solvent extracts (section 2.7.4). The discs were dried in the fume hood to evaporate all traces of the solvent and placed onto agar plates seeded with test bacteria. The plates were incubated at 37°C overnight. The diameters of growth inhibition zones were measured.

2.7.6 Growing isolates for stab culture antimicrobial testing

Agar plates were inoculated by picking an actinomycete colony using a toothpick and stabbing the agar in a central position. The plates were incubated at room temperature for 14 days.

2.7.7 Sloppy agar overlays for stab culture antimicrobial testing

Sloppy agar was prepared by adding 7g of agar to 1000ml of Luria broth and boiling the mixture. Six ml volumes of the molten agar were dispensed into test tubes. The test tubes were capped and autoclaved. The appropriate volume of test organism culture to be added to the 6ml sloppy agar was calculated using the formula OD_{600} X µl = 160 for all the bacteria except *E. coli*, for which the formula was OD_{600} X µl = 4. The appropriate amount of culture was pipetted into the sloppy agar tube and vortexed gently. The sloppy agar was poured onto the stab inoculated plates and gently swirled to distribute evenly over the surface of the plate surrounding the actinomycete colony. Once the sloppy agar overlay was set, the plates were incubated overnight at 37°C. Plates that did not have lawns after the overnight incubation were incubated for a further 24 hours.

2.8 Analysis of the antimicrobial agents

2.8.1 Screening for antibiotic biosynthetic genes.

The genomic DNA of all isolates was screened for the biosynthetic genes involved in the production of glycopeptide antibiotics and Type-II polyketides. This was achieved by PCR amplification of these genes using specific primers as described by Wood *et al* 2006. For the glycopeptide antibiotics, the forward primer Foxy: 5′CTGGTCGGCAACCTGATGGAC3′ and the reverse primer Roxy: 5′CAGGTACCGGATCAGCTCGTC3′ which amplify the *oxyB* gene were used. To amplify the genes involved in the production of Type-II polyketides, the forward primer ARO-PKS-F: 5′GGCAGCGGITTCGGCGGITTCCAG3′ and the reverse primer ARO-PKS-R: 5′CGITGTTIACIGCGTAGAACCAGGCG3′ [I = inosine] were used. PCR reactions (50µl) contained 2 mmol l^{-1} of $MgCl_2$, 0.5 mol l^{-1} of each primer, 0.6 mmol l^{-1} of dNTPs, 0.1 U

Taq polymerase and 2µl template DNA. The PCR cycling programme was an initial denaturation (96°C for 2 min) followed by 30 cycles of denaturation (96°C for 45 s), annealing (60°C for oxyB/64°C for the KSa–KSb gene pair, for 30 s), and extension (72°C for 2 min) and a final extension period of 72°C for 5 min. The PCR products were electrophoresed on 1% agarose gels, cleaned and sequenced.

2.8.2 Thin layer chromatography

Thin layer chromatography was performed on 5 X 10 silica gel $60F_{254}$ plates (Merck) for all solvent extracts that showed antimicrobial activity. The solvent systems contained a mixture of methanol and ethyl acetate in different proportions (60:40 and 40:60 then 90:10 and 10:90 methanol: ethylacetate). The chromatography tanks were prepared by lining the sides of a 2-liter glass beaker with filter paper prior to the addition of 80 ml of the appropriate solvent mixture. The beaker was tightly covered with aluminum foil and the solvent was allowed to migrate up the filter paper for at least 1 h before use to saturate the atmosphere within the "tank." 20, 10 and 5µl volumes of the samples were spotted approximately 1.0 cm apart at an origin which was1.5 cm from the bottom edge of the chromatogram sheet. The spots were air dried and the sheet was placed into the "tank" in such a way that the origin was in the solvent but the spots were not washed off. The foil lid was replaced and the chromatogram was developed until it reached the pre-drawn solvent front approximately 0.5cm from the top edge of the silica plate. Plates were placed in the fume hood to evaporate the solvent. After all the solvent had evaporated, appropriate reagents were dabbed onto the plates and air dried prior to treatment with reagents which would allow for the development of spots. The reagents used were ninhydrin, Fehling's

reagent and cerium (IV) ammonium sulphate. Spots were visualized after gently heating of the plates until the spots appeared.

2.8.3 Bioautography

Bioautography was performed on plates similar to those used for TLC. The plates were processed as described above except that the chromatograms were not developed for spot formation. For boiautography, after the solvent had evaporated the plates were seeded with an actively growing culture of *Micrococcus luteus* (OD_{600} = 0.5). Each seeded plate was placed on a wet paper towel in a petri dish and incubated at 37°C overnight. Plates were developed by dabbing 0.25% MTT (methylthiazotetrazolium in sterile water) and incubating at 37°C for an hour (Schillaci *et al.*, 2005).

2.9.4 High performance liquid chromatography

200µl samples of crude solvent extracts of HA8 fermentation cultures were chromatographed by HPLC using a Nucleosil C_{18} column in a Dionex Ultimate 3000 model HPLC system. The column was eluted at a flow-rate of 0.8 ml/min using a gradient from 10 to 100% acetonitrile in water. A total of 40 0.8ml fractions were collected. Fractions were concentrated by evaporating the solvent and the remaining residues were spotted on a disc for use in a disc diffusion analysis as outlined in section 2.7.5.

Chapter 3: Isolation and identification of actinomycetes from Antarctic soils: Results and Discussion

3.1 Isolation from soil samples.

The successful isolation of organisms from environmental samples requires an understanding of the environmental factors affecting their growth. Nutritional conditions found in Antarctica are not easy to recreate *in vitro* and the development of ideal conditions is challenging. Actinomycetes were isolated from various Antarctic soil samples by plating 100µl of serial dilutions of the soil suspension on selective media agar. All isolation media was supplemented with cycloheximide to suppress fungal growth. Cycloheximide is a protein synthesis inhibitor that acts specifically on the 60S subunit of eukaryotic ribosomes (Obrig *et al.,* 1971). Fungi were the most abundant contaminating microorganisms and they quickly covered the agar surface, making it impossible to pick any actinomycete colonies from any plates that were not supplemented with cycloheximide. Plates were incubated at different temperatures (4, 16, 30 and 37°C). Most colonies were obtained from plates incubated at 30°C. Actinomycete colonies only appeared after 2 months at 16°C and after 3 months at 4°C. Due to time constraints 30°C was selected as the ideal temperature for the routine maintenance of actinomycete isolates.

Although non-actinomycete bacteria predominated on the isolation plates, the selection of actinomycete colonies was aided by their unique morphological features. Actinomycetes are filamentous and when growing on a solid substratum, for example agar, the branching network of vegetative hyphae grows both on the surface of the substratum (aerial mycelium) and penetrates

into it to form a substrate mycelium (Prescott, 1990). The aerial mycelium gives the colonies a powdery look which makes them easy to identify. Figure 3.1 shows typical actinomycete colonies growing amidst non actinomycete colonies on a 7H9 agar isolation plate.

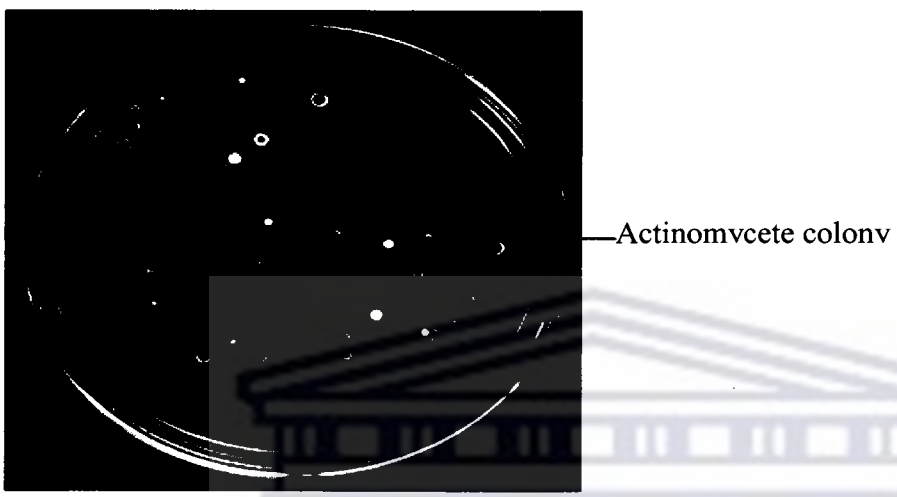

Figure 3.1: A 7H9 agar isolation plate (10^{-4} dilution after 14 days) showing actinomycete colonies

It was noted that the ratio of actinomycete colonies to other bacterial colonies was low. Soil samples may be enriched for isolation of actinomycetes. The methods that are commonly used for enrichment are treatments with heat and phenol. Researchers have also used methods such as preliminary exposure of soil suspensions to UV light and treatment of soil samples with antibiotics (Galatenko and Terekhova, 1990), and the use of specific actino- or bacteriophage (Kurtböke and French, 2007). Heat treatment of samples was omitted in this study because it selects against actively growing actinomycetes that are present in soil samples as mycelia and is selective for actinomycetes present as heat-resistant spores.

As streptomycetes have been extensively studied, it is more likely that novel compounds will be found in rare genera of actinomycetes rather than in streptomycetes. Soil samples can be treated

with antibiotics such as tobramycin and novobiocin which favour the growth of these rare genera. Tobramycin is especially favourable for detection of isolates belonging to the *Micromonospora, Amycolatopsis, Streptosporangium* and *Nocardiopsis* genera, whilst novobiocin selects for isolates belonging to *Amycolatopsis* and *Micromonospora* (Terekhova *et al.,* 1991). However these methods have been seen to greatly reduce the total numbers of actinomycetes isolated from soil samples (Hayakawa e*t al.,* 2004, Alferova *et al.,* 1989). This would be favourable in soil samples likely to have numerous culturable microorganisms. In this study, these methods were not employed as the total culturable microbial diversity of the Dry Valley soils was expected to be low (Cowan *et al.,* 2002). A total of 23 colonies were collected for culturing from the selective media agar plates, two of which were isolated from a plate grown at 4°C and did not resemble any other colonies from the other plates.

Actinomycete isolates were inoculated into liquid broth and contamination was monitored by Gram staining (Figure 3.2).

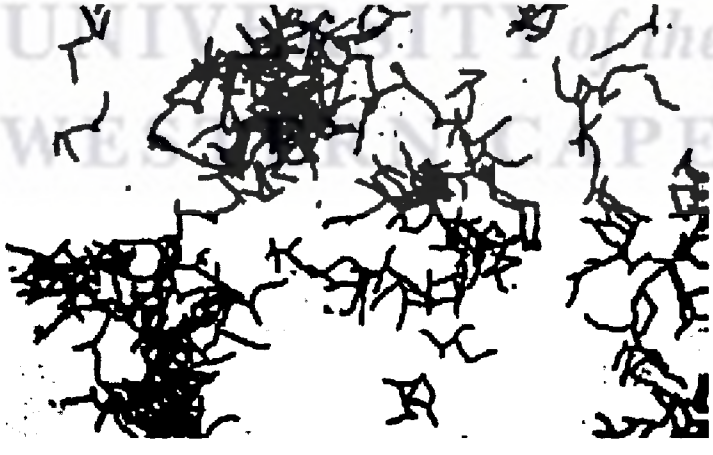

Figure 3.2: Photomicrographs of isolate SAS (Gram stained) as seen under the light microscope (magnification 1000x /1.25 oil)

3.2 Determining optimum growth temperatures

Microorganisms isolated from extremely cold environments can either be obligate psychrophiles or psychrotolerant mesophiles. Psychrophiles are capable of growing at or below 0°C, but are unable to grow above 20°C. Psychrotolerant organisms, whilst capable of growth at 0°C, are able to grow well at temperatures above 20°C (Morita, 1975). In order to determine to which group the isolated actinomycetes should be assigned, as well as to establish their optimal growth conditions, the effect of temperature on growth was determined. In this study, growth was observed over a period of 21 days. 100ml cultures were grown in 1L flasks with shaking. At 3 days intervals, 5ml samples were removed and the dry weight of the cells was determined. Despite attempts to work aseptically, the frequency at which the culture was opened made it difficult to avoid contamination. After numerous attempts, this method was abandoned in favour of the one described in section 2.2.3. The main disadvantage of the modified protocol was that as the culture volumes were small, the nutrients might have been depleted. The growth curves that were obtained in the different media were very similar showing that observed growth patterns were due to the different temperatures and not due to media compositions. All isolates showed the best growth in the modified *Streptosporangium* media, which is a rich medium containing several carbon sources. All subsequent fermentations were carried out in this medium.

Analysis of the growth curves obtained resulted in the isolates being grouped into 4 categories. Isolate HA8 was the sole member of the first group. Isolate HA8 had an optimum growth temperature of 30°C but showed very poor growth at 37°C. Intermediate growth was observed at 4 and 16°C (Figure 3.3).

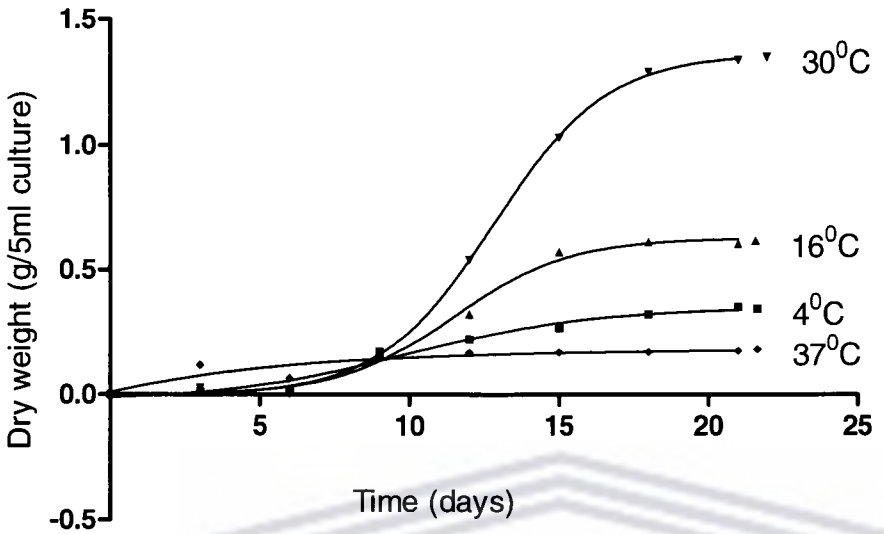

Figure 3.3: Temperature growth curves of isolate HA8 in modified *Streptosporangium* medium

HA8 had a very long lag phase with the exponential phase of the growth starting only after day 6. The growth profile of this isolate makes it difficult to classify it as a psychrotolerant mesophile (because of the poor growth at 37°C) or as true psychrophile because the optimum growth temperature was above 20°C. It could be speculated that the organism is not an obligate psychrophile but might be a strain that has evolved from a mesophile but has lost the ability to adapt to a wider temperature range. The isolate could also be a psychrotolerant mesophile with a narrow growth temperature range.

The second group of isolates consisted of isolates MVS and SAS. These strains had similar growth curves (Figure 3.4 and 3.5). Both isolates had an optimum growth temperature of 30°C. However, unlike strain HA8, growth of SAS at 4°C was very poor.

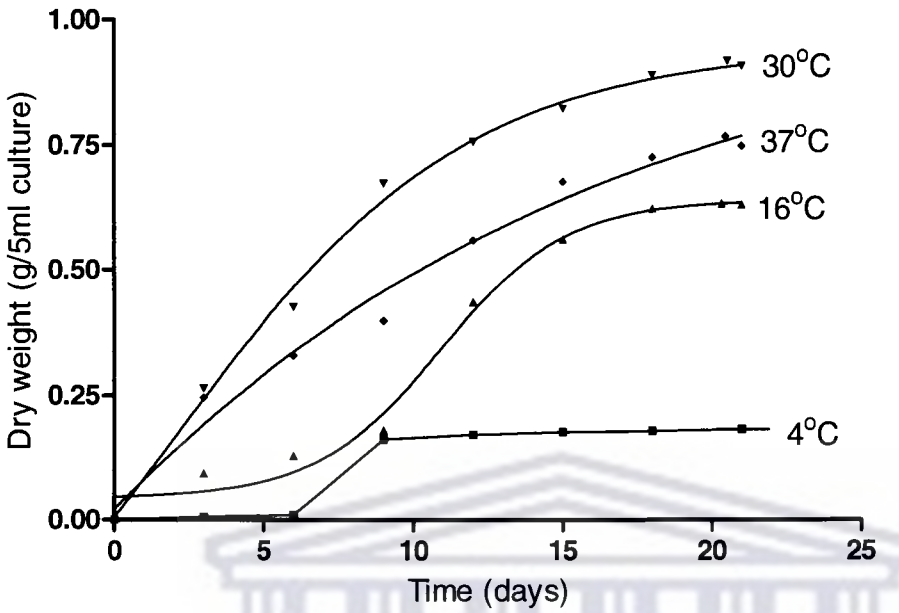

Figure 3.4: Temperature growth curves of isolate MVS in *Streptosporangium* medium

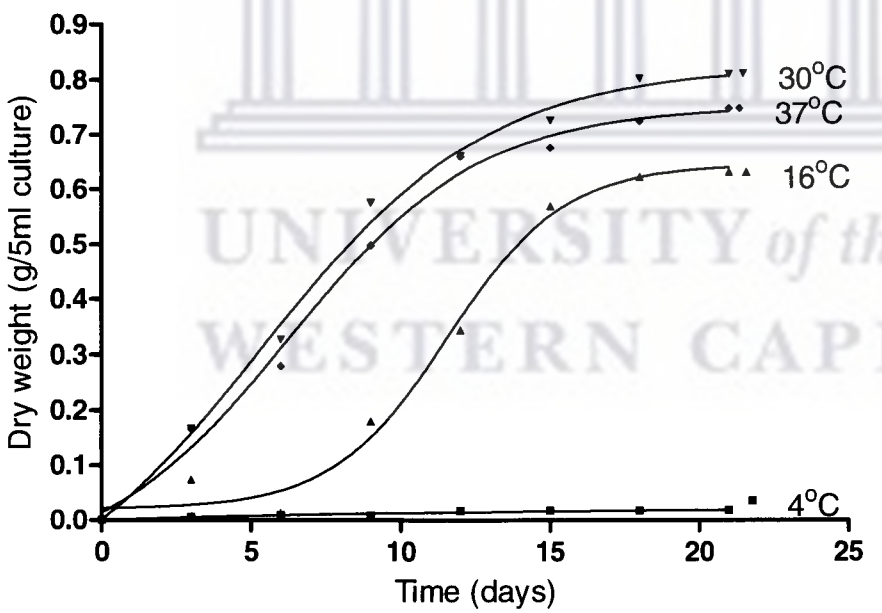

Figure 3.5: Temperature growth curves of isolate SAS in *Streptosporangium* medium

It is likely that the isolate SAS survives the cold environment as dormant spores but is not capable of active growth whilst MVS can grow very slowly at low temperatures. Both of these isolates could be considered to be mesophiles. The reduced lag phase at 30°C and 37°C also supports the assumption that these isolates are mesophiles which have survived the extremely low temperatures. Like some bacteria found in cold environments, they are probably not cold adapted but may have developed mechanisms of protection such as producing a thick capsule which protects against freeze shock, or producing spores which can resist desiccation and freezing (Margesin and Schinner 1999).

Isolate MVD had an optimum growth temperature of 37°C. This isolate grew very poorly at 4°C (Figure 3.6).

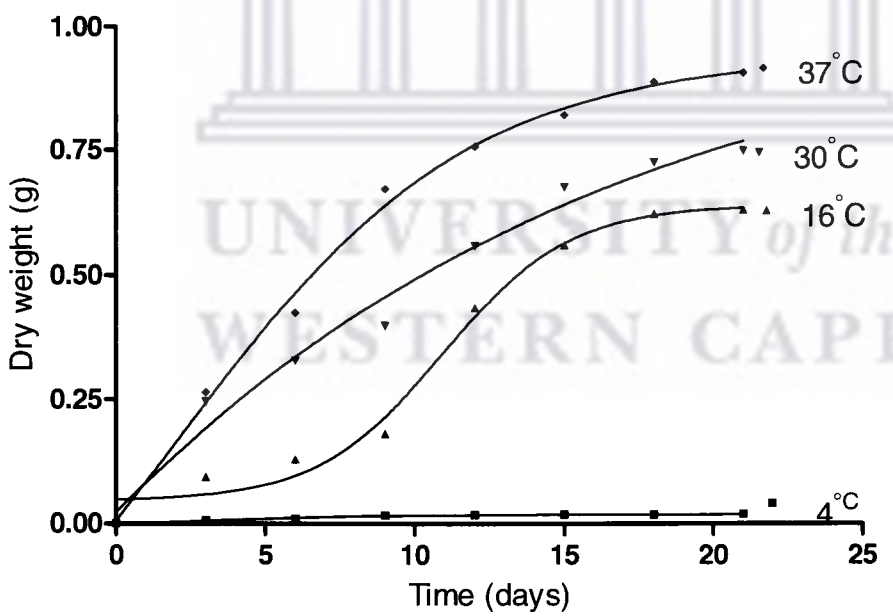

Figure 3.6: Temperature growth curves of isolate MVD in *Streptosporangium* medium

MVD was isolated from a soil sample that was collected from under a seal. Figure 3.6 illustrates that isolate MVD shows no growth at 4°C. The isolate might therefore be metabolically inactive at this temperature. 16Sr RNA gene analysis revealed that this isolate was a *Nocardia* species. Some members of the genus *Norcadia* are medically important pathogens and some have been identified as pathog This isolate might have been a pathogen present in the seal which survived the low temperature, probably as spores. enic to aquatic animals. An example is *Nocardia salmonicida* which is pathogenic to fish (Brown - Elliot *et al.,* 2006).

Three isolates, HA3, HA5 and HA15 grew poorly at all temperatures and in all media tested in this study. These isolates showed a growth optimum of 16°C (Figure 3.7- 3.9). The total biomass of these isolates was notably lower than all other isolates studied.

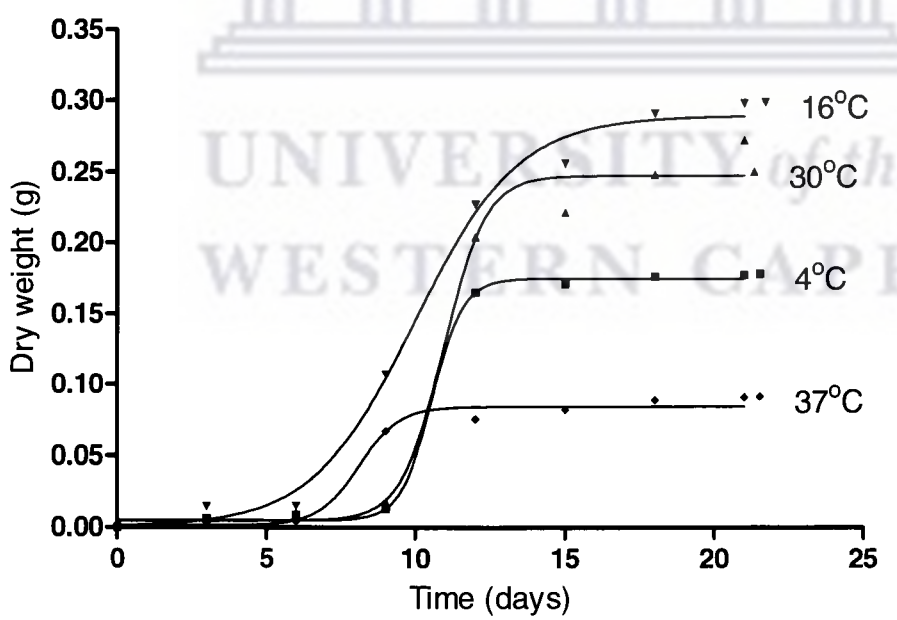

Figure 3.7: Temperature growth curves of isolate HA15 in *Streptosporangium* medium

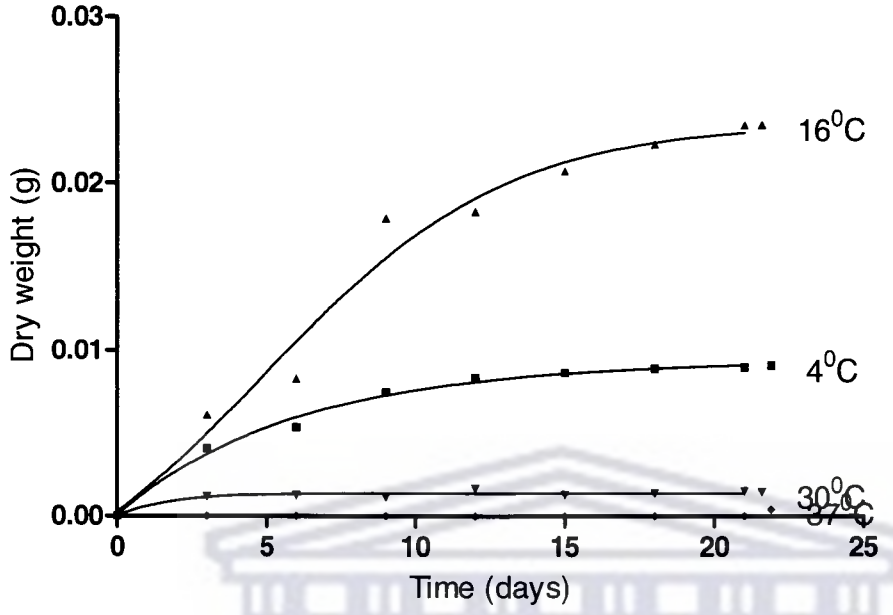

Figure 3.8: Temperature growth curves of isolateHA3 in *Streptosporangium* medium

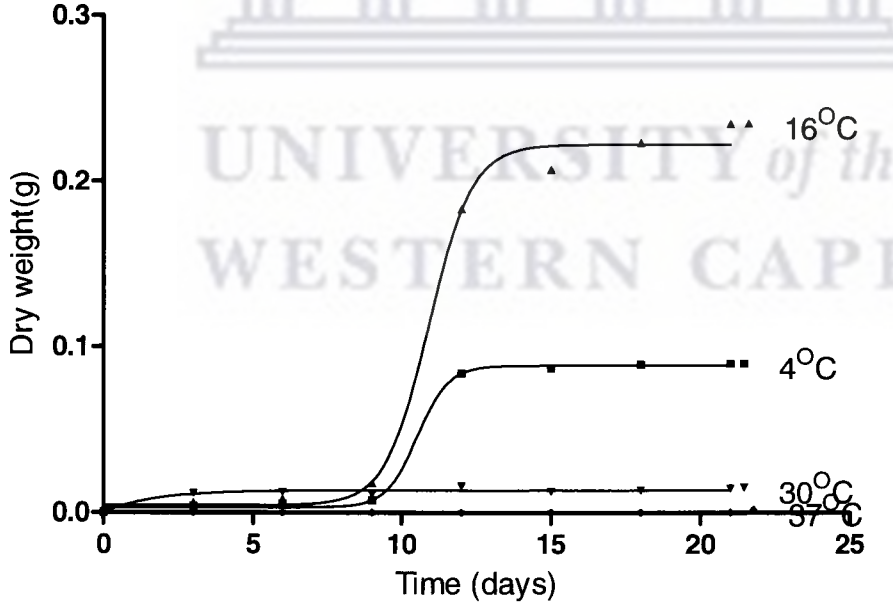

Figure 3.9: Temperature growth curves of isolate HA5 in *Streptosporangium* medium

These isolates may be considered as psychrophiles according to the definitions given (Morita, 1975) based on the observed poor growth at temperatures above 16°C. Antarctic soil is known to contain low nutrient levels (Vishniac and Mainzer, 1972). The poor growth exhibited by these isolates in relatively rich media might imply that they are oligotrophs and hence reflects their adaptation to the low nutrient environment. Oligotrophs are characterized by slow growth, low rates of metabolism, and generally low population density (Semenov, 1991). These organisms are usually present in environments with low levels of nutrients (Koch, 2001) such as the Antarctic soils. They are divided into two categories, the facultative oligotrophs which are capable of growth at both low and high concentrations of organic substances, and the obligate oligotrophs which can only grow at low concentrations of carbon (Ishida and Kadota, 1981).

Two colonies were isolated from plates incubated at 4°C. These isolates were successfully sub-cultured but grew weakly as pure isolates on agar plates and failed to grow in liquid culture. Changes made to growth conditions in an attempt to obtain growth in liquid media included different shaking speeds, different culture volumes (from 3-20% of the total volume capacity), different aeration methods and the use of soil extract media. These isolates were then considered to be unculturable in the scope of this study. One possible reason for failing to culture these isolates might be is that a required nutrient or "growth factor" was absent from the prepared media. These isolates may also have been obligate oligotrophs and the high nutrient levels in prepared media could have inhibited growth (Ishida *et al.*, 1986). It is suggested that further studies involving isolation of actinomycetes from oligotrophic environments should include media with low nutrient concentrations.

3.3 DNA extraction and PCR

Isolates were grown for 14 days at their optimal temperatures. DNA was extracted from uncontaminated cultures and the purified genomic DNA was amplified using the universal 16S rRNA primers E9F and U1015R. The amplicons obtained were estimated to be approximately 1400bp (Figure 3.10). DNA extracted from cells that were grown in broth containing humic acid failed to amplify directly (see lanes 4, 6, 7, 10, 14, 16 and 17 in Figure 3.10). This may be due to the fact that humic acid is a potent inhibitor of *Taq* polymerase, decreasing the efficiency of primer binding and chelating Mg^{2+} ions (Tsai and Olson, 1992).

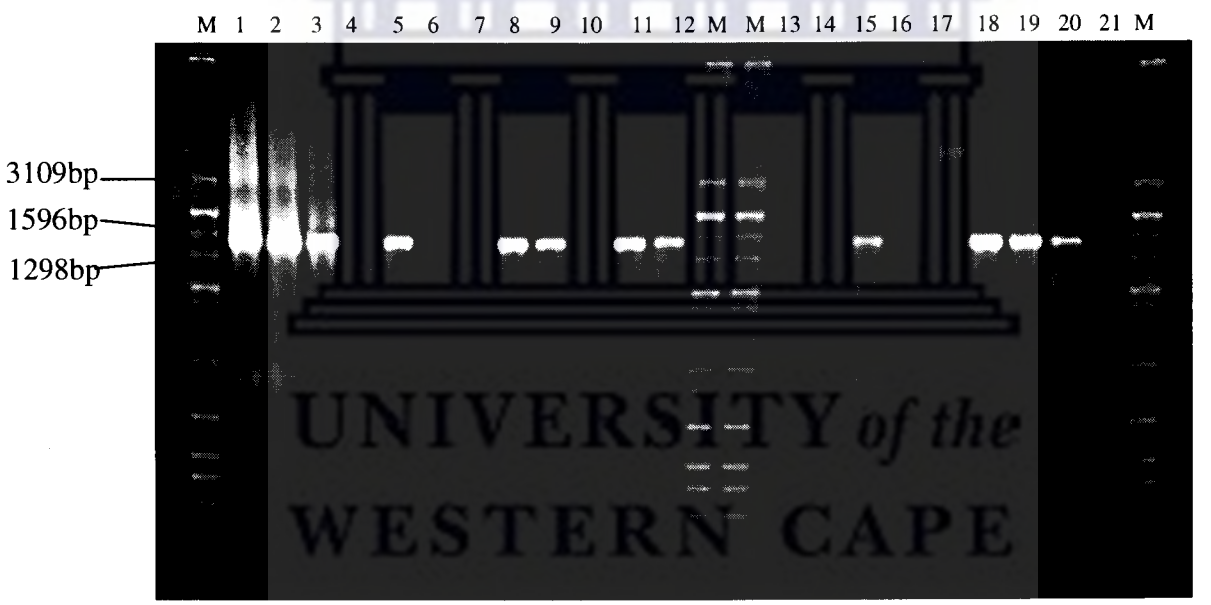

Figure 3.10: Agarose gel (1%) showing PCR amplification of the 1S rRNA genes of 20 actinomycete isolates using primers E9F and U1510R. Lanes M: DNA molecular weight marker (λ DNA cut with Pst 1). Lanes 1-12 and 13-20: actinomycete isolates. Lane 21: negative control.

DNA obtained from the humic acid isolates was further purified using PVPP mini columns. While the purifying of DNA in this way resulted in easy amplification by PCR, a significant decrease in DNA concentration was observed (Figure 3.11).

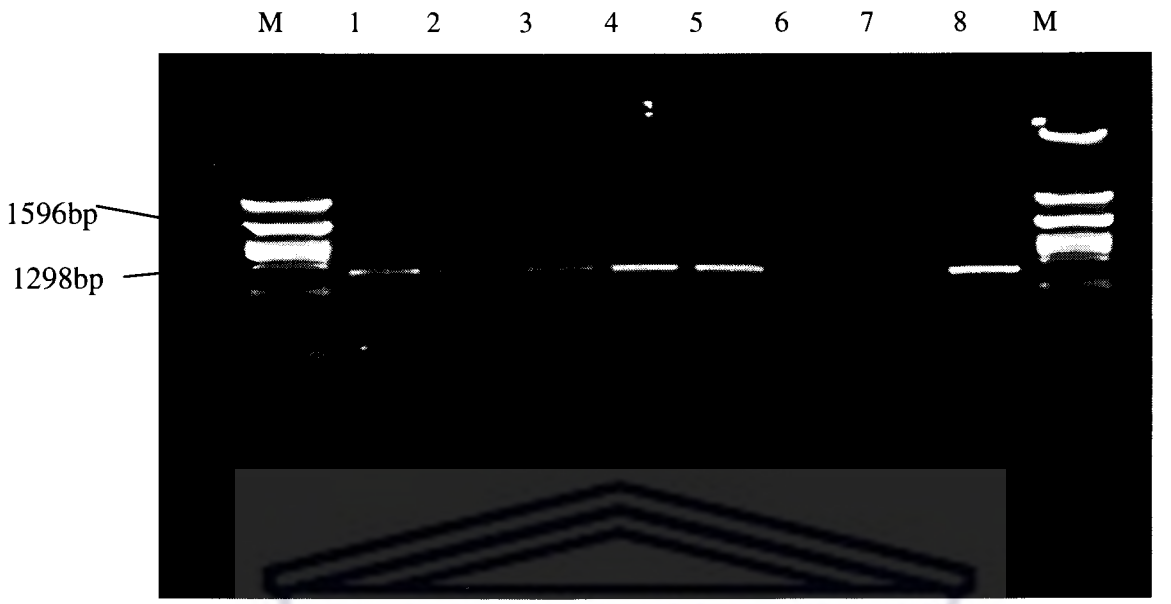

Figure 3.11: PCR amplification of the 16S rRNA genes of actinomycetes isolated on HA plates. The primers used were E9F and U1510R. Lanes M:DNA molecular weight marker (λDNA cut with Pst 1).

The purified PCR products were sequenced and the sequence chromatograms were edited using Bio-Edit software. Colony PCR was attempted on the two isolates that could not be grown in liquid culture. Colonies were also resuspended in TE buffer, boiled for 10 minutes and the supernatant used as a template for PCR. Both methods failed to yield positive results.

Despite all measures to ensure that the cultures from which DNA was isolated were pure, isolate MVD repeatedly gave sequences with overlapping peaks at specific base loci on the sequence chromatogram, resulting in ambiguous bases which could not be resolved. This led to the speculation that the isolate possessed multiple (different) copies of the 16S rRNA gene. The presence of multiple different copies of the 16S rRNA gene has been demonstrated for some genera and species of bacteria, with Actinomycetes such as *Nocardia nova* having at least three different operons (Conville and Witebisky, 2007). The 16Sr RNA gene PCR product of the MVD

isolate was cloned into the p-Gem T vector and used to transform competent *E. coli* cells. Each positive (white) colony can only contain one insert and thus the multiple copies of the gene would have been separated. The colony PCR using T7 primers amplified the insert giving a fragment of approximately 1400bp (Figure 3.12).

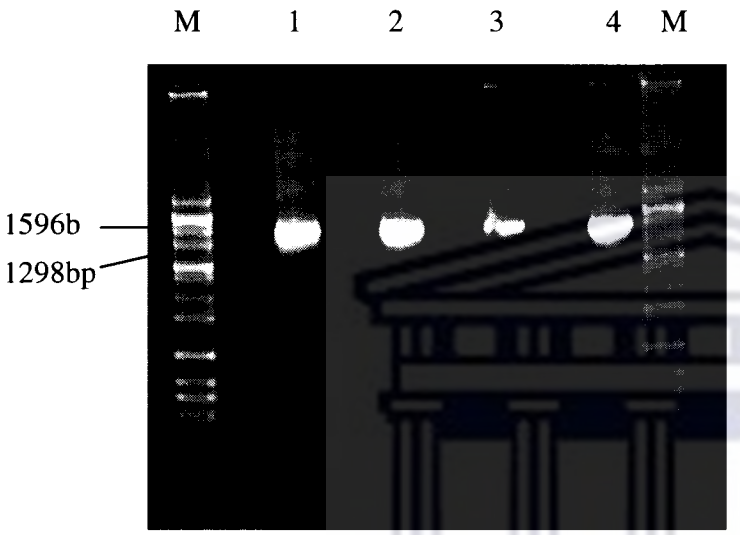

Figure 3.12: Colony PCR amplification of the 16S rRNA gene of the MVD isolate using T7 primers. Lanes M: DNA molecular weight marker (Pst1 digest of λ DNA), lanes 1-4: PCR products from four colonies transformed with the MVD 16S rRNA gene.

The resulting PCR products were purified for sequencing by electrophoresing the reaction volume on 1% agarose gel and excising the appropriate fragment for purification using a GFX kit.

3.4 Phylogenetic analysis

In this study the 16S rRNA gene sequences for the seven isolates were compared to existing sequences in the NCBI nucleotide database using BLAST (blastn).

Table 3.1 Blast results of the 16S rRNA sequences for all isolates showing the five closest relatives (type strains).

Isolate	Seq length	Closest relative	% identity	Accession
HA5	1442	*Streptomyces flavofungini*	98	EU273540.1
		Streptomyces violascens	98	EU273550.1
		Streptomyces argenteolus	98	EU048540.1
		Streptomyces sampsonii	98	EU273539.1
		Streptomyces levis	98	EU124569.1
HA3	1451	*Streptomyces viridochromogenes*	99	EU812168.1
		Streptomyces coeruleorubidus	99	EU841625.1
		Streptomyces griseorubens	99	EU593700.1
		Streptomyces tendae	99	EU570499.1
		Streptomyces pseudogriseolus	99	AB184516.1
HA8	1444	*Streptomyces chryseus*	98	EU593575.1
		Streptomyces helvaticus	97	AB184367.1
		Streptomyces albidochromogenes	97	AB249953.1
		Streptomyces flavidovirens	97	AB184270.1
		Streptomyces flaveus	97	AB184749.2
HA15	1308	*Streptomyces chryseus*	98	EU593575.1
		Streptomyces helvaticus	97	AB184367.1
		Streptomyces albidochromogenes	97	AB249953.1
		Streptomyces flavidovirens	97	AB184270.1
		Streptomyces flaveus	97	AB184749.2

MVS	1102	*Streptomyces atrolaccus*	99	AB184450.2
		Streptomyces tubercidicus	99	EF371435.1
		Streptomyces hygroscopicus	99	AB184818.1
		Streptomyces libani	99	AB184414.1
		Streptomyces nigrescens	99	AB184225.1
SAS	1131	*Streptomyces microflavus*	99	EU841675.1
		Streptomyces flaveus	99	AB184082.2
		Streptomyces lavendulae	99	AB184079.2
		Streptomyces anulatus	99	EU570444.1
		Streptomyces flavofuscus	99	EF178690.1
MVD	1112	*Nocardia fluminea*	99	EU593589.1
		Nocardia cummidelens	99	AF277202.1
		Nocardia soli	99	AF430051.1
		Nocardia salmonicida	99	AF430050.1
		Nocardia ignorata	99	AJ303008.1

The seven isolates were shown to be between 97 and 99% identical to reference sequences found in the database. Although a sequence identity value of 97% may be considered to confirm species designation (Stackebrandt and Goebel, 1994), confirmatory biochemical, physiological and morphological testing should be conducted before species identity of isolates is confirmed.

A partial 16S rDNA sequence for each isolate and the reference sequences obtained from the database were aligned with Clustal W featured in Bioedit version 6.0.5. Two neighbour joining phylogenetic trees (Figure 3.4 for the streptomycetes) and (Figure 3.5 for *Nocardia* species) were constructed using Mega version 4 (Tamura *et al.,* 2007). The scale bar of all trees represents a 0.1% difference in nucleotide sequences. Bootstrap values provide a measure of the reliability of the phylogenetic analysis.

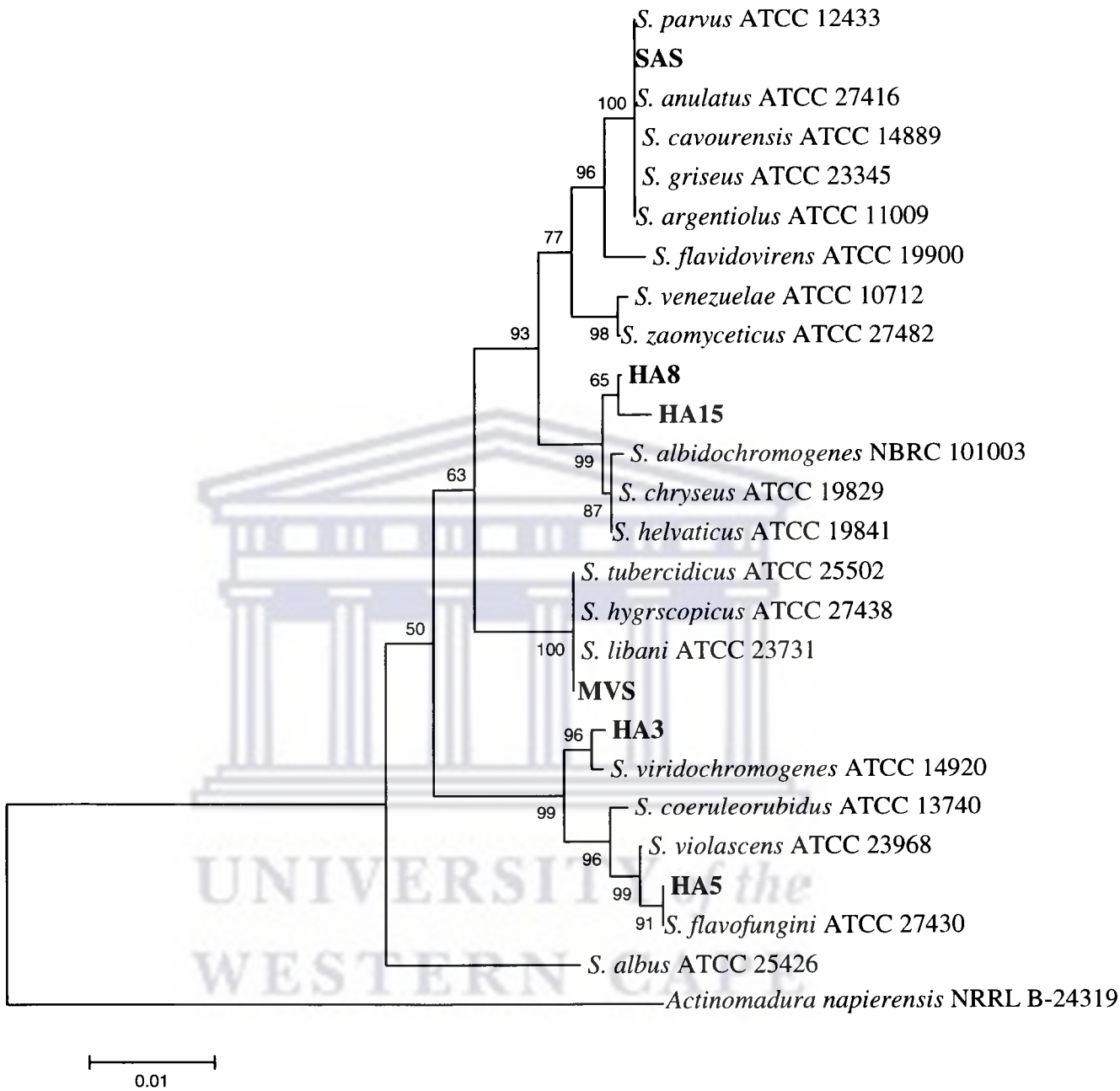

Figure 3.13: Neighbour joining distance tree using the aligned partial16S rRNA gene sequences of the six Streptomyces isolates and the type strains of the most closely related genera. Sequences from this study are shown in bold face. Boot strap values (in percent are shown at the nodes. *Actinomadura napierensis* was used to position the root of the tree.

Cluster analysis confirmed the results obtained from the BLAST search. The isolates HA3 and HA5 clustered together in the same clade. HA15 clustered with HA8, suggesting that these two isolates were the same. From the phylogenetic analysis it appeared that the isolates HA8 and HA15 formed a distinct arm in the *S. chryseus* clade, which was considered to be reasonably robust with a bootstrap value of 65%. Considering that the similarities of these isolates to their closest relatives were between 98 and 97%, it was suggested that the isolates HA8 and HA15 might be representatives of a new species.

Cluster analysis of the *Nocardia* tree demonstrated that the isolate MVD clustered in the *Nocardia salmonicida* clade which contains *Nocardia cummidelens, Nocardia fluminea, Nocardia salmonicida* and *Nocardia soli* (Figure 3.14). The species *N. salmonicida* is a known fish pathogen (Brown - Elliot *et al.*, 2006). The clustering of MVD in this clade is therefore not surprising as it was isolated from a soil sample collected from underneath a seal carcass. The isolated bacterium could have been a seal pathogen deposited into the soil underlying the carcass. The optimum growth temperatures obtained for MVD also strengthens the argument that the isolate might have been of seal origin. The failure to grow at 4°C suggests that the bacterium would not be metabolically active at Antarctic temperatures. The survival of the isolate would presumably be linked to spore formation.

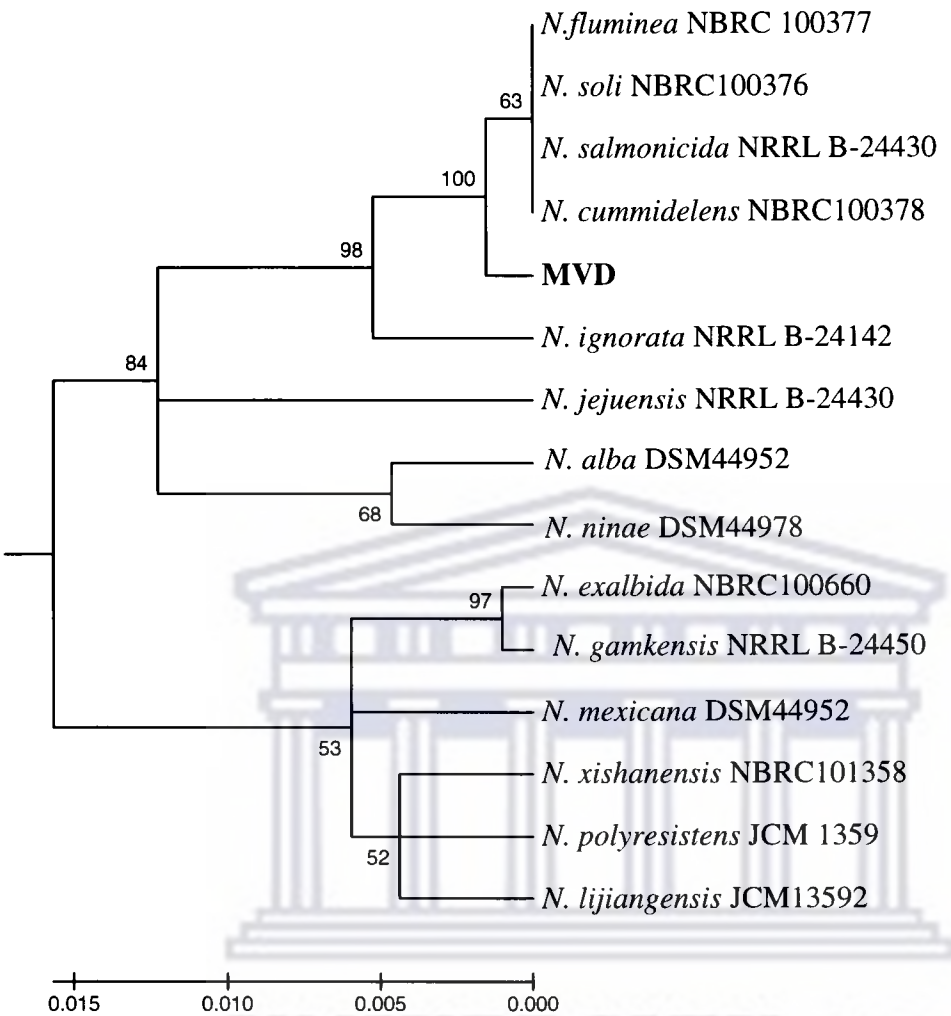

Figure 3.14: Phylogenetic tree showing position of MVD in the Nocardia tree based on the aligned partial 16S rRNA gene sequence analysis of all members of the genus Nocardia (only showing a sub-tree). Sequences from this study are shown in bold face. Boot strap values (in percent) are shown at the nodes.

3.5 Morphological and physiological characterization

The seven actinomycete colonies were characterized morphologically and physiologically according to methods outlined by the International *Streptomyces* Project (ISP) (Shirling and Göttlieb, 1966).

TABLE 3.2: Morphological and Physiological characterization of the isolates.

Characterstic	HA3	HA5	HA8	HA15	MVD	MVS	SAS
Spore morphology	Straight	Straight	Straight	Straight	Spiral	Spiral	Rectiflex us
Colour of aerial mycelium	Cream	White	White	Cream	Brown	Grey	Greyish yellow to yellowish brown
Colour of substrate mycelium	Cream	Cream	Cream	Cream	Yellow	Cream	Orange
Diffusable pigments	–	–	–	–	–	–	–
Melanin	–	–	–	–	–	–	+
Utilization as sole carbon source:							
D-mannitol	–	Wk+	–	–	+	+	++
Raffinose	–	–	–	–	–	+	–
Arabinose	Wk+	Wk+	–	–	+	–	–
Xylose	–	–	–	–	++	Wk+	+
Innositol	Wk+	–	+	Wk+	++	+	–
Sucrose	Wk+	–	–	–	Wk+	+	–

Key 1: for morphological observations: - characteristic absent
+ characteristic present

2: for carbon utilization - no growth observed
+ growth similar to positive control (glucose)
++ growth better than positive control
Wk+ growth present but less than positive control

The morphological and physiological characteristics of the isolates are shown in Table 3.2. These characteristics were compared to the published characteristics of their closest relatives. Isolate HA5 was shown by 16S rRNA phylogenetic analysis to be most closely related to *Streptomyces flavofungini* with a sequence homology of 98% (Table 3.1 and Figure 3.13). The morphological characteristics of this isolate were compared to those described for *S. flavofungini* (Shirling and Göttlieb, 1968). The colours of both the aerial and substrate mycelia of HA5 were very similar to those described. HA5 was observed to sporulate poorly on both ISP4 and ISP5 media, which usually stimulates prolific sporulation, behavior also reported for *S. flavofungini*. HA5 does not produce diffusible or melanoid pigments, findings consistent with the findings of Shirling and Göttlieb (1968) that only 66% of *S. flavofungini* strains produced pigment.

There was however a notable difference between the carbon utilization results reported for *S. flavofungini* and those observed for HA5. While *S. flavofungini* was reported to utilize glucose, arabinose, xylose, inositol, mannitol and raffinose, HA5 did not utilize inositol, raffinose and xylose and only weakly utilized arabinose and mannitol. Very little growth was observed with sucrose, which is similar to that observed with *S. flavofungini*. The difference in the carbon utilization results may be as result of adaptation of the different isolates to different environments. Xylose is a sugar found in the embryos of most edible plants whilst raffinose is a trisaccharide composed of galactose, fructose, and glucose which is found in many vegetables and whole grains. Since there are no higher plants in the Dry Valleys of Antarctica from which the soil samples for this study were collected, and with the knowledge that the soils are oligotrophic, it can be assumed that these sugars are not available in the environment. The genes

that code for the enzymes which degrade these sugars may have been inactivated or lost. With the advent of whole genome sequencing studies, it has become apparent that as organisms adapt to the environment, deterioration and loss of many genes that are not required in the environment occurs and there are very few genes that are conserved (Casjens, 1998, Cases *et al.*, 2003).

The morphological characteristics of isolate MVS showed great similarity to those described for the type strains *S. tubercidicus, S. hygroscopicus* and *S. libani,* all of which had 99% 16S rRNA sequence similarity to the isolate (Table 3.1). Although MVS is also closely related to *S. atroluccus,* the morphological and physiological information for this strain was not available for comparison. The isolate and its closest relatives all formed a very robust clade with a bootstrap value of 99% (Figure 3.13). Neither phylogenetic nor morphological characteristics observed in this study could distinctly classify the isolate MVS to the correct species.

Not all isolates were morphologically similar to the type strains that had been identified as their closest relative. Phylogenetically, HA3 is most closely related to *S. viridochromogenes* (Table 3.2). The spore chain morphology of *S. viridochromogenes* was reported to be spiral (Shirling and Gottlieb, 1968) while HA3 has straight spore chains (Table 3.2, Figure 3.15). Similarly, the colony colour observed for HA3 also differed greatly from the one described for *S. viridochromogenes* (Table 3.1). While the colonies colour of *S. viridochromogenes* were reported to be in the green, orange, yellow or blue colour series, HA3 colonies were pale cream with white aerial mycelia in all media (Table 3.2). *S. viridochromogenes* was reported to produce a green pigment in oatmeal agar (Shirling and Gottlieb, 1968), a characteristic also absent in HA3 colonies grown on the same medium (Table 3.2).

HA3 was also shown as being closely related to *S. coeruleorubidus, S. griseorubens, S. tendae* and *S. pseudogriseolus* all with 99% similarity (Table 3.2). Other than *S. griseorubens,* the were reported to have spiral spore chains (Shirling and Gottlieb, 1968).

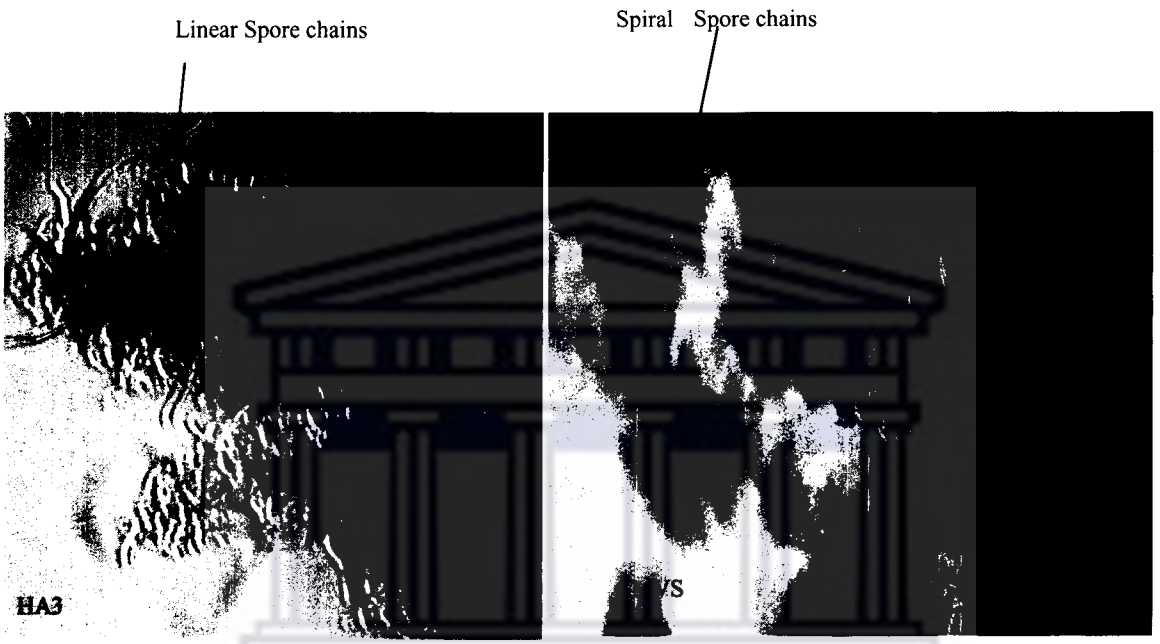

Figure 3.15: Spore chain morphology of HA3 and MVS as seen under a light microscope (x400 magnification).

It is therefore possible that the isolate HA3 does not belong to any of the 5 species that were identified as its closest relative.

Another isolate which exhibited different characteristics from those of its closest relative was SAS. The 16S rRNA gene sequence BLAST results identified *Streptomyces microflavus* as being the closest relative with 99% homology (Table 3.1). Colonies of SAS were grayish yellow to yellowish brown on yeast agar, oatmeal agar, salt starch agar and glycerol- asparagine agar when viewed from the underside of the colony. The aerial mycelia of *S. microflavus* are, however,

reported to be grayish yellow (Shirling and Gottlieb, 1968) whilst the isolate SAS had bright orange aerial mycelia. No melanoid pigments are reported for *S. microflavus*, but melanin was clearly produced in SAS (Figure 3.16).

Figure 3.16: Investigating melanin production by isolate SAS. Plate (A): Isolate SAS growing on ISP6 medium. Plate B: negative control.

Comparison between the physiological observations and 16S rRNA gene sequence analysis was therefore considered inconclusive in assigning the isolate to *S. microflavus*. The isolate also showed 99% similarity to *S. flaveus, S. lavendulae, S. anulatus* and *S. flavofuscus*. The morphological characteristics for the type strain *S. flaveus* could not be obtained for comparison, so the isolate was compared to the type strain *S. lavendulae* (Shirling and Gottlieb, 1968). The observed characteristics of SAS were very similar to those reported for *S. lavendulae* and thus the isolate might be a member of this species.

The isolates HA8 and HA15 showed 98% similarity with *S. chryseus*. The type strain was reported to have spiral spore chains (Shirling and Gottlieb, 1968) and yet the spore chain morphology of both HA8 and HA15 were observed to be straight (Table 3.2). Unlike *S. chryseus*

colonies, the colony colours of neither HA8 nor HA15 were in the red colour series. The observed morphology bore some resemblance to the characteristics described for *S. helvaticus* (Shirling and Gottlieb, 1968), but because the percentage similarity from phylogenetic analysis was 97%, it was speculated that these isolates might be a new species. The type strains *S. chryseus* and *S. helvaticus* were obtained for direct comparison with these two strains. There were notable differences between the type strains and the isolates, including the colony morphologies, and the appearance of aerial mycelia (data not shown).

The comparison of the results obtained from phylogenetic analysis to those obtained from morphological studies showed that it is not sufficient to use a single method when trying to identify actinomycetes to species level. However, it is not uncommon for the two methods that were used in this study to yield different results. Although the genus *Thermoactinomyces* had been classified as thermophilic actinomycetes on the basis of morphological characteristics, such as production of aerial and substrate mycelia, this genus was reported to be more closely related to *Bacillus* species than to actinomycetes by analysis of 16Sr RNA sequences (Song *et al.*, 2001).

Both methods used in this study (morphological characteristic and phylogenetic analyses) have limitations. One of the limitations of 16S rRNA analysis is that it is not always possible to find variable regions that can be used to distinguish different species in the same genus. As more sequence information becomes available, it is becoming evident that the resolution power of 16S rRNA sequences is limited when closely related organisms are being studied (Stackebrandt and Goebel, 1994).

Traditionally, chemotaxomy was an important tool in the classification of actinomycetes. The methods detect differences in cell wall and cell envelop structures and include cell wall chemotype, phospholipid type, menaquinone and fatty acid composition, as well as the presence of and type of mycolic acids present (Naumova *et al.,* 2000). These methods are however not routinely used because they are time consuming, cumbersome and are considered to have limited diagnostic value. Other techniques that have also been used in accurate identification of actinomycetes include DNA- DNA hybridization and ribotyping (Laurent *et al.,* 1996).

3.6 Distribution of HA8 in different soil samples

Isolate HA8 was interesting, not only because it appeared to be novel, but because it appeared to be abundant on all isolation plates. The distribution of this isolate in different continental (Antarctic, Marion Island and South African) soil samples was investigated. HA8 species specific primers were designed by aligning the 16S rRNA gene sequences of HA8 and its closest relatives. The alignment revealed that HA8 has a section of 14 nucleotides which were not present in any of the closest relatives. This region was used to design the forward primer 5′CGACTGGGTTTTCCCGGACG3′ which binds to positions 382-401 on the HA8 16S rRNA gene (Figure 3.17). Analysis of this primer using the database BLAST showed that it was very specific to the isolate HA8. The *Streptomyces* specific primer Strep F 5′ACGTGTGCAGCCCAAGACA3′ was used as the reverse primer.

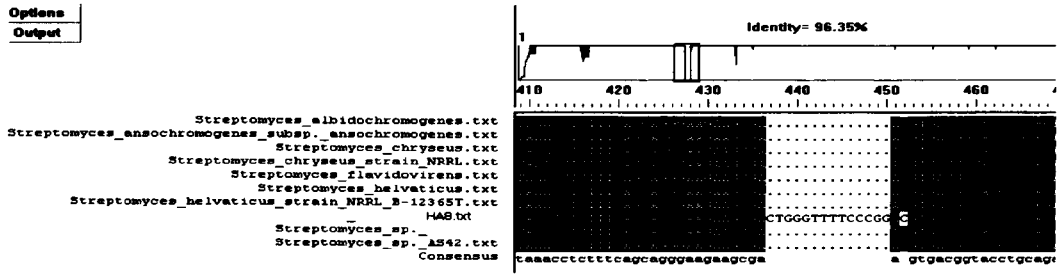

Figure 3.17: Alignment of the 16S rRNA gene sequences of HA8 and its closest relatives.

Metagenomic DNA was isolated from soil samples and 16Sr RNA genes were amplified using universal primers (E9F and U1510R). Bacterial DNA was amplified from all soils (Figure 3.18).

Figure 3.18: PCR amplification of the 16S rRNA genes from soil metagenomic DNA. The primers used were the universal primers E9F and U1510R. Lane M: λ DNA cut with Pst1. Lanes 1-2: Soil samples from the University of the Western Cape grounds. Lanes 3-8: Antarctic Dry Valley soil samples. Lanes 9-12: Marion Island soil samples.

The PCR products were purified by excising the appropriately sized band (= 1500bp) and using a GFX PCR and Gel Band purification kit. The purified products were used as templates for a nested PCR using the HA8 specific primers (Figure 3.19).

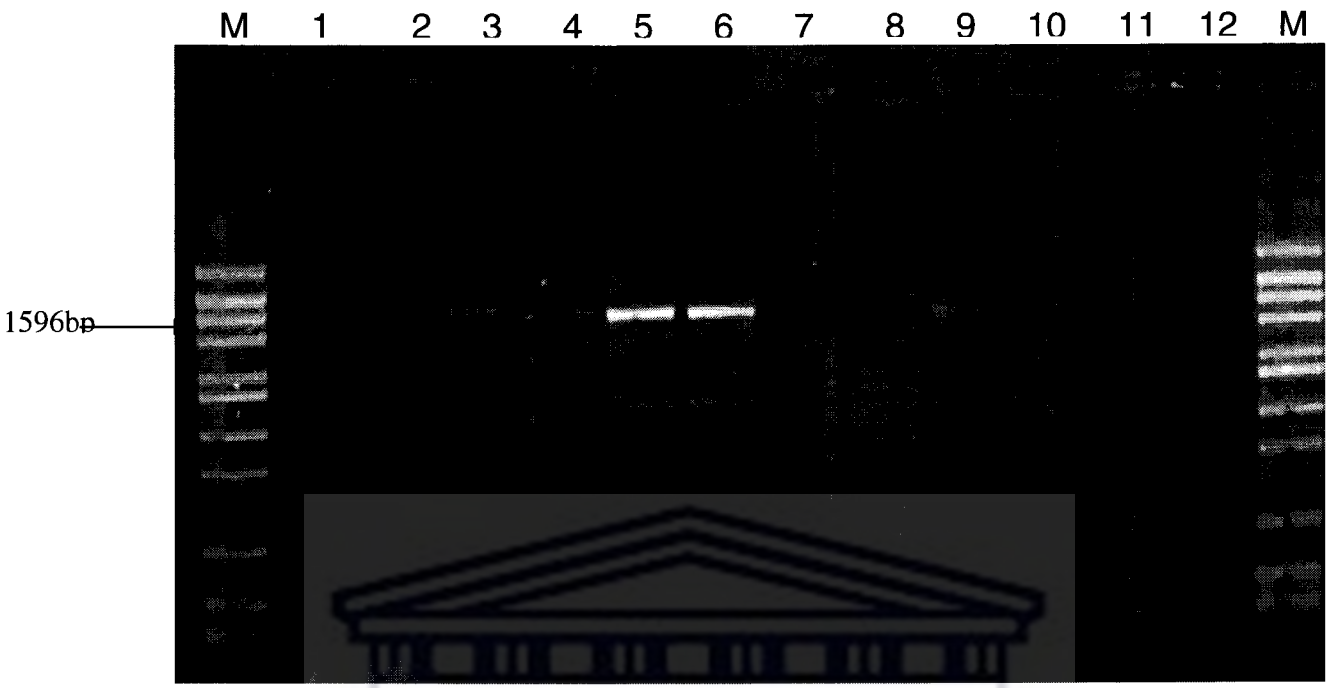

Figure 3.19: 16S rRNA amplicons of PCR using HA8 specific primers. The primers used were the HA8 specific forward primer and Strep F reverse primer. Lane M: λ DNA cut with Pst1. Lanes 1-2: Soil samples from the University of the Western Cape grounds. Lanes 3-8: Antarctic Dry Valley soil samples. Lanes 9-12: Marion Island soil samples.

No amplification was observed in soil samples collected from the University of the Western Cape grounds. Four of the six Antarctic soil samples (Figure 3.19 lanes 3-6) showed successful amplification of the HA8 16S rRNA gene. One soil sample from Marion Island showed positive results (Figure 3.19 lane 9).

The products of the nested PCR reactions were cloned into *E. coli* using the pGEMT cloning vector. The PCR reactions that had not shown any amplification were used as negative controls. As expected, no positive (white) colonies were observed on the negative control plates. Positive transformants were observed on all Antarctic and Marion Island samples. Colony PCR was

performed on positive transformants using M13 primers in order to amplify the insert DNA.

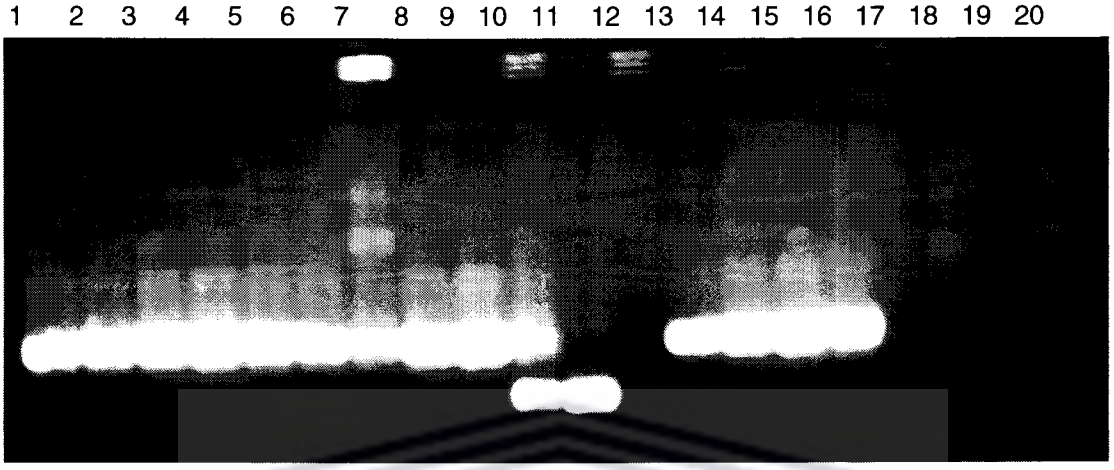

Figure 3.20: Colony PCR amplicons of randomly chosen colonies following cloning of 16S rRNA genes obtained from metagenomic samples using HA8 specific primers. Lanes 1-16: positive transformants. Lanes 17-20: negative controls.

The results obtained (Figure 3.20) showed that the insert was successfully amplified. The PCR amplicons in lanes 10 and 11 were smaller than expected. The PCR products were purified and sequenced. The sequence results confirmed that the amplicons were 100% similar to those previously obtained for the HA8 16S rRNA gene sequences. HA8 was therefore considered to be widely distributed in Antarctic and Marion Island soils. This wide distribution of the isolate implies that the isolate is not a newly introduced contaminant but could possibly be indigenous to that region or has been in the region long enough to be widely dispersed.

Chapter 4: Antibiotic screening

4.1 Screening isolates for antimicrobial activity

Actinomycetes produce a variety of antimicrobial agents including many of those important in medicine, such as aminoglycosides, anthracyclines, chloramphenicol, β-lactams, macrolides, tetracyclines etc. (Gupte *et al.*, 2002). The isolates obtained in this study were screened for antimicrobial activity against 10 bacterial strains. Isolates were grown on different liquid media and on agar plates and antimicrobial activity was observed after 7, 14, and 21 days. Results of the screening suggested that one of the seven isolates (HA8) showed good activity against eight of the ten bacterial strains tested. It was noted that isolate SAS produced compounds that inhibited the growth of an unidentified fungal contaminant (data not shown).

Isolate HA8 exhibited antimicrobial activity against all the test bacteria using the solvent extracts from the fermentation broths in the disc diffusion method (Figure 4.1). Stab cultures grown on agar plates did not exhibit any antibacterial properties against the test bacteria. The antimicrobial agents were extracted using solvents with different polarities. Polar compounds would be extracted using methanol. These could only be extracted from the cells.

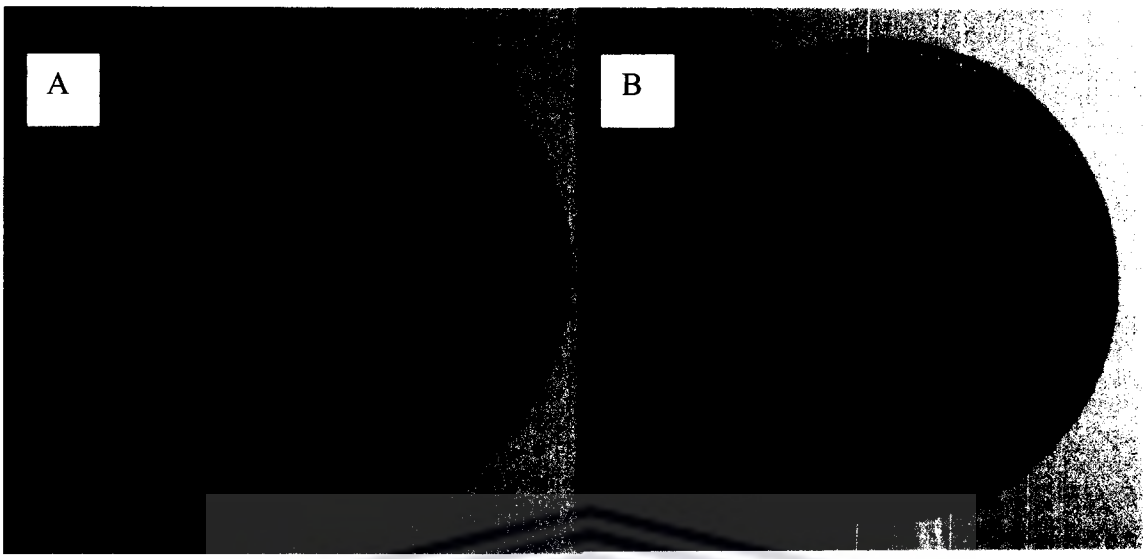

Fig 4.1: Disc diffusion plates showing zones of inhibition on bacterial lawns of (A) *Proteus mirabilis* strain 87 grown in *Streptosporangium* medium and (B) *Klebsiella pneumoniae* K11 grown in 7H9 media. The disc in the center of the plate is impregnated with Ampicillin (positive control).

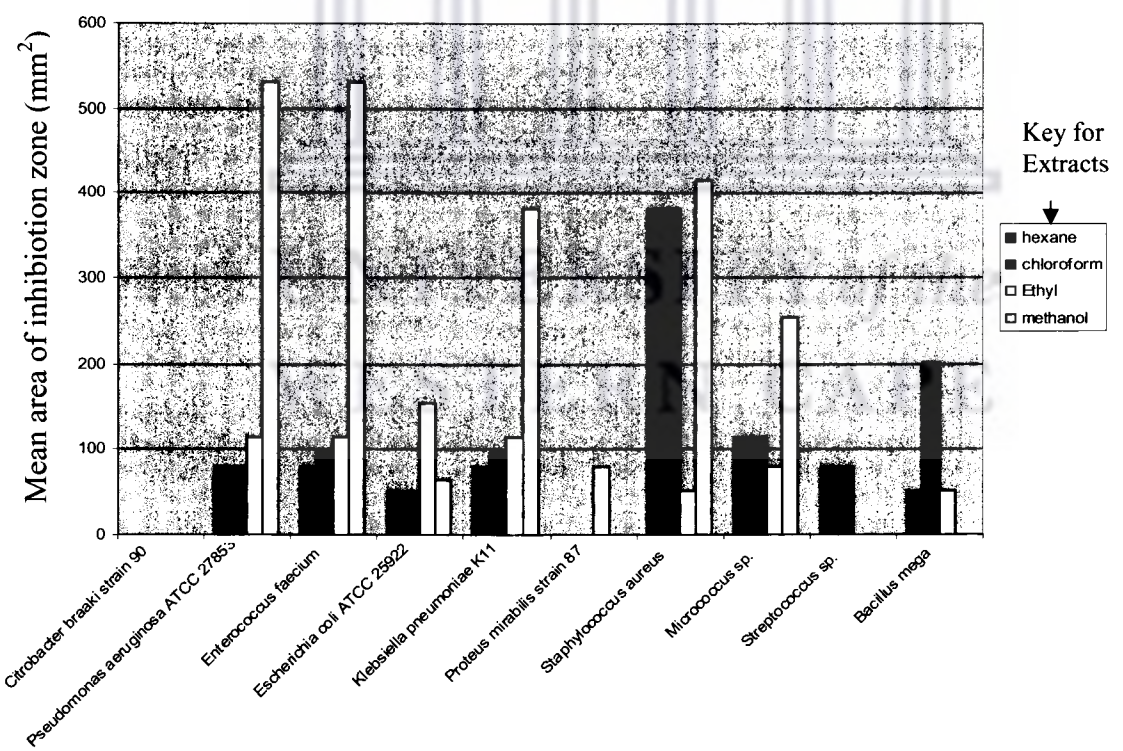

Figure 4.2: Antimicrobial activity of the solvent extracts from isolate HA8 culture filtrates on the test organisms. The mean area of inhibition zones from two replicate plates are reported.

Table 4.1: Antimicrobial activity of HA8 against the test bacteria in different culture media. The results are reported as diameters (mm) of inhibition zones.

Test organisms	Growth media used															
	HA media				ISP5 media				7H9 media				*Streptosporangium* media			
	Hexane	Chloroform	Ethylacetate	Methanol	Hexane	Chloroform	Ethylacetate	Methanol	Hexane	Chloroform	Ethylacetate	Methanol	Hexane	Chloroform	Ethylacetate	Methanol
Citrobacter braaki strain 90	10	-	11	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa ATCC 27853	-	11	-	12	10	10	10	8	10	10	8	23	10	10	12	26
Enterococcus faecium	10	12	10	16	12	10	11	13	12	12	12	23	10	11	12	26
Escherichia coli ATCC 25922	-	9	-	8	8	12	10	9	-	-	-	-	8	8	14	9
Klebsiella pneumoniae K11	16	10	8	-	14	-	9	16	-	-	16	-	10	11	12	22
Proteus mirabilis strain 87	-	9	-	-	-	10	-	-	9	10	-	-	-	16	10	-
Staphylococcus aureus	16	12	18	22	14	10	12	12	8	-	10	12	22	10	8	23
Micrococcus sp.	18	18	10	18	24	22	10	20	14	16	16	18	12	16	10	28
Streptococcus sp.	-	10	-	14	9	8	8	9	25	10	8	20	10	12	-	-
Bacillus megaterium	-	-	-	-	-	10	-	-	10	-	-	-	8	16	8	-

Key: - means no inhibition zone observed
8 mean diameter of inhibition zone= 8mm c

The patterns of antimicrobial activity exhibited by the different solvent extracts from HA8 culture filtrates on different test organisms (Figure 4.2) suggests the presence of more than one compound, one polar and the other non-polar. Activity against *P. mirabilis, Streptococcus* sp, and *B. megaterium* was observed with non-polar extracts, with weak activity observed against *E. coli*. The chloroform extracts showed to be most active against *Proteus mirabilis* (Table 4.1) and in some fermentation broths for example HA and ISP5 media, activity was only observed in the chloroform extracts. The absence of activity against *Streptococcus* in both the methanol and the ethyl acetate extracts from *Streptosporangium* media also supports the suggestion that there was more than one compound present.

The results from both Table 4.1 and Figure 4.2 indicate that methanol extracts had larger inhibition zones than other extracts, possibly implying either higher concentration of polar compounds or greater potency. The absence of activity against *E. coli* in all extracts obtained from 7H9 suggested that the media has an effect on production of antimicrobial compounds. Extracts obtained from *Streptosporangium* broth were observed to exhibit better activity that other media. This might be due to the variety of carbon sources present in this medium, as compared to the other media which contained only one source. Studies have shown that the carbon source available affects the antibiotic yield. Glycerol and glucose (both of which are present in *Streptosporangia* medium) have been reported as ideal carbon sources for optimum production of a variety of antibiotics (Pandeyi *et al.,* 2005, Bhattacharyya *et al.,* 1998, Vasavada *et al.,* 2006). Sporulation has also been linked to antibiotic production in actinomycetes (Martinez *et al.,* 2005, Hoskisson *et al.,* 2001). The *Streptosporangium* medium was observed to give the

best sporulation (section 3.2) and this may be correlated to the higher yields of antibiotic production.

Citrobacter braaki showed highest resistance to the antimicrobial compounds produced by isolate HA8. This led to the speculation that the antimicrobial agents produced were structurally related to either cephalosporin or penicillin, as *Citrobacter* species are Gram -negative bacteria that are often resistant to cephalosporins due to the overexpression of their chromosomal ß-lactamase genes (Pepperell *et al.,* 2002).

4.2 Antibiotic biosynthetic genes

Studies of the biosynthesis pathways involved in production of natural products have been an important strategy in estimating the biosynthetic potential of streptomycetes (Webe *et al.,* 2003). Glycopeptides such as vancomycin and teicoplanin and type-II polyketides such as oxytetracycline, tetracenomycin and erythromycin are important classes of antibiotics produced by actinomycetes (Cox *et al.,* 1997). The total genomic DNA of the isolates in this study was screened for the biosynthetic genes involved in the production of glycopeptide and Type-II polyketide antibiotics, in order to investigate their potential for production of those antibiotics.

Screening for the genes involved in the production of glycopeptide antibiotics was achieved by PCR amplification of the *oxy B* gene. *OxyB* is a cytochrome P450 enzyme that is responsible for catalyzing the cross-linking of amino acid residues during glycopeptide antibiotic biosynthesis (Donadio *et al.,* 2005, Pootoolal *et al.,* 2002). The *oxyB* gene is therefore an important gene in

strains that produce glycopeptide antibiotics. The primers used were designed using the *oxyB* sequences of *Amycolatopsis balhimycina, Amycolatopsis orientalis* and *Streptomyces toyocaensis* (Wood *et al.,* 2006). Sequence alignment using DNA Man showed that the primers bind to position 383-400 and 1065-1079 of the *Streptomyces toyocaensis oxyB* gene, respectively and amplify a 696bp region. The oxyB gene was not successfully amplified in genomic DNA from HA8 (data not shown).

Isolates were also screened for the Type II polyketide gene clusters. Type II polyketide synthases (PKSs) are a family of multi-enzyme systems that catalyze the biosynthesis of aromatic natural products such as actinorhodin (produced by *Streptomyces coelicolor),* tetracenomycin (produced by *Streptomyces glaucescens*) and oxytetracycline (produced by *Streptomyces rimosus*) (Shapiro, 1989). PCR screening for the polyketide biosynthetic genes was performed using primers designed to amplify the ketosynthase alpha (KSα) and ketosynthase beta (KSβ) tandem (Figure 4.3) (Wood *et a.,l* 2006, Ichinose *et al.* 2003). Amplification by PCR was expected to yield a fragment of 492–630 bp (Wood *et al.,* 2006).

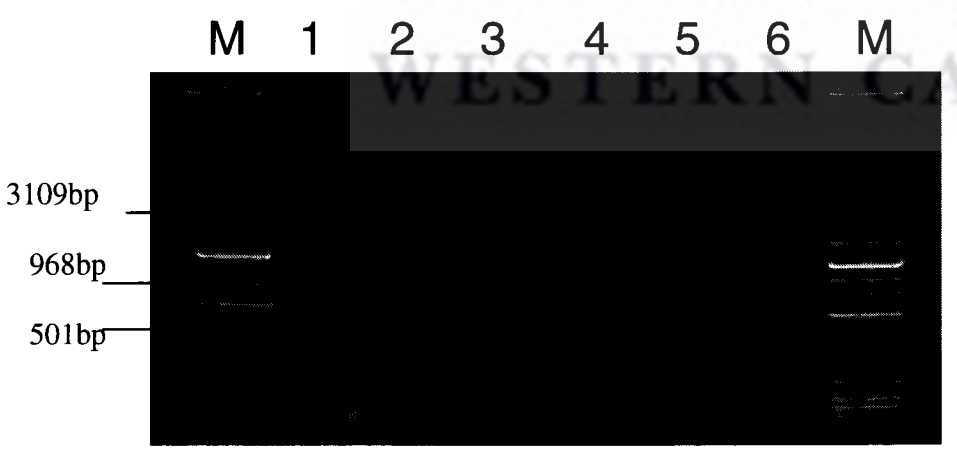

Figure 4.3: PCR amplification of polyketide synthase tandem-gene (KSα and KSβ) of actinomycete isolates. Lanes M: Molecular weight maker (λDNA cut with PstI). Lane 1: HA3. Lane 2: HA5. Lane3: HA8. Lane 4: HA15. Lane 4: MVD. Lane 5: MVS. Lane 6 SAS.

PCR screening for the polyketide synthase tandem gene indicated that the gene was not present in isolate HA8 (Figure 4.3 Lane 3). However, amplification of the required gene fragment from isolate HA3 (lane 1) was observed. Isolate HA3 did not exhibit any antimicrobial activity against any of the test bacteria. This positive amplification may mean that isolate HA3 produced other agents that are active against microorganisms not used in this study. These could include antifungal compounds. It should also be noted that the primers used to amplify the gene may also amplify a KSα–KSβ fragment from genes involved in spore-pigment synthesis. The observed signal might therefore be amplification of the gene involved in spore pigment synthesis and not antibiotic synthesis. These primers have been reported to amplify a 510-bp fragment from the spore-pigment polyketide synthase gene cluster of *Streptomyces avermitilis* ATCC 31267T, AB070937 (Wood *et al.*, 2006).

4.3 Thin layer chromatography and bioautography

Thin layer chromatography on silica gel was used as a tool for separating the constituents of the crude extracts of isolate HA8. The coupling of thin-layer chromatography with microbiological detection (bioautography) has been used for the identification and quantification of several antibiotics (Neidert *et al.*, 1987). The main benefit of bioautography is that it combines chromatographic separation and in-situ activity determination, thus providing information about antimicrobial activities of substances separated from a mixture. In this study direct bioautogrphy was used. A developed TLC plate (Figure 4.4) was dipped in the suspension of the microorganism growing in LB broth. The plate was incubated and microorganisms grew directly on it. For location and visualization of antibiotic compounds, the tetrazolium dye 3-{4,5-

dimethylthiazol-2-yl}-2,5-diphenyltetrazolium bromide (MTT) was used. Tetrazolium salts are converted by the dehydrogenases of living microorganisms to intensely coloured formazan (Schillaci *et al.*, 2005). Where antimicrobial compounds are present, the bacteria are killed and colour is not produced. Zones of inhibition show as pale areas on a coloured background (Figure 4.5).

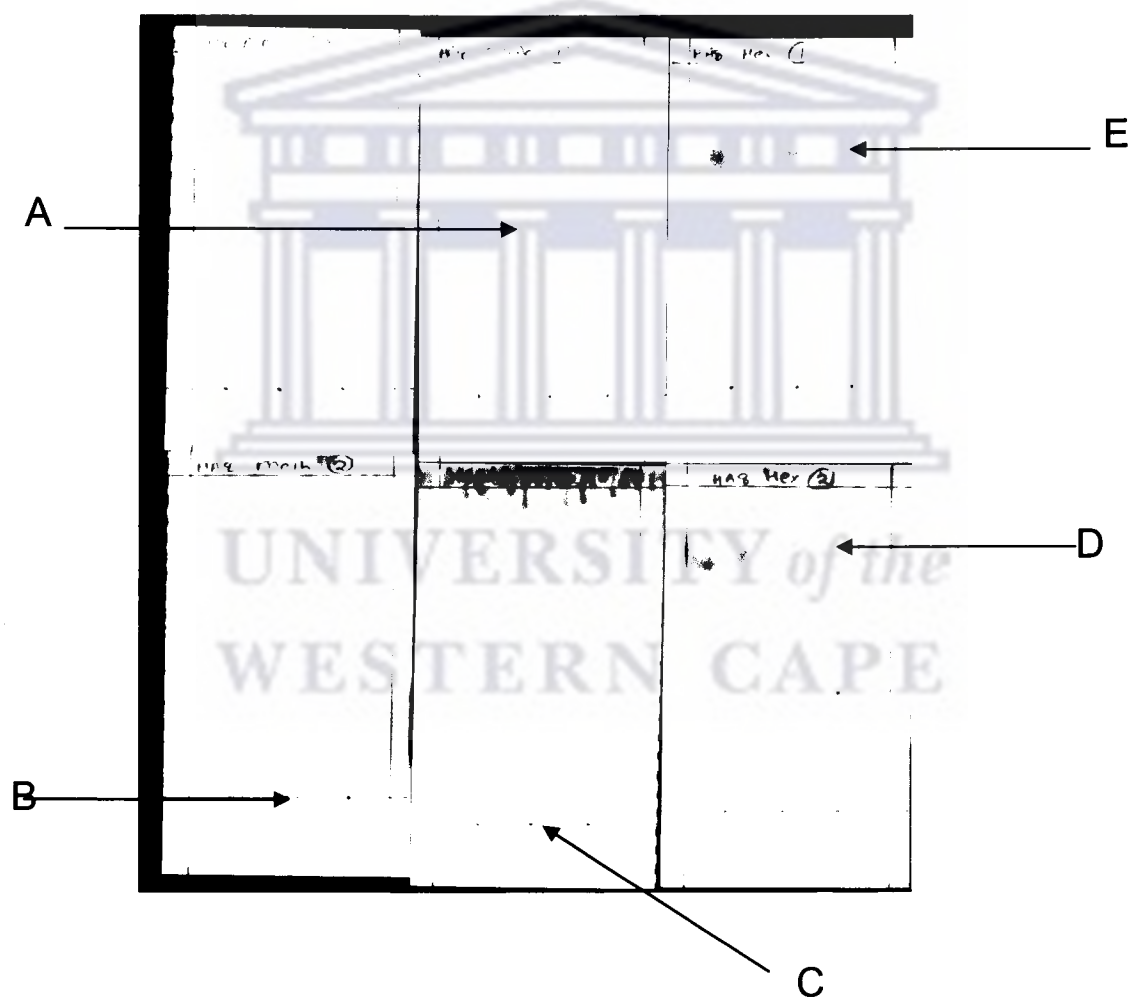

Figure 4.4: Thin layer chromatograms of HA8 solvent extracts developed using cerium (IV) ammonium sulphate. The arrows A to E point to the areas where spots were observed.

The bioautography results were compared to the thin layer chromatograms in an attempt to identify potential antimicrobial agents. However, regions where cells were inactive did not correspond to regions of the chromatogram where TLC spots were observed hence positive identification of the antimicrobial agents was not possible. This could be because the antimicrobial compounds in the crude extracts comprised only a very small fraction of the total extract.

The results of the TLC and the bioautography might have been more comparable if more specific reagents like phenylhydrazine sulfonate, which detects a variety of antibiotics or sodium azide which detects penicillins and cephlosporins had been used (Jork *et al.*, 1990).

4.5 High performance liquid chromatography

High performance liquid chromatography was used in attempt to purify the antimicrobial compounds from the crude solvent extract of isolate HA8 culture filtrate. Forty fractions were collected manually over the course of the run. The profile (Figure 4.6a) shows that purification of the constituents of the crude extracts was not successful. The broad based peaks in Figure 4.6a imply that a variety of compounds were eluted together.

Chapter 5: General discussion and concluding remarks

Antarctic Dry Valley soils are characterized by low water contents ranging from 0.2-5.0% w/w (Horowitz *et al.*, 1972), mean annual temperatures of <-20°C, desiccating winds, diurnal freeze-thaw cycles, and high seasonal UV radiation (Wynn-Williams, 1990). It was predicted that the Antarctic terrestrial environment would be a rich source of both novel bacterial species and rare genera of Actinobacteria, an assumption supported by recent phylogenetic studies (Rodriguez-Valera, 2004). This study aimed to isolate, characterize and screen novel actinomycetes from Antarctic soil samples. It is believed though that only 1% of terrestrial microorganisms have been cultured using conventional methods (Kennedy *et al.*, 2008). It is therefore necessary to modify culture based methods in order to access organisms which do not readily grow on standard media. In this study, different isolation media including *Streptosporangium* media modified for Antarctic isolates and media containing humic acid were used. In addition, isolation plates were incubated at various temperatures, including at 4°C, in order to isolate the obligate psychrophiles. Studies have shown that metagenomic based approaches also offer great potential in gaining access to protein-coding genes (Cowan *et al.*, 2005).

The isolation strategies in this study yielded 23 isolates belonging to seven different actinomycete species, six *Streptomyces* and one *Nocardia*. For the purpose of screening novel bioactive molecules in future studies, pretreatment methods such as the use of heat, phenol and media specific for rare actinomycete genera such as *Actinoplanes, Micromonospora, Microbispora*, and *Streptosporangium* must be considered.

It was interesting to note in this study that most of the strains isolated showed optimum growth at 30^0C. This has been reported in various other studies, were microorganisms isolated from Antarctic environments were mesophilic and not psychrophilic. In one study a novel actinomycete *Micromonospora endolithica* was isolated and characterized as having a temperature growth range of 8–39 °C, and an optimum of 27–29 °C (Hirsch *et al.,* 2004). In another study six actinomycete strains producing antibiotics were characterized. All six were observed to grow optimally at 25^0C (Moncheva *et al.,* 2002). One study has also reported the isolation of nine thermophilic and ten mesophilic actinomycete strains from Antarctic soil samples on agar medium (Gushterova *et al.,* 2005).

The identification of the actinomycetes isolated in this study was attempted using two methods, 16S rRNA gene sequence analysis and morphological and physiological characterization. Analysis of 16S rRNA gene sequences has been demonstrated to be a powerful method for investigating phylogeny of microorganisms (Cho and Tiedje, 2001). Morphological and physiological characterization is one of the traditional methods used in classification of bacteria (Shirling & Gottlieb, 1966). Other traditional methods of classification include chemotaxonomy based on characteristics such as cell wall chemotype, phospholipid type, menaquinone and fatty acid composition, as well as presence and type of mycolic acids (Ward and Goodfellow, 2004). These methods, however, are labour intensive and time consuming (Wang *et al.,* 1999) and were not attempted in this study.

The screening of isolates revealed one isolate, HA8, which produced antimicrobial agents active against eight of the ten bacterial strains tested. It was, however, observed that one isolate, SAS,

produced compounds that inhibited the growth of a contaminated unidentified fungus when growing on agar plates. In further studies, isolates should be screened for antimicrobial activity against a wider variety of test organisms including fungi.

The antimicrobial compounds produced by isolate HA8 could not be positively identified in this study. The amounts of antimicrobial compounds produced in this study were insufficient for purification. Analysis of the antimicrobial compounds in the crude extracts was difficult because the compounds might have been a very small proportion of the crude extracts. In further studies, the cultures need to be fermented in larger quantities so that larger amounts of the compounds can be produced and subsequently purified.

The PCR screening of isolates for genes involved in the synthesis of antibiotics can assist with information on the antibiotic biosynthetic potential of a strain. The technique can, however, only screen for genes involved in the biosynthesis of known antibiotic classes for which the biosynthetic gene sequences are publicly available. Antibiotics for which PCR screening primers cannot be designed will therefore be outside the scope of the screen. The design of PCR primers to detect characteristic genes for antibiotic biosynthesis should be based on a gene that is unique to the synthesis of that antibiotic class. Furthermore, the product of the target gene should be involved in the synthesis of the core antibiotic structure, so that the PCR primers will be universal for that class of antibiotic. This makes the applications of this technique limited.

In this study, actinomycetes were successfully isolated from Antarctic soil samples. However it was evident that a larger number of non-*Streptomyces* strains could have been obtained by using

enrichment methods. The isolate HA8 produced at least two different antimicrobial compounds that were active against both Gram positive and Gram negative bacteria. At least one other isolate SAS produced compounds that inhibited fungal growth. It is evident that isolates should also be screened for activity against various fungal strains.

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