Actinomycete biodiversity assessed by culture-based and metagenomic investigations of three distinct samples in Cape Town, South Africa

by

Muhammad Saeed Davids

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Abstract

Three independent study samples were used, two of which were aquatic and the third terrestrial. The first aquatic sample was taken from the Big Bay beach, Cape Town. Three individual samples were taken from this beach namely, rock-pool sand (RP), ocean sand (OS) and shore-line sand (SL). After employing the conventional culture-based isolation method on these three samples no actinomycete colonies were found. The second aquatic sample was taken from the University of Cape Town (UCT) freshwater dam. Two samples were taken from this area, surrounding sand (SS) and dam sand (DS). Again conventional culture based isolation techniques were used and no actinomycete colonies were seen. Attempts to isolate actinomycetes from aquatic samples were abandoned after this, and a terrestrial sample was chosen.

A sample was taken from the Rhodes Memorial at the foot of Devils peak, Cape Town. This sample yielded a total of fifty two (52) actinomycete colonies. All isolates were subjected to antibiotic screening tests and a rapid identification method. The results of which were analysed and 8 isolates were chosen for further characterisation. Four belonged to the genus *Streptomyces*, three of which (strains TZC2, TMC9 & UCZ4) were chosen based on high antibiotic activity, and the fourth (strain UCZ14) was chosen because it was initially thought to be a non-*Streptomyces* strain. The remaining four isolates were chose as they were non-*Streptomyces* strains. Three strains (TMB1, TMB2 and UCZ5) belonged to the genus *Amycolatopsis* and the last (TY1) belonged to the genus *Micromonospora*. Of the eight strains characterised, all the *Streptomyces* strains require further study, including DNA-DNA hybridization, to determine if any belong to new species. *Amycolatopsis* isolates TMB1 and TMB2 appear to belong to the same, novel species. The remaining two non-*Streptomyces* isolates, *Micromonospora* strain TY1 and *Amycolatopsis* strain UCZ5, both require further study (as well as DNA-DNA hybridization) to determine if they are a new species.

All of the samples used for actinobacterial isolation were subjected to a culture-independent (metagenomic) study. The results provided an explanation for why no actinomycetes were found in the aquatic samples, as all of the sequenced clones were shown to be most closely related to uncultured bacteria. In the terrestrial sample, a total of 120 clones were obtained and all were sent for sequencing. Of these clones, the majority (76%) were most closely related to uncultured bacteria. The most common genus was *Streptomyces* (8% of the clones), followed closely by *Micromonospora* (7% of the clones). Interestingly, no *Amycolatopsis* clones were identified, despite three *Amycolatopsis* strains being isolated from this source. Of all the clones obtained, three genera were particularly interesting, as they have very few described species and are thus rare. These genera were *Luedemannella*, *Actinophytocola* and *Blastococcus*.

CHAPTER 1

INTRODUCTION

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Introduction

1.1 Actinomycetes

Soil is a rich source of bacteria and fungi. In this study the most important are those from the class *Actinobacteria* (El-Tarabily & Sivasithamparam, 2006). Actinobacteria are Gram positive bacteria that respire aerobically, however there are cases of members which can grow under anaerobic conditions. Actinomycetes are filamentous actinobacteria, which have G+C rich DNA, form filamentous hyphae and asexual spores (Prescott *et al,* 2005) and have become a great source of active antimicrobial compounds (Hopwood, 2006). An example is streptomycin, which led to the further discovery of leading pharmaceutical drugs (Lazzarini *et al*, 2000).

Mycelia are a morphological feature of this order of bacteria and can be substrate mycelia, aerial mycelia or both. Conidia singly, in pairs or in chains are the asexual spores found in this group of bacteria and can be arranged in a variety of ways. *Micromonospora* and *Thermomonospora* represent two genera where the conidia are single. Pairs of conidia can be found in the genus *Microbispora*. Finally the conidia can also form short or long chains (up to 20 spores is considered short) as found in *Streptomyces* and *Amycolatopsis* among others (Goodfellow, 1989).

Actinomycetes have been a great source of antibiotics in the past (Duraipandiyan *et al*, 2010) and have provided almost half the discovered bioactive secondary metabolites (Lam, 2006). The order *Actinomycetales* belongs to the class *Actinobacteria* along with eight other orders. Within the order *Actinomycetales* there are 13 sub-orders and within these sub-orders there are over 42 families (Zhi *et al.*, 2009). In order to correctly identify actinomycetes to the genus level, 16S-rRNA gene phylogenetic analysis, morphology, physiology and chemotaxonomy are used. The phylogenetic analysis is used to determine the genus to which an isolate belongs and also provides an indication of the most closely related species in the genus. Physiological characteristics help with further identification and can be from a variety of features. The ability to grow under different conditions (pH, temperature and salinity) is one such test. Another test to differentiate species is the use of sole carbon and sole nitrogen sources. Enzymatic activity is also used and is determined by degradation of a variety of substrates (e.g. starch, cellulose and adenine). Diffusible pigment and melanin production, growth in the presence of inhibitory substances and antibiotic activity against different test organisms are other tests that can also be carried out to help with identification and differentiation (Williams *et al*, 1989).

Chemotaxonomic data is useful in differentiating between the genera of actinobacteria and can also be used to show differences between closely related species (e.g. fatty acid analysis). The whole-cell sugar pattern and isomer of diaminopimelic acid (DAP) in the peptidoglycan are widely determined characteristics. It should be noted that, although DAP occurs commonly as the diagnostic diamino acid in actinobacterial peptidoglycan, other diamino acids also occur, namely, L-lysine (e.g. *Arthrobacter* and *Promicromonospora*), 2,4-diaminobutyric acid (e.g. *Leifsonia*) and ornithine (e.g. *Microbacterium*). The presence or absence of mycolic acids, the cell membrane fatty acid profile, the menaquinone profile and the phospholipid profile are the other chemotaxonomic characteristics used in identification. These tests have limitations, in that they are mainly useful in identification to the genus level. As a result, chemotaxonomy is routinely used to confirm genus appointment based on 16S ribosomal RNA (rRNA) gene analysis (Williams *et al*, 1989; Lechevalier, 1989).

Because of the limitations mentioned above, the 16S-rRNA gene is used as a much more accurate way of identification, with the morphological, physiological and chemotaxonomic tests used to supplement this identification. The analysis of the 16S-rRNA gene can quickly identify an isolate down to the genus level along with its closest relatives (Goodfellow, 1989). 16S-rRNA gene analysis is also used to differentiate between species within a genus. If the 16S-rRNA gene sequence similarity between two strains is below the accepted set threshold of 97% and there are phenotypic differences between them, then they are considered to belong to different species (Stackebrandt & Goebel, 1994). Recently, Stackebrandt and Ebers proposed that the minimum 16S-rRNA gene sequence similarity threshold be raised to 98.7% before DNA-DNA hybridization (DDH) methods are employed (Stackebrandt & Ebers, 2006) to determine whether the strains belong to different genomic species. In cases where 16S rRNA gene sequence similarity between two strains is above 98.7%, DDH is used to determine whether these two strains belong to different genomic species. In DDH, species need to display phenotypic differences as well as a DNA relatedness of less than 70% (with a ΔT_m >5°C) in order to be considered different genomic species (Wayne *et al*, 1987). Besides the 16S-rRNA gene other protein coding genes have been proposed to allow species differentiation. One such gene is the gyrase β-subunit encoding gene, *gyrB*. It has already been successfully implemented in the discrimination of the closely related strains in the genus *Pseudomonus*, it has also been shown that the gyrB-based grouping of the genera *Acinetobacter* and *Micromonospora* are consistent with that of DNA-DNA hybridisation results (Kasai *et al.*, 2000). The reasoning behind the use of this gene is owed to its high evolution rate thus allowing better discrimination and resolution between closely related species (Volokhov *et al.*, 2007).

Actinomycetes are commonly found in soil (Oskay *et al*, 2004), however they have also been isolated in other places, such as from marine and freshwater samples. Since such sources have not been as extensively studied as soil, they offer a much better chance of discovering new species of actinomycetes which, in turn, means a higher chance of discovering potentially new antibiotics. For this reason, marine samples offer an opportunity to isolate new bioactive molecules. The diversity of terrestrial life is undoubtedly high, however in terms of actinomycete discovery there has been a definite decrease in the discovery of novel species and a rise in re-isolations (Lam, 2006). Haefner (2003) stated that there is possibly more biodiversity in the oceans than in terrestrial environments (oceans make up 70% of the planet's surface) Furthermore, the marine environment offers unique environmental circumstances, which could lead to unique compounds being synthesised by the microorganisms that live there. For example, any compound made and released by a microorganism in the sea is rapidly and greatly diluted and so must therefore be highly potent to counteract this effect.

It is clear that the marine world offers a great reservoir of potentially novel and highly active bioactive molecules. Examples of this potential can clearly be seen by the new marine genera that have been discovered such as *Salinispora* and *Demequina* (Bull & Stach, 2007). Along with those two described genera there are others which are awaiting formal taxonomic description, e.g. '*Marinispora*', '*Lamerjespora*' and '*Solwaraspora*' (Bull & Stach, 2007; Lam, 2006). The most exciting of these discoveries is *Salinispora* since it is the first obligatory marine actinomycete to be described. It has been shown by Fenical's research group (Jensen *et al*, 2005) that this genus is widespread in oceanic sediments. It has also been found in the Great Barrier Reef and to date two species from this genus have been described, namely, *Salinispora arenicola* and *Salinispora tropica* (Euzéby, 2010; Maldonado *et al*, 2005). As stated before, the benefits of searching for actinomycetes from aquatic samples has the potential to yield novel antibiotics. This point can be illustrated by briefly discussing what antibiotics have been discovered from marine actinobacteria.

Four compounds are of interest, abyssomicin C, diazepinomicin, salinosporamide A and the sporolides. Abyssomicin C was isolated from a marine *Verrucosispora* strain and is a polycyclic polyketide antibiotic. It has potent activity against Gram-positive bacteria as well as activity against multiple-resistant and vancomycin-resistant strains of *Staphylococcus aureus*. Diazepinomicin is a farnesylated dibenzodiazepinone, which is produced by a marine *Micromonospora* strain and shows antibacterial, anti-inflammatory and antitumour activity. Salinosporamide A is produced by *S. tropica* and is a β-lactone-γ-lactam and seems to be a potent anticancer drug. What makes it even more special is that it is the first antibiotic from an obligate marine actinomycete to enter clinical trials *S. tropica* also produces two unique macrolides, sporalides A and B. These two compounds have yet to show antibacterial or

antibiotic activity, but represent the potential of marine actinomycetes to produce novel secondary metabolites (Lam, 2006). Discovery of potentially novel antibiotics has become very important to today's world with the emergence of drug resistant bacteria.

1.2 Characteristics of selected actinomycete genera

1.2.1 The genus *Streptomyces* (suborder *Streptomycineae*, family *Streptomycetaceae*)

The genus *Streptomyces* was proposed by Waksman & Henrici (1943). *Streptomyces* is the type genus of the family *Streptomycetaceae* and is the most common genus of the order *Actinomycetales.* This is clearly evident by the number of described species, which currently stands at 576 (Euzéby, 2010) this is more than ten times the number seen in the genera *Micromonospora* and *Amycolatopsis*. Streptomycetes have been extensively screened for natural antimicrobial agents because of their propensity to produce secondary metabolites and their abundance in the soil and ease of cultivation (Seong *et al*, 2001). A few *Streptomyces* species are pathogenic to man and animals (e.g. *Streptomyces somaliensis* and *Streptomyces sudanensis*) (Quintana *et al*, 2008), while others are phytopathogenic (e.g. *Streptomyces ipomoeae*, *Streptomyces scabies* and *Streptomyces acidiscabies*) (El-Tarabily & Sivasithamparam, 2006; Loria *et al*, 1997).

Streptomycetes are aerobic, Gram positive and non-acid fast bacteria that have an oxidative type of metabolism, and can use various carbon and nitrogen sources for growth. They generally reduce nitrates to nitrites and can degrade a variety of substrates with casein, gelatin and starch being examples. *Streptomyces* species produce extensively branched mycelium that rarely fragments. Morphologically, colonies begin with a somewhat smooth, leathery surface, but later develop aerial mycelium that may appear floccose, granular, powdery or velvety. Streptomycetes can be chemotaxonomically diagnosed by the presence of LL-diaminopimelic acid (LL-DAP) and glycine in the cell wall. There are no diagnostic whole-cell sugars for this genus. Streptomycetes lack mycolic acids and the predominant menaquinones found are hexa- or octa-hydrogenated with nine isoprene units. The phospholipid content of the cell membrane typically contains diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylinositol mannosides (PIM) (Williams *et al.*, 1989; Anderson & Wellington, 2001). The majority of discovered natural antimicrobial agents have come from the genus *Streptomyces* (El-Tarabily & Sivasithamparam, 2006). Since *Streptomyces* is such a common genus, the frequency of isolation is high, which in turn makes the discovery of potentially novel antibiotics difficult as the same species are being isolated repeatedly.

1.2.2 The genus *Amycolatopsis* (suborder *Pseudonocardineae*, family, *Pseudonocardiaceae*)

This genus was proposed by Lechevalier *et al* (1986), to accommodate species that previously belonged to the genus *Nocardia*. They were previously assigned to the genus *Nocardia* based solely on morphological characteristics, in particular the tendency of their vegetative hyphae to fragment. However, this characteristic is seen in other genera in the order *Actinomycetales*, so has not been a very good criterion to distinguish genera. Taxonomists have thus turned to chemotaxonomic methods of differentiation, and so the chief characteristic of *Amycolatopsis* is the presence of *meso*-diaminopimelic acid (*meso*-DAP), arabinose and galactose in the cell wall and the absence of mycolic acids. The predominant menaquinone is MK-9 (H_4). The phospholipid profile contains PE with or without phosphatidylmethylethanolamine (PME) and variable amounts of PIMs, PI and DPG (Mertz & Yao, 1993; Lee & Hah, 2001).

Amycolatopsis is one of the rarer genera with only 10 novel species being discovered in the first 13 years of its existence (Ding *et al*, 2007) and currently consists of 45 species with validly-published names (Euzéby, 2010). *Amycolatopsis* has also been important in drug discovery over the years. Most notable are *Amycolatopsis mediterranei* and *Amycolatopsis rifamycinica*, which are producers of the rifamycin group of antibiotics (ansamycin type antibiotics). The rifamycin group is important due to the fact that one of its derivatives, rifampicin, is used extensively in the treatment of tuberculosis (Bala *et al*, 2004). Another notable antibiotic produced by a member of this genus is the glycopeptide antibiotic, vancomycin, which is produced by *Amycolatopsis orientalis* and is used in the treatment of infections caused by Gram-positive bacteria. Incidences of resistance to this antibiotic have arisen over the last few years (Wink *et al*, 2003; Srinivasan *et al*, 2002). Another compound, avoparcin, which is vancomycin-like, has been used as an additive in growth promoting feeds in agriculture and is produced by *Amycolatopsis coloradensis* (Labeda, 1995). Three species from this genus are also known animal pathogens namely *Amycolatopsis pretoriensis*, *Amycolatopsis kentuckyensis* and *Amycolatopsis lexingtonensis*, which were isolated from the placentas of mares suffering from equine placentitis (Labeda *et al*, 2003).

1.2.3 The genus *Micromonospora* (suborder *Micromonosporineae*, family *Micromonosporaceae*)

This genus, the type genus of the family *Micromonosporaceae*, was proposed by Ørskov in 1923 and currently contains 44 members with validly-published names (Koch *et al*, 1996b; Euzéby, 2010). Micromonosporae are Gram-positive, non-acid fast bacteria with a well developed, branched, septate mycelium, which does not fragment easily. Aerial mycelium is absent. Morphologically, colonies may initially appear pale yellow or orange, turning a darker colour as the colonies mature. This occurs due to the production of brown-black, green-black or black spores. The spore surface ornamentation of

Micromonospora species has been described as smooth, rough, warty or blunt spiny and spore ornamentation has consequently become a useful diagnostic tool to differentiate between species. The cell wall of *Micromonospora* species contains *meso*-DAP and glycine, with xylose and arabinose as the diagnostic whole-cell sugars. The following menaquinones can be found in the members of this genus in varying amounts, tetra-, hexa- and/or octa-hydrogenated menaquinones with 9, 10, and/or 12 isoprene units, but MK-10(H_4) and MK-10(H_6) are the major components in many strains. Mycolic acids are absent and the predominant phospholipids are PE, PI and PIMs. This genus seems to be rather rare in soil, making the occurrence of isolation low. The opposite is true for aquatic samples where micromonosporae can be prevalent in high numbers (Kawamoto, 1989; Koch *et al*, 1996a).

The history of *Micromonospora* and antibiotic discovery is an interesting one. This genus, which is not difficult to cultivate, was never extensively studied in terms of antibiotic production. This all changed when gentamicin, an aminoglycoside antibiotic, was discovered in 1963 by the Schering group in broths of *Micromonospora purpurea* (subsequently reclassified as *Micromonospora echinospora*). Since then, it has been found that *Micromonospora* species produce representatives from almost every chemical family of antibiotics (Wagman & Weinstein, 1980).

1.3 Culture-independent technique (Metagenomics)

The microbial diversity of soils is truly overwhelming, with rough estimates of the number of species per gram of soil being in the several thousands to millions. It has been deduced that by using traditional, cultivation-based techniques of isolation only approximately 1-5% of bacteria can be isolated (Elsas *et al*, 2008). This means that there is a great cache of untapped biotechnological potential which can be exploited, but how can this be done effectively?

The method used to tap into this microbial diversity has become known as metagenomics. The term is derived from the concept of meta-analysis (the process of statistically combining separate analyses) and genomics (Schloss & Handelsman, 2003). Metagenomics is the study of the complete genome of an environmental sample and has become a very powerful tool in the exploration of microbial biodiversity. Metagenomics is a culture-independent procedure where the whole bacterial genome of a sample (metagenome) is extracted and cloned into vectors, thus producing a metagenomic library. Once this is done, the DNA sequences are studied either by expression or sequencing methods (Schloss & Handelsman, 2005). This is very useful in situations where the environment of a sample is extreme and the bacteria which do grow require a very strict set of conditions to grow. It is also these bacteria that are often the ones targeted by scientists for the unique products produced in order for them to survive.

In order to better understand how metagenomics can work as a study tool, the general procedure and possible issues must be mentioned. In order to create the clone library, the metagenome must be extracted from the sample, so the first step is normally cell lysis. There are a few methods used, such as chemical lysis or mechanical lysis each with its advantages and disadvantages. Chemical lysis is gentler, but is not effective across all cell wall structures. Mechanical lysis is more effective across the different cell wall structures, but is also harsher on the DNA, which could lead to shearing. Mechanical lysis is still the preferred method, especially for soil samples, due to its effectiveness. The next step involves DNA extraction of the metagenome, which is normally done using classical deproteinisation in organic solvents. Following this, different procedures are carried out to purify the metagenomic DNA, which include removal of inhibitors, agarose gel purification and gel filtration. The metagenomic DNA is now ready for further study, which could involve any of the following: identification of functional genes, estimation of the microbial diversity in the environmental sample, understanding the population dynamics of a whole community or assembly of the complete genome of an uncultured organism. In each of these studies, the DNA is cloned into some form of vector, which is best suited to the size of DNA fragments needed in the study (Rajendhran & Gunaskaran, 2008). With a clone library constructed, the metagenomic study can continue with further analysis.

Two different approaches to metagenomic studies have arisen and are known as the 'sequence-driven' and 'function-driven' approaches. The function-based approach is based on the identification of a desired trait and the subsequent sequencing and biochemical analyses of that clone/vector which expresses it. This method has certain limitations, the main one being that expression must occur at a high level for the target protein to be detected, which means that the entire gene cluster must have been cloned (and the genes must be expressed in the chosen cloning host). This limitation is being overcome by the development of sensitive assays and screening techniques that can detect low levels of activity in a sample.

The sequence-driven approach relies on the use of conserved DNA sequences as probes for desired gene clusters in a metagenomic clone library. An example of the usefulness of this approach is the discovery of a gene from a seawater clone, which shows similarity to the bacteriorhodopsin gene, which was previously thought to only occur in the archaea. A limitation of the sequence-based approach is its randomness. How is one to decide which genes to search for? Furthermore, it is possible that a sample yields nothing, which would be a waste of time and money. This however does not discredit its usefulness (Schloss & Handelsman, 2003). Figure 1 illustrates the whole metagenomic process.

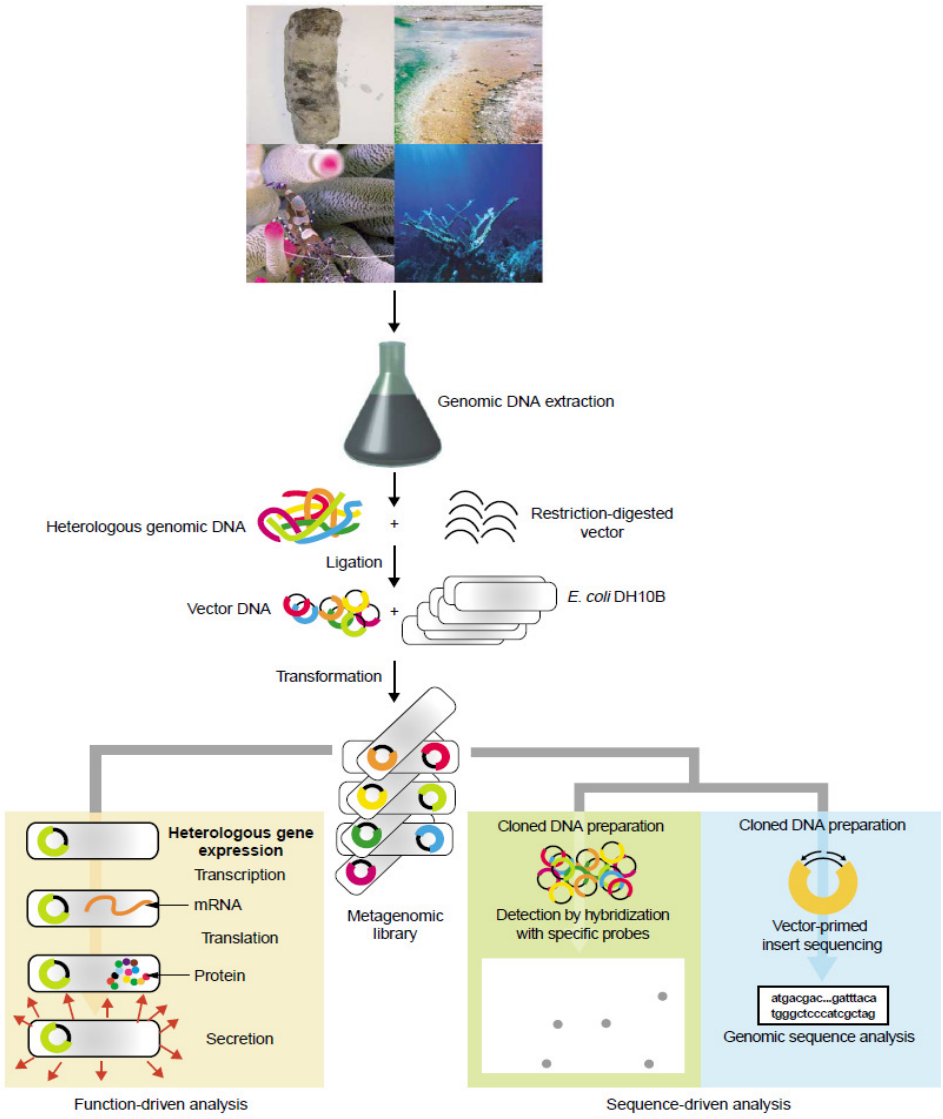

Fig 1. Graphical representation of the metagenomic method, showing the different steps performed from sampling to the different methods of analysis (function-driven or sequence-driven). Taken from Schloss & Handelsman (2003).

Metagenomics has also been applied to marine samples. As stated earlier, the marine environment offers a greatly understudied source of potentially new bioactive molecules. Proof of this method being viable in marine samples was shown by Webster *et al* (2001) who discovered that the number of actinomycetes present was a lot higher within the sponge species *Rhoploeides odorabilethan* than was evident from cultivation-based techniques (Newman & Hill, 2006). This study shows that without metagenomics many potentially useful actinomycetes, which are well known secondary metabolite producers, would never be found and thus their products would be lost. For the reasons mentioned, metagenomics must be seen as an important technique for use in further studies. All of the methods described above are focused on the isolation of novel actinomycetes in an attempt to discover potentially new antimicrobial agents. This is important due to the growing problem of drug resistant bacteria.

1.4 Drug resistance and tuberculosis (TB)

Drug resistant bacteria are becoming a great problem, arising from the prolonged and improper use of antibiotics. By using antibiotics at a too low concentration or for an inadequate amount of time, bacteria are allowed to adapt to these pressures through natural selection (Tan *et al*, 2000), resulting in an increase of drug-resistant strains. In South Africa, the biggest problem presented by drug-resistant bacteria is that of the causative agent of pulmonary tuberculosis (TB), *Mycobacterium tuberculosis*. The problem worsens with HIV co-infection, as HIV is a known risk factor and hastens the progression of TB (Johnson *et al*, 2001). The latest's statistics released by the World Health Organisation show that in 2007 there were 9.27 million new cases of TB and approximately 1.78 million deaths. South Africa clearly has a problem with TB as it was ranked fifth based on the number of total cases in 2007 with 0.46 million (WHO, 2009). Furthermore, there has been the emergence of multi-drug-resistant tuberculosis (MDR-TB), which is classified as being resistant to isoniazid and rifampicin (Telenti & Iseman, 2000), two of the front-line drugs used in treating TB. Recently, along with MDR-TB, there has been a further classification of resistant *M. tuberculosis*, known as extensively-drug-resistant tuberculosis (XDR-TB). XDR-TB is characterised by strains which are resistant to the same drugs as MDR-TB, as well as to fluoroquinolones and to one or more of amikacin, capreomycin and kanamycin (Jones *et al*, 2008). For these reasons, we seem to be losing the battle against drug resistant bacteria and, if examples of such resistant bacteria can already be found, then it is just a matter of time before more "superbugs" appear. To combat this ever worsening problem, novel antibiotics must be discovered.

1.5 Aims of this study

The aim of this study was to isolate actinomycetes from three different sample types, namely a marine sample, a freshwater sample and a soil sample, and to screen these isolates against *Mycobacterium aurum* strain A+ for antimycobacterial antibiotics. *M. aurum* A+ is a non-pathogenic bacterium that has a similar antibiotic susceptibility profile to *M. tuberculosis* (Chung *et al*., 1995). Isolates which showed antimycobacterial activity were chosen for identification (phylogenetic and phenotypic characterization) to determine whether they represent new species. In parallel, a metagenomic study of each of the three environmental samples was done to gauge the actinobacterial biodiversity of the different sampling locations and to compare this biodiversity with that of the antibiotic-producing genera isolated on the agar plates.

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Chapter 2

Isolation of actinobacteria from sea sand, dam mud and mountain soil

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2.1 Abstract

Three sand samples were collected from Big Bay beach, Cape Town, from different parts of the beach. By using standard culture-based techniques, no actinobacterial colonies were obtained. Marine isolations were abandoned and a freshwater sample was used. Two separate samples were collected from the dam situated on the University of Cape Town, Upper Campus. No actinobacterial colonies were obtained from the dam samples.

A terrestrial soil sample was collected from the Rhodes Memorial at the foot of Devil's Peak, Cape Town. A total of 52 actinobacterial isolates was obtained from this sample based on colony morphologies. All 52 isolates were subjected to antibiotic screening using the test bacterium *M. aurum* A+ and varying levels of activity were seen. All isolates were subjected to a rapid genus identification method to identify each one to the genus level. Forty isolates were shown to belong to the genus *Streptomyces*, while the remaining 12 consisted of six non-*Streptomyces* strains and six isolates which could not be recovered from freezer stocks. The sequencing of the 16S-rRNA genes of the non-*Streptomyces* isolates showed that three were from the genus *Amycolatopsis* (strains TMB1, TMB2 & UCZ5), one was from *Micromonospora* (strain TY1), one was from *Nocardia* (strain UCZ15) and the last was in fact a *Streptomyces* strain (strain UCZ14). Isolate UCZ15 could not be grown from freezer stocks, so strains TMB1, TMB2, UCZ5, TY1 and UCZ14, along with three of the *Streptomyces* isolates with very strong anti-*M. aurum* A+ activity (strains TCZ2, TMC9 & UCZ4) were chosen for further phenotypic and genetic characterisation.

2.2 Introduction

Soil has been the major source of actinomycete discovery in the past (El-Tarabily & Sivasithamparam, 2006) and, as a result, re-isolation of already described species has occurred. This presents a problem to drug discovery, especially with the emergence of drug resistant bacteria. Newer antibiotics need to be discovered, but since soil has been, and continues to be extensively studied, this task may prove difficult. There is, however, an environment which could possibly solve this, namely, the aquatic environment. This can refer to either marine or fresh water, as both have not been very well studied. The studies which have been done on these environments show great potential, as stated in Chapter 1 (section 1.1). Thus, studying aquatic environments should possibly take precedence over soil, as they are the most likely to

yield new species and possibly new genera (e.g. *Salinispora*), and thus increase the possibility of new bioactive compounds. It must however be mentioned that cultivation of marine actinomycetes using the cultivation based method can prove difficult. The reason for this is mostly likely due to the specialised growing conditions required by these actinomycetes. Unlike their terrestrial counterparts, who have been well studied and nutrient requirements well known, marine actinomycete nutrient requirements are largely unknown and isolation cannot be guaranteed using the traditional terrestrial media (Zotchev, 2011).

2.3 Materials and Methods

2.3.1 Sample collection, treatment and media

The marine samples were collected from three different sites on Big Bay beach, Cape Town in February, 2009 (Fig 2.1). One from a rock pool (RP), the other from the shore line sand (SL) (covered during high tide) and lastly from approximately 10m into the water (OS) (always covered by water). This was done to observe any possible similarities or differences between the three zones, since they are linked to each other. The rock pool is isolated from the ocean, except when occasionally the seas are rough and water washes into them. The shoreline area has times when it is moist and others when it is exposed to the sun and possibly dries out, which could affect the ability of microorganisms to survive.

Fig 2.1 Aerial photograph of Big Bay Beach, Cape Town. Clearly indicating where the three different samples were collected, OS – ocean soil, SL – shore-line sand and RP – rock pool sand. Photograph was taken from Google maps.

The marine samples did not undergo any form of treatment and a sterile 2% (w/v) NaCl solution was used for the dilutions. For each sample, 1 g (wet weight) was weighed out and suspended in 1 ml of sterile 2% (w/v) NaCl solution. It was then vortexed for approximately 5 min and allowed to settle. Each suspension was then serially diluted to 10^{-5} using sterile 2% (w/v) NaCl.

All of the dilutions, from undiluted to 10^{-5}, were spread plated in 100µl volumes on four different types of sterile media: starch-casein agar (pH 7.5) supplemented with 2% (w/v) NaCl (Kim *et al.* 2003), Yeast Extract Malt Extract agar (International *Streptomyces* Project (ISP#2)) (pH 7.5) supplemented with 2% (w/v) NaCl (Shirling & Gottlieb, 1966), Seawater Starch agar (pH 7.8) supplemented with 2% (w/v) NaCl (Pathirana *et al.*, 1991) and Difco Marine Medium made according to the manufacturer's specifications. All of the media contained cycloheximide (50 µg/ml) and nalidixic acid (10 µg/ml). All plates were incubated for a period of 4 weeks at 20-25°C, with regular inspections. A second set of plates was made later using autoclaved seawater instead of 2% (w/v) NaCl. A third batch of plates was made exactly as the first, but supplemented with amphotericin B (25 µg/ml). The second batch of plates was incubated at 30°C for 2 weeks.

A sample was taken from the UCT fresh water dam in April, 2009 (Fig 2.2). Two separate samples were taken, one from the surrounding soil (SS), which had previously been submerged when the water level was higher. Another sample was taken from sediment under water (DS), approximately 1 m from the edge of the water. This was done to observe the similarities and differences between the two samples, since it can be expected that they may have similar microbiological diversity. However, each experienced different forms of environmental pressures, which could have had an effect on their bacterial diversity.

Fig 2.2 Aerial photograph of the University of Cape Town dam, Cape Town, showing where the two different samples were collected, SS – surrounding soil, DS – dam soil. Photograph was taken from Google earth.

One gram of each sample was pre-treated by incubating at 60°C for one hour. For both the SS and DS samples, a treated and untreated dilution series was made and plated. For the SS sample (treated and untreated), 0.1 g was suspended in 1 ml of sterile distilled water and vortexed for approximately 5 min and allowed to settle. The suspensions were then serially diluted to 10^{-4} using sterile distilled water. The same procedure was carried out with the DS sample, except that 0.5 g of soil was suspended in 1 ml of sterile distilled water.

All of the dilutions, from undiluted to 10^{-4}, were spread plated on four different types of sterile media: Difco Middlebrook 7H9 (MB) agar prepared according to the manufacturer's specifications and supplemented with 10 mM glucose (albumin and catalase omitted), ISP#2 at pH 7.3 (Shirling & Gottlieb, 1966), Czapek Solution agar (CZ) at pH 7.3 (Atlas, 2004) and Modified Czapek solution agar (MC) at pH 7 (Nonomura & Ohara, 1971). All of the media contained cycloheximide (50 µg/ml), nalidixic acid (10 µg/ml) and amphotericin B (25 µg/ml). All plates were incubated for a period of 4 weeks at 30°C, with regular inspections.

The third sample was collected in June 2009 from the Rhodes Memorial, which is at the foot of Devil's Peak (MS) (Fig 2.3). Rhodes Memorial is just above and to the north-west of UCT's Upper Campus. The soil sample was taken from a shady area covered by trees, approximately 5cm below the surface of the soil.

Fig 2.3 Aerial photograph of Rhodes Memorial, Cape Town. Clearly indicating where the sample was collected, MS – Mountain soil. Photograph was taken from Google maps.

One gram of soil was pre-treated at 60°C for one hour. Both the treated and untreated samples were serially diluted in sterile distilled water: 0.1 g of both treated and untreated sample was suspended in 1 ml of sterile distilled water and vortexed for approximately 5 min and allowed to settle. The suspensions

were then serially diluted to 10^{-4} using sterile distilled water. A second suspension of the soil was used to determine the pH of the soil.

All of the dilutions, from undiluted to 10^{-4}, were spread plated on four different types of sterile media: MB agar pH adjusted to 8.3, ISP#2 agar at pH 8.3 (Shirling & Gottlieb, 1966), CZ agar at pH 8.3 (Atlas, 2004) and MC agar at pH 8.3 (Nonomura & Ohara, 1971). All of the media contained cycloheximide (50 µg/ml) and nalidixic acid (10 µg/ml). All plates were incubated for a period of 3 weeks at 30°C, with regular inspections. Colonies were selected, based on morphological characteristics, and sub-cultured onto fresh plates of the same medium on which they had been isolated in order to obtain pure cultures. The isolates were then named according to their relevant treatments and medium of isolation and were given a colony number. All selected colonies were grown in ISP#2 broths at 30°C with shaking until sufficient growth was seen and were then Gram stained to ensure no contamination. Stock cultures were made using glycerol at a final concentration of 15% (v/v) and stored at -20°C.

2.3.2 Antimicrobial activity determination

The selected actinomycete isolates were stab inoculated onto ISP#2, MB, MC and CZ media and incubated for 9 days at 30°C. The optical density (OD_{600nm}) of a 16 hour culture of the test bacterium, *M. aurum* A+, grown at 37°C, was then determined using a Beckman DU530 UV spectrophotometer. This was to determine the volume of *M. aurum* A+ culture to add to each overlay medium such that 160 = OD_{600}x µl. The calculated volume of *M. aurum* A+ was added to 6 ml of sloppy Luria-Bertani (LB) agar (Sambrook *et al.*, 1989), mixed and overlaid onto the stab-inoculated actinomycete plates. These plates were incubated for a further 2 days at 37°C to allow the test bacterium to grow. The areas of the zones of inhibition were then calculated (by subtracting colony area from inhibition area) and assigned an arbitrary strength according to Table 2.1. The isolates chosen for further study were subjected to further antibacterial testing against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. Again the ODs of 16 hour 37°C cultures were measured and the amount to be added to sloppy-agar tubes was calculated using the formulae 4 = OD_{600}x µl for *E. coli* and 160 = OD_{600}x µl for *S. aureus*. This was done to ensure that approximately the same number of bacterial cells was added to each experiment so that the results would be comparable. The same procedure was followed as with *M. aurum A*+, but the incubation period was shortened to 16 hour at 37°C.

Table 2.1 Arbitrary assignment of antimicrobial strength based on the area of the zone of inhibition

Antimicrobial activity	Area of zone of inhibition (mm^2)
Very Weak	<100
Weak	100-1000
Moderate	1001-2000
Strong	2001-3000
Very Strong	>3000

2.3.3 DNA extraction

For DNA extraction, the boiling method described by Cook & Meyers (2003) was used. Where the boiling method was not successful, the following was done: isolates were grown in ISP#2 broths at 30°C for between 7 and 14 days with shaking. Gram stains were performed to determine the purity of all cultures. Genomic DNA was extracted as described by Wang *et al*. (1996), incorporating the modifications of Everest & Meyers (2008).

2.3.4 16S-rRNA gene PCR amplification

The PCR amplifications were performed in 50 µl volumes containing: 100ng diluted DNA (50 ng/µl), 0.5U Super-Therm *Taq* polymerase (JMR Holdings, USA), 2 mM $MgCl_2$, 150 µM dNTPs and 0.5 µM of each primer, F1 and R5 (Cook & Meyers, 2003). All primers used were synthesised by the UCT MCB DNA Synthesising unit. The conditions of the PCR were as follows: initial denaturation for 2 min at 96°C followed by 30 cycles of denaturation at 96°C for 45s, annealing at 56°C for 30s and extension at 72°C for 2 min, with a final extension for 5 min at 72°C. All PCR reactions were performed in a Techne TC-512 thermal cycler.

2.3.5 Restriction endonuclease digestions (Rapid Identification Method)

Identification of isolates to the genus level was accomplished by using the rapid identification method in which the 16S–rRNA gene DNA is digested singly with different restriction endonucleases (Cook & Meyers, 2003). The digestions were performed in 20 µl volumes containing 10U of the appropriate restriction endonuclease and incubated for 16 hours at 37°C. Digestions were performed with the restriction endonucleases, *Mbo*I (*Sau*3A isoschizomer), *Vsp*I (*Asn*I isoschizomer), *Hind*III, *Sph*I, *Kpn*I and *Pst*I as per the protocol of Cook & Meyers (2003). For those isolates where the digests failed or were hard to interpret, the DNA was sequenced.

All digests were electrophoresed at 90V on 1.2% (w/v) agarose gels containing 0.8 µg/ml ethidium bromide along with a λ-*Pst*I size marker. Gels were visualised using a GelDoc System at 254 nm (Gel Doc XR, Bio-Rad laboratories Inc.).

2.3.6 DNA sequencing and sequence analysis

The amplified 16S–rRNA gene DNA product was purified using the MSB Spin PCRapace clean up kit (Invitek, Germany) and the DNA was sequenced using F1, F3, F5, R1, R3 and R5 (Weisburg et al, 1991) universal bacterial primers by Macrogen Inc (Seoul, Korea). Sequences were edited using Chromas (version 2.01, Technelysium) and analysed using DNAMAN (version 5.2.9, Lynnon Biosoft, 2001). The Basic Local Alignment Search Tool (BLAST) was used to determine the closest sequence matches by using the BLASTn search tool in the GenBank database (Altschul *et al.* 1997). All sequences used in construction of phylogenetic trees were downloaded from the GenBank database and compiled in DNAMAN. All sequence alignments were done using ClustalW in MEGA 4 (Tamura *et al.*, 2007) and neighbour-joining and maximum-parsimony trees were constructed.

2.3.7 Physiological and morphological characterisation

All ISP media were prepared according to Shirling & Gottlieb (1966). Morphological characterisation (aerial mycelium and spore chain morphology) were determined on ISP#4 (inorganic salts-starch agar). Production of a soluble pigment was determined on ISP#5 (glycerol-asparagine agar), with the production of melanin tested on ISP#6 (yeast extract-iron agar) and ISP#7 (tyrosine agar). Growth at different temperatures (30°C, 37°C and 45°C), in the presence of NaCl (0%, 1%, 3%, 5% and 7% w/v) and at different pHs (4.3, 7 and 9) was tested on Bennett's medium (Atlas, 2004), as described by Locci (1989). Physiological media for testing the degradation of adenine, allantoin, casein, cellulose, gelatin, guanine, hypoxanthine, starch, L-tyrosine, xanthine and xylan were made and used as described by Locci (1989). The hydrolysis of aesculin, arbutin and pectin, the production of H_2S and nitrate reduction were tested on media prepared as described by Locci (1989). The degradation of urea and the degradation of Tween 80 were tested as described by Gordon *et al.* (1974) and Sierra (1957), respectively. Nitrogen source utilisation media were prepared as described by Locci (1989). Carbon source utilisation media were made as described by Shirling & Gottlieb (1966). All carbon sources and nitrogen sources were filter sterilized. Carbon sources were tested at a concentration of 1% and nitrogen sources at 0.1%. Plates were incubated at 30°C, unless otherwise stated, and checked after the recommended periods (Locci, 1989; Shirling & Gottlieb, 1966).

2.3.8 Scanning Electron Microscopy (SEM)

Selected isolates were plated on ISP#4 and incubated for 2 weeks at 30°C, after which a section of the agar containing actinomycete growth was cut out (1x1 cm) using a sterile scalpel. This sample was then fixed using 100 mM sodium cacodylate buffer containing 2% (v/v) glutaraldehyde (pH 8.0) overnight at 20-25°C. The samples were then washed twice for 30 min each in 100 mM sodium cacodylate buffer at pH 8.0 without glutaraldehyde. Post-fixing of all samples was done in 100 mM sodium cacodylate buffer at pH 8.0 containing 1% osmium tetroxide for 2h at 20-25°C. The samples were washed once with 100 mM sodium cacodylate buffer (pH 8.0) and twice with sterile distilled water for 10 min each. An increasing alcohol gradient was used (30, 50, 70, 85, 95 and 100% v/v ethanol at 10 min each) to dehydrate the fixed samples, with the 100% ethanol wash step being performed thrice in order to ensure dehydration. The samples were stored in 100% ethanol. All prepared samples were critical-point dried and sputter coated with gold palladium and viewed under a *Nova NanoSEM 230* Scanning Electron Microscope (Electron Microscope Unit, University of Cape Town).

2.3.9 Chemotaxonomy

In order to get cell mass to perform the chemotaxonomic tests, the selected isolates were grown in 100 ml of ISP#2 broth at 30°C until a large cell mass was seen. The cell mass was collected by centrifugation at 10 000 x g for 10 min, after which the pellet was washed twice with sterile distilled water (100 ml). The cell mass was then resuspended in approximately 20 ml of sterile distilled water and transferred into a round-bottomed flask for freeze drying.

Approximately 10 mg of the freeze dried cells were used to determine the isomer of DAP present in the cell wall peptidoglycan by the method of Hasegawa *et al.* (1983). The whole cell sugar patterns were also determined as described by Hasegawa *et al.* (1983), using 100 mg of freeze dried cells and with a change in the thin-layer chromatography (TLC) solvent system to ethyl acetate: pyridine: distilled water (100:35:25).

2.3.10 *gyrB* gene amplification, sequencing and analysis

The following primers were used to amplify the *gyrB* gene: GgyrB-F1, GgyrB-R1 and 7G-gyrB-R described by le Roes *et al.* (2008), 7G-gyrB-F described by Everest & Meyers (2009) and KgyrB-R1892 and KgyrB-R described by Kirby *et al.* (2010). All PCR conditions for amplification were the same as described in section 2.3.4 (with the exception that the $MgCl_2$ concentration was 4 mM). Sequencing was carried out as for the 16S-rRNA genes (section 2.3.6) using the *gyrB* PCR primers in the sequencing reactions. All analysis was carried out as mentioned in section 2.3.6; *gyrB* genetic distances were calculated in MEGA, using Kimura's 2-parameter model (Kimura, 1980).

2.4 Results and discussion

2.4.1 Marine and freshwater isolations

Plates were incubated for four weeks at 20-25°C. This lower temperature was selected due to the lower temperatures found in the environments where the samples were collected (i.e. generally < 20 °C). After four weeks of incubation, the first set of plates showed no actinomycete growth, based on colony morphologies. Other colonies did form, showing that there were microorganisms present in the sample. The plates made with the sea water also produced non-actinobacterial colonies, but contained no actinomycete colonies after 4 weeks of incubation at 20-25°C. After these three attempts at isolating actinomycetes from the sample, it was determined that the actinomycetes present are mostly likely very difficult to culture because they require highly specialised growing conditions (Newman & Hill, 2006) and are possibly unculturable. This is supported by the data gathered from the metagenomic study on all three samples, which are presented in Chapter 3.

After 2 weeks of incubation at 30°C, small colonies formed from the dam samples, which seemed to have the morphological characteristics of actinomycete growth. These colonies were subcultured and incubated at 30 °C. After 2 weeks of incubation, it became evident that the colonies were most likely not actinomycetes, as the morphological characteristics changed from what is expected from actinomycetes colonies (hard and leathery) to shiny and mucous-like in texture. Furthermore, no further growth occurred on the isolation plates. There are a number of possible reasons for the lack of actinomycete isolation from the dam samples, such as pH, nutrients present in growth medium and reasons similar to those explaining no actinomycete colonies being found in the marine samples.

2.4.2 Rhodes Memorial isolations

The results are presented for each strain isolated and characterised. Firstly the more common *Streptomyces* isolates are presented, followed by the less common non-*Streptomyces* isolates belonging to the genera *Amycolatopsis* and *Micromonospora*.

2.4.2.1 Initial isolations and characterisation

A total of 52 isolates was chosen from the MS sample isolation plates (section 2.3.1), the majority of which belonged to the genus *Streptomyces.* There were also three *Amycolatopsis* strains, one *Micromonospora* strain and a *Nocardia* strain. The cycloheximide and nalidixic acid were able to limit the number of colonies of Gram-negative bacteria and fungi which grew on the plates, making actinomycete colony identification easier. The untreated soil-sample plates were mostly overgrown on the lower dilutions and a total of 29 isolates came from these plates. The heat-treated soil sample showed

much less growth of undesired bacteria and so actinomycete colonies could be isolated from the lower dilutions as well as the higher dilutions. A total of 23 isolates was isolated from the heat-treated sample plates. The heat pre-treatment had no effect on the number of actinomycete colonies isolated, which was surprising, as the treatment was meant to kill the non-sporulating bacteria thus improving the isolation of the sporoactinomycetes.

All of the isolates were subjected to antibiotic screening against *M. aurum* A+ on ISP#2, CZ, MC and 7H9 plates. Thirteen (13) isolates showed very strong activity (>3000 mm^2; Table 2.1), eight of which cleared the plate when incubated on ISP plates. Unfortunately, 6 isolates could not be re-grown from stocks, despite multiple attempts with different growth conditions. Five of these lost isolates were from the group which showed very strong activity. All antibiotic activity results can be seen in appendix A.

The rapid identification method was used to identify the isolates down to the genus level. Almost all (40) were indicated to belong to the genus *Streptomyces*, while the remaining were indicated to belong to non-*Streptomyces* genera. These 6 non-*Streptomyces* isolates were sent for 16S-rRNA gene sequencing (using just the F1 primer) in order to determine the genus to which each belonged. The results showed that three belonged to the genus *Amycolatopsis*, one to the genus *Micromonospora*, one to the genus *Nocardia* and one to the genus *Streptomyces*. This shows that the results from the rapid identification method should be interpreted with caution.

It was decided that all six of these isolates, along with the three isolates with the highest anti-*M. aurum* activity, would be characterised further. Unfortunately, the *Nocardia* isolate would not grow from stocks, which left eight isolates for characterisation: four *Streptomyces* strains (UCZ4, UCZ14, TMC9 and TCZ2), three *Amycolatopsis* strains (TMB1, TMB2 and UCZ5) and one *Micromonospora* strain (TY1). The rest of this section deals with the further characterisation of these 8 isolates, starting with the *Streptomyces* strains and then moving on to the *Amycolatopsis* strains and finally the *Micromonospora* strain.

2.4.2.2 *Streptomyces* isolates

2.4.2.2.1 *Streptomyces* strain UCZ14

Streptomyces strain UCZ14 was isolated from the untreated soil from Rhodes Memorial on a CZ plate. It was initially thought that this strain was a non-*Streptomyces* isolate based on the rapid genus-identification method, however, 16S-rRNA gene analysis of the 1421-nt sequence showed that it belonged to the genus *Streptomyces*. Its closest evolutionary relative according to the GenBank database is *Streptomyces lydicus* strain NBRC 13058^{T} with a 99% sequence similarity (this was confirmed using DNAMAN). The neighbour joining tree (Fig. 2.4) shows that strain UCZ14 grouped with the type strains of *S. lydicus* and *Streptomyces chattanoogensis* with a low bootstrap value of 56%. The gene sequence similarity between *S. chattanoogensis* and strain UCZ14 is 99.44%. The whole-cell hydrolysate of strain UCZ14 contained LL-DAP and no diagnostic sugars, supporting its genus assignment. Strain UCZ14 produced no diffusible pigments and the aerial mycelium was grey. Strain UCZ14 has straight spore chains; the spore surface cannot be determined as the aerial mycelium is immature in the electron micrograph (Fig 2.5). Strain UCZ14 showed very strong activity against *M. aurum* A+ (Appendix A), but no activity against *E. coli* and weak activity against *S. aureus* (Appendix A), suggesting that the antibiotic produced is effective against mainly Gram-positive bacteria. The physiological test results and the ability of strain UCZ14 to utilise sole carbon and nitrogen sources are shown in Tables 2.2 and 2.3. DNA-DNA hybridization will be needed before this isolate can be classified as a new species. Strain UCZ14 should also be compared to *S. lydicus* and *S. chattanoogensis* (based on published data) to see whether it can be distinguished from them phenotypically.

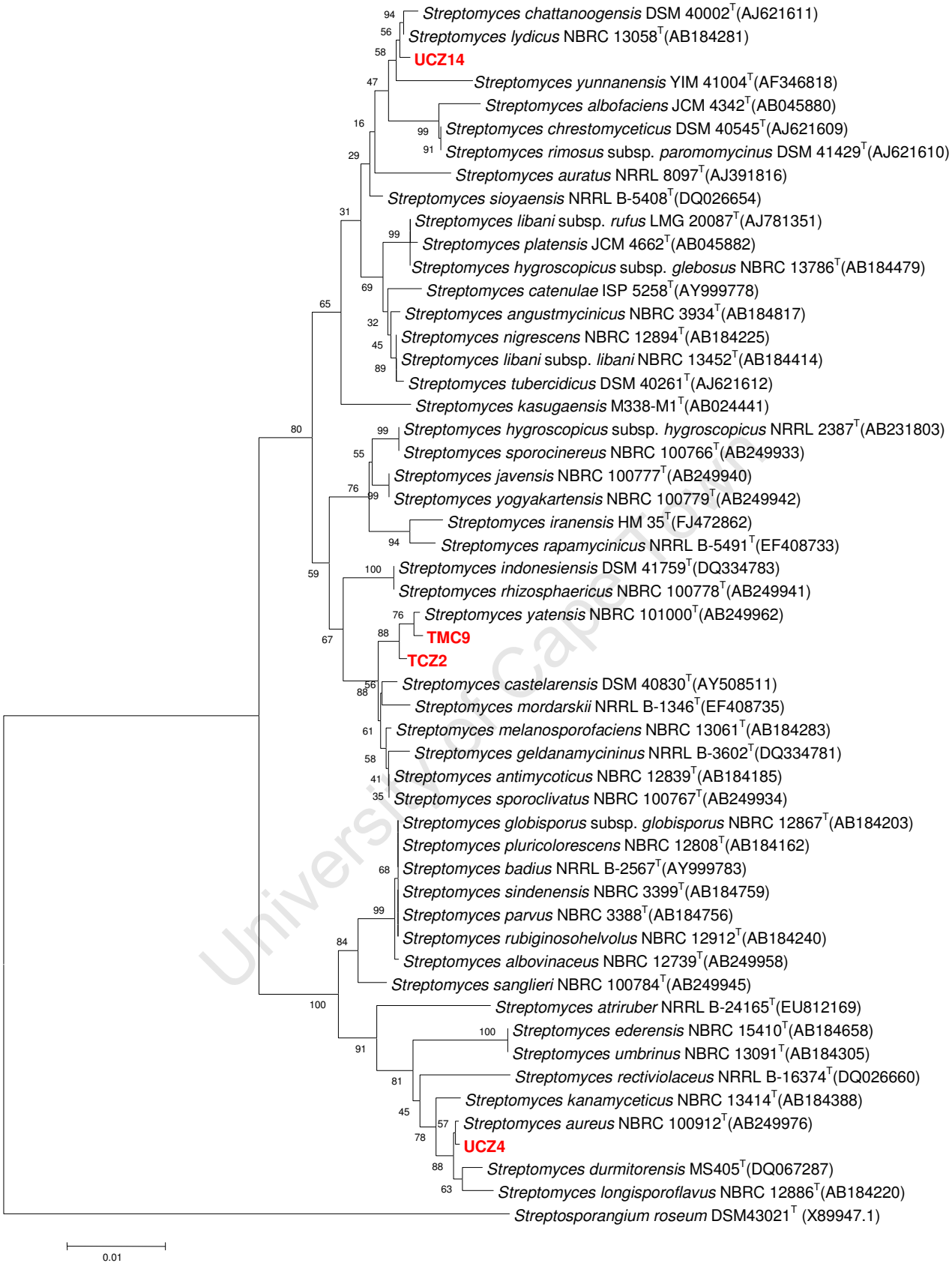

Fig 2.4 Unrooted 16S-rRNA gene neighbour-joining tree showing the phylogenetic relationships of *Streptomyces* strains UCZ4, UCZ14, TCZ2 and TMC9 and selected members of the genus that have validly published names. The tree was constructed based on 1402nt of common sequence. *Streptomyces* strains UCZ4, UCZ14, TCZ2 and TMC9 are shown in red. Numbers at the nodes are the percentage bootstrap values of 1000 replications. The scale bar represents 1 nucleotide substitution per 100 nucleotides.

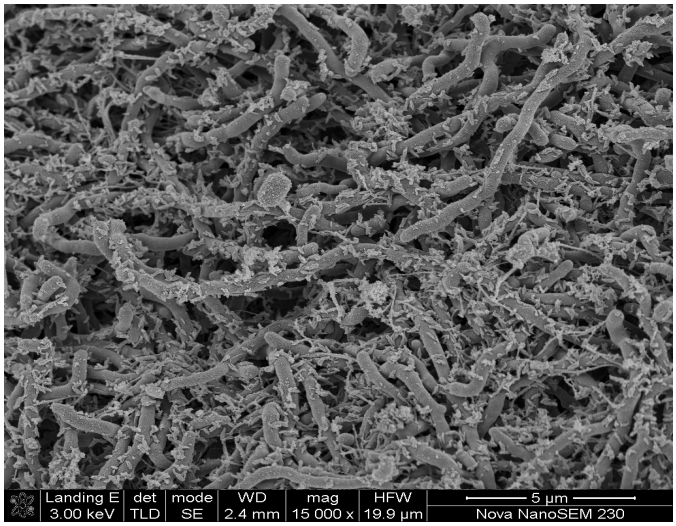

Fig 2.5 Scanning electron micrograph of *Streptomyces* strain UCZ14 showing straight spore chains; spore surface ornamentation cannot be determined as aerial mycelium is still immature. Bar = 5 µm.

Table 2.2 Sole carbon and nitrogen utilization test results for the *Streptomyces* isolates

Test	UCZ14	UCZ4	TMC9	TCZ2
Carbon Sources				
D-Glucose	+	+	+	+
Negative Control	-	-	-	-
L-Arabinose	+	++	++	++
Sucrose	-	+	-	-
D(+) Xylose	+	++	weak+	+
D-Galactose	-	++	++	++
meso-Inositol	+	++	weak+	+
D(-) Mannitol	+	++	+	+
L-Rhamnose	-	++	+	+
Raffinose	+	++	-	-
Mannose	+	++	weak+	+
D(-) Fructose	-	++	++	++
Salicin	-	+	-	-
Nitrogen sources				
L-Asparagine	+	+	+	+
Negative Control	-	-	-	-
L-Histidine	-	++	++	++
DL-α-Amino-n-Butyric Acid	-	++	+	++
L-Cysteine	+	+	+	weak+
L-Hydroxyproline	-	-	+	-
L-Methionine	+	+	-	-
Potassium Nitrate	weak+	++	+	++
L-Phenylalanine	-	+	++	+
L-Serine	+	++	++	++
L-Threonine	+	++	++	++
L-Valine	weak+	+	-	+

Notes: -, no growth; weak+, growth between - & +; +, good growth; ++ very good growth

Table 2.3 Physiological test results for the *Streptomyces* isolates

Test	UCZ14	UCZ4	TMC9	TCZ2
H_2S Production	-	+	weak+	-
Nitrate reduction	-	+	-	-
Adenine Deg.	+	+	+	-
Guanine Deg.	-	-	-	-
Hypoxanthine Deg.	+	+	+	+
Xanthine Deg.	+	weak+	weak+	-
Cellulose Deg.	-	-	-	-
Gelatin Deg.	+	+	+	+
Starch Deg.	+	+	+	+
L-Tyrosine Deg.	weak+	+	-	weak+
Xylan Deg.	-	-	-	-
Casein Deg	+	+	+	+
Tween 80 Deg.	+	+	+	+
Urea Deg.	-	+	weak+	-
Allantoin Deg.	-	+	weak+	+
Aesculin Deg.	+	+	+	+
Arbutin Deg.	+	+	+	+
pH 4.3	++	-	-	-
pH 7	++	++	++	++
pH 9	++	++	+	+
0% NaCl	++	++	++	++
1% (w/v) NaCl	++	++	+	+
3% (w/v) NaCl	++	+	+	-
5% (w/v) NaCl	+	-	-	-
7% (w/v) NaCl	+	-	-	-
30°C	++	++	++	++
37°C	++	+	+	+
45°C	-	-	-	-
ISP#5	-	-	-	-
ISP#6	-	-	-	-
ISP#7	-	-	-	+
Pectin	-	-	-	-

Notes: -, no growth/degradation; weak+, growth between - & + (or a weak reaction); +, good growth/degradation; ++ very good growth; for ISP#5-7, + indicates melanin production and – indicates no production; Deg. = Degradation

2.4.2.2.2 *Streptomyces* strain UCZ4

Streptomyces strain UCZ4 was isolated from the untreated Rhodes Memorial soil sample on a CZ plate. It produced no diffusible pigments and grew as orange colonies before sporulation. The spore mass was grey-white. A GenBank database search of the 1418-nt 16S-rRNA gene sequence showed that its closest evolutionary relative is *Streptomyces aureus* strain NBRC 100912^T with a 99% sequence similarity (this was confirmed using DNAMAN). In the neighbour joining tree (Fig 2.4), strain UCZ4 groups with the type strain of *S. aureus* with a bootstrap value of 57%. The electron micrograph (Fig 2.6) of *Streptomyces* strain UCZ4 shows that it has straight spore chains and a smooth spore surface ornamentation. The whole cell hydrolysate of strain UCZ14 contained LL-DAP and no diagnostic sugars, supporting its genus

assignment. Strain UCZ4 showed very strong activity against *M. aurum* A+ (Appendix A), but no activity against *S. aureus* and *E. coli*. The physiological test results and the ability of strain UCZ14 to utilise sole carbon and nitrogen sources are shown in Tables 2.2 and 2.3. With such a high 16S-rRNA gene sequence similarity, DNA-DNA hybridisation will be needed to determine whether strain UCZ4 is a new species.

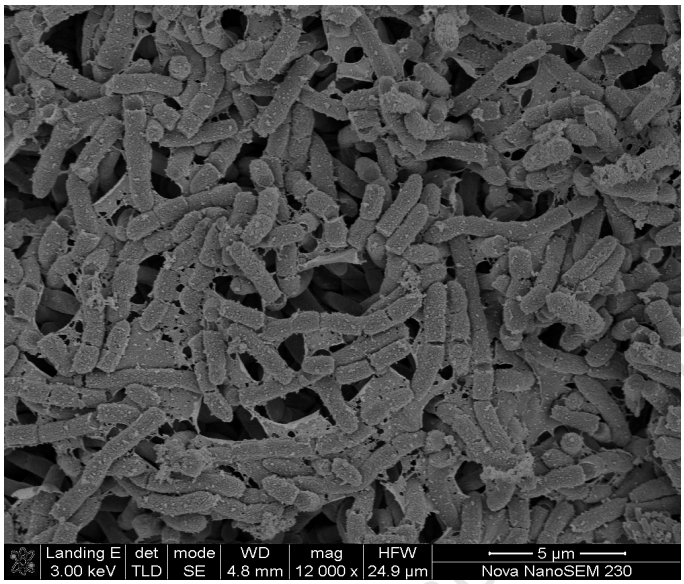

Fig 2.6 Scanning electron micrograph of *Streptomyces* strain UCZ4 showing straight spore chains with smooth spore surface ornamentation. Bar = 5 µm.

2.3.2.2.3 *Streptomyces* strain TMC9

Streptomyces strain TMC9 was isolated from the treated Rhodes Memorial soil sample on an MC plate. It produced melanin and grew as pale yellow translucent colonies on ISP#4. A GenBank database search using the 1391-nt 16S-rRNA-gene sequence showed that *Streptomyces yatensis* NBRC 101000^T is the closest evolutionary relative of *Streptomyces* strain TMC9 with a sequence similarity of 99% (confirmed using DNAMAN). This was supported by the neighbour joining tree (Fig 2.4), which showed that strain TMC9 grouped with *S. yatensis* NBRC 101000^T, supported by a moderate bootstrap value of 76%. It is interesting to note that strain TMC9 also grouped with *Streptomyces* strain TCZ2, which will be discussed next. The electron micrographs (Fig 2.7 A & B) of *Streptomyces* strain TMC9 show that it produces spiral spore chains with spiny spore surface ornamentation. The whole cell hydrolysate of strain TMC9 contained LL-DAP and no diagnostic sugars, as expected for this genus. Strain TMC9 showed very strong activity against *M. aurum A+*, but no activity against *E. coli* and weak activity against *S. aureus* (Appendix A), suggesting that the antibiotic produced is effective against mainly Gram-positive bacteria. The physiological test results and the ability of strain TMC9 to utilise sole carbon and nitrogen sources are shown in Tables 2.2 and 2.3. With such a high 16S-rRNA gene sequence similarity, DNA-DNA hybridisation will be needed to conclude whether TMC9 is a new species.

A) B)

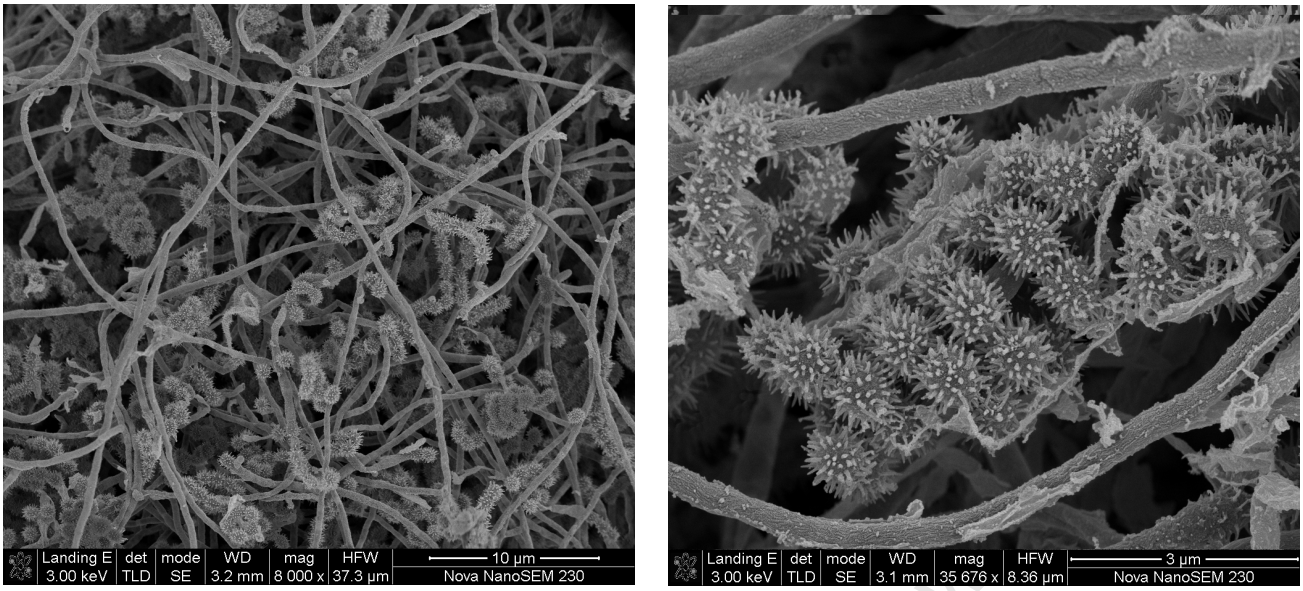

Fig 2.7 Scanning electron micrographs of *Streptomyces* strain TMC9 showing (A) spiral spore chain with (B) spiny spore surface ornamentation. Bars = 10 µm (A) and 3 µm (B).

2.3.2.2.4 *Streptomyces* strain TCZ2

Streptomyces strain TCZ2 was isolated from the treated Rhodes Memorial soil sample on a CZ plate. It produced melanin and grew as yellowish translucent colonies on ISP#4, very similar characteristics to isolate TMC9. Strain TCZ2 showed very strong activity against *M. aurum* A+, but no activity against *E. coli* and weak activity against *S. aureus* (Appendix A), suggesting that it is only effective against Gram-positive bacteria. The whole cell hydrolysate contained LL-DAP and no diagnostic sugars. The physiological test results and the ability of strain TCZ2 to utilise sole carbon and nitrogen sources are shown in Tables 2.2 and 2.3. A 1491-nt 16S-rRNA gene GenBank database search showed that its closest evolutionary relative is *S. yatensis* NBRC 101000^T. This was supported by the neighbour joining tree (Fig 2.4), where *Streptomyces* strain TCZ2 grouped with isolate TMC9 and *S. yatensis* NBRC 101000^T with a bootstrap value of 88%. The sequence similarity between strains TCZ2 and TMC9 is 99.78% based on a 16S-rRNA gene sequence alignment. The sequence similarity between strain TCZ2 and *S. yatensis* NBRC 101000^T is 99.66% based on DNAMAN. This suggests that *Streptomyces* strains TCZ2 and TMC9 may belong to the same species. The electron micrographs (Fig 2.8 A & B) of strain TCZ2 showed that it has spiral spore chains with a spiny spore surface ornamentation. However, there are only a few differences between strains TCZ2 and TMC9 in the physiological test results (Tables 2.2 and 2.3). DNA-DNA hybridization will be required to determine whether strain TCZ2 is a distinct genomic species from the type strain of *S. yatensis* and whether strains TCZ2 and TMC9 belong to the same genomic species.

A) B)

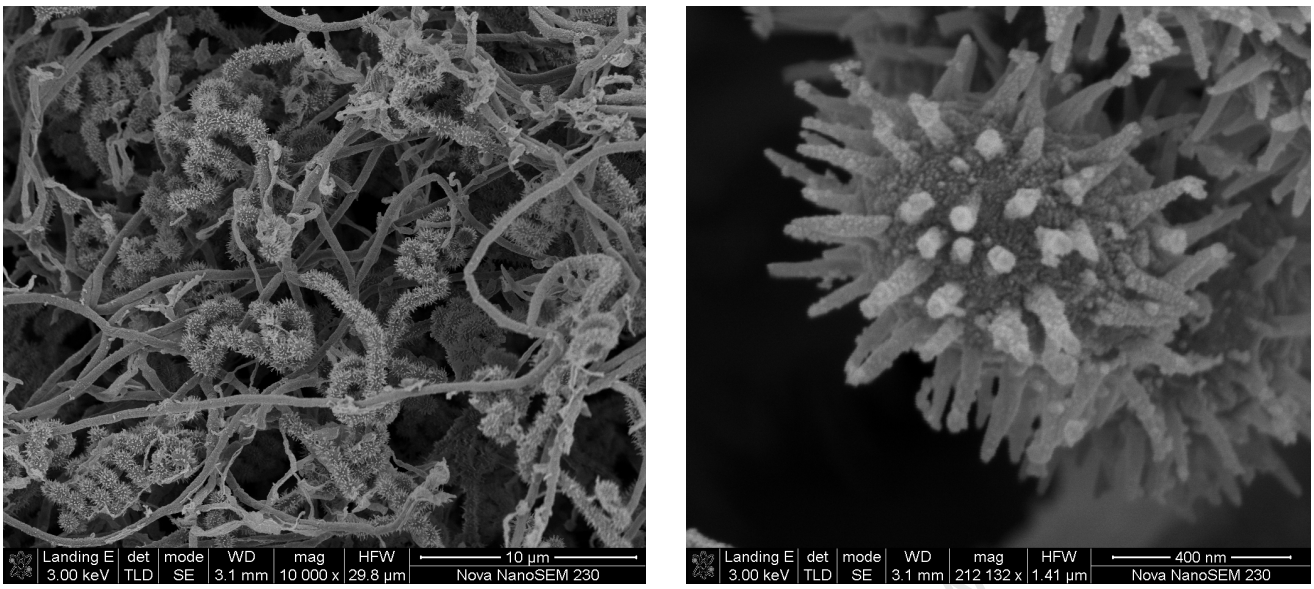

Fig 2.8 Scanning electron micrographs of *Streptomyces* strain TCZ2 showing (A) spiral spore chain with (B) spiny spore surface ornamentation. Bars = 10 µm (A) and 400 nm (B).

2.4.2.3 Non-*Streptomyces* isolates

2.4.2.3.1 *Amycolatopsis* strain TMB1

Amycolatopsis strain TMB1 was isolated from the treated Rhodes Memorial soil sample on an MB plate. It produced no pigments and showed a white flaky spore mass when grown on ISP#4. Strain TBM1 showed no antibacterial activity against any of the test bacteria (Appendix A). A GenBank BLAST search of the 1420-nt 16S-rRNA gene sequence showed that its closest evolutionary relative is *Amycolatopsis sacchari* DSM 44468^T with a sequence similarity of 97%. The neighbour joining tree (Fig 2.9) showed that strain TMB1 grouped with the type strain of *A. sacchari*, but with low bootstrap support (64%). The sequence similarity between strain TMB1 and *A. sacchari* DSM 44468^T is 97.04%, using DNAMAN. The whole cell hydrolysate contained *meso*-DAP, as well as arabinose and galactose, as expected for *Amycolatopsis*. Strain TMB1 was compared with *A. sacchari* by using physiological tests and the ability to utilize sole carbon and nitrogen sources. This data is presented in Tables 2.4 & 2.5. The physiological differences between strain TMB1 and *A. sacchari* include hypoxanthine degradation, xanthine degradation, allantoin degradation and arbutin degradation. Differences were also seen under two of the growth conditions tested, namely, 5% NaCl and 45°C, where *A. sacchari* DSM 44468^T grew better than strain TMB1. The low sequence similarity along with the physiological data suggests that strain TMB1 could be a new species. This argument is further strengthened by the *gyrB* sequence analysis in section 2.4.2.3.3

2.4.2.3.2 *Amycolatopsis* strain TMB2

Amycolatopsis strain TMB2 was isolated from the same plate as strain TMB1. It shows very similar, if not identical, morphology to strain TMB1. For this reason, it was suspected to be the same species. Strain TMB2 showed no antibacterial activity against any of the test bacteria (Appendix A). A GenBank BLAST search of the 1414-nt 16S-rRNA gene sequence showed that its closest evolutionary relative is *A. sacchari* DSM 44468^T with a sequence similarity of 97%. The sequence similarity between strains TBM1 and TMB2 is 100% over 1414-nt (determined using DNAMAN). The neighbour joining tree (Fig 2.9) shows that TMB1 and TMB2 grouped together with a bootstrap value of 100, with *A. sacchari* as the next closest relative. The whole cell hydrolysate of strain TMB2 contained *meso*-DAP, as well as arabinose and galactose. Strain TMB2 was also compared against *A. sacchari* DSM 44468^T by using physiological testing and the ability to use sole carbon and nitrogen sources (Tables 2.4 & 2.5). Strains TMB1 and TBM2 had exactly the same results in all the tests. This, together with the very high sequence similarity, strongly suggests that strains TMB1 and TBM2 belong to the same species.

Table 2.4 Sole carbon and nitrogen utilization test results for the *Amycolatopsis* isolates and their closest relatives

Test	TMB1	TMB2	*A. sacchari* DSM 44468^T	UCZ5	*A. balhimycina* DSM 44591^T	*A. rifamycinica* DSM 46095^T
Carbon Sources						
D-Glucose	+	+	+	+	+	+
Negative Control	-	-	-	-	-	-
L-Arabinose	-	-	++	++	++	++
Sucrose	weak+	weak+	+	+	+	+
D(+) Xylose	-	-	+	+	+	+
D-Galactose	+	+	++	++	++	++
meso-Inositol	-	-	+	+	+	+
D(-) Mannitol	+	+	+	+	+	+
L-Rhamnose	-	-	weak+	weak+	weak+	+
Raffinose	-	-	-	weak+	+	-
Mannose	+	+	+	+	+	+
D(-) Fructose	++	++	++	++	++	++
Salicin	-	-	-	-	-	+
Nitrogen sources						
L-Asparagine	+	+	+	+	+	+
Negative Control	-	-	-	-	-	-
L-Histidine	-	-	++	++	++	++
DL-α-Amino-n-Butyric Acid	++	++	++	++	+	-
L-Cysteine	weak+	weak+	+	+	+	+
L-Hydroxyproline	-	-	+	++	+	+
L-Methionine	weak+	weak+	+	+	+	+
Potassium Nitrate	++	++	+	++	++	++
L-Phenylalanine	+	+	+	+	+	+
L-Serine	++	++	++	++	++	++
L-Threonine	++	++	++	++	+	+
L-Valine	+	+	++	+	+	+

Notes: -, no growth; weak+, growth between - & +; +, good growth; ++ very good growth

Table 2.5 Physiological test results for the *Amycolatopsis* isolates and their closest relatives

Test	TMB1	TMB2	*A. sacchari* DSM 44468^T	UCZ5	*A. balhimycina* DSM 44591^T	*A. rifamycinica* DSM 46095^T
H_2S Production	+	+	+	+	+	+
Nitrate reduction	weak +	weak +	weak +	+	weak +	weak +
Adenine Deg.	-	-	-	-	-	-
Guanine Deg.	-	-	-	-	-	-
Hypoxanthine Deg.	-	-	+	+	+	+
Xanthine Deg.	-	-	+	-	-	-
Cellulose Deg.	-	-	-	-	-	-
Gelatin Deg.	-	-	-	+	+	+
Starch Deg.	-	-	-	-	-	-
L-Tyrosine Deg.	+	+	+	+	+	-
Xylan Deg.	-	-	-	-	-	-
Casein Deg	+	+	+	+	+	+
Tween Deg.	+	+	+	+	+	+
Urea Deg.	+	+	+	weak+	+	weak+
Allantoin Deg.	-	-	+	+	+	-
Aesculin Deg.	+	+	+	+	+	+
Arbutin Deg.	+	+	weak+	weak+	+	weak+
pH 4.3	+	+	+	+	+	+
pH 7	++	++	++	++	++	++
pH 9	++	++	++	+	++	++
0% NaCl	++	++	++	++	++	++
1% (w/v) NaCl	++	++	++	+	++	+
3% (w/v) NaCl	++	++	++	-	++	-
5% (w/v) NaCl	+	+	++	-	-	-
7% (w/v) NaCl	+	+	+	-	-	-
30°C	++	++	++	++	++	++
37°C	++	++	++	++	++	++
45°C	-	-	++	-	-	+
ISP#5	-	-	-	-	-	-
ISP#6	-	-	-	-	-	-
ISP#7	-	-	-	-	-	-
Pectin	-	-	-	-	-	-

Notes: -, no growth/degradation; weak+, growth between - & + (or a weak reaction); +, good growth/degradation; ++ very good growth; for ISP#5-7, + indicates melanin production and – indicates no production; Deg. = Degradation

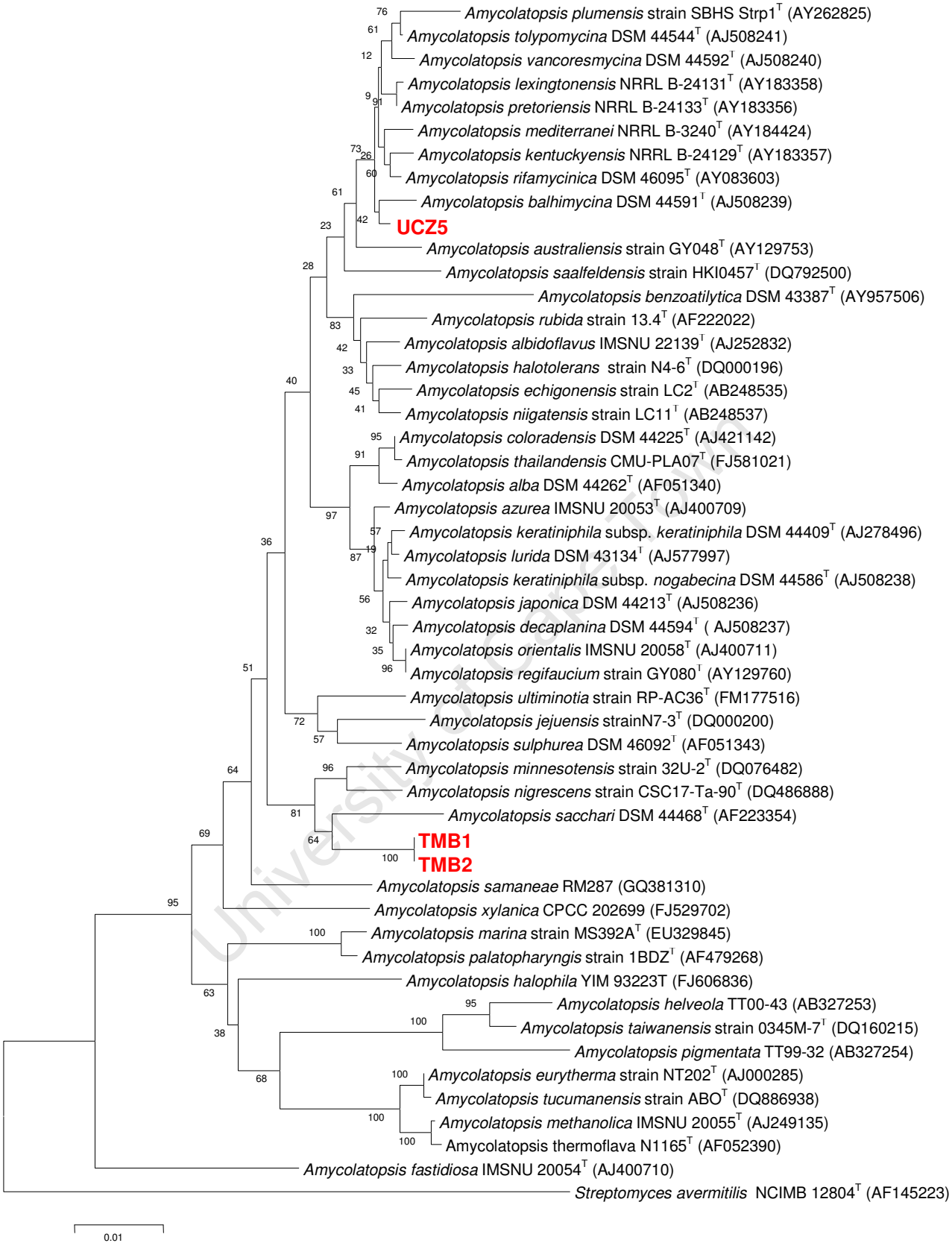

Fig 2.9 Unrooted 16S-rRNA gene neighbour-joining tree showing the phylogenetic relationships of *Amycolatopsis* strains UCZ5, TMB1 and TMB2 and all the members of the genus that have validly published names. The tree was constructed based on 1381nt of common sequence. *Amycolatopsis* strains UCZ5, TMB1 and TMB2 are shown in red. Numbers at the nodes are the percentage bootstrap values of 1000 replications. The scale bar represents 1 nucleotide substitution per 100 nucleotides.

2.4.2.3.3 *gyrB* gene sequences of *Amycolatopsis* strains TMB1 and TMB2

gyrB gene amplification was attempted on all the non-*Streptomyces* strains, using the primer pairs seen in Table 2.6. Only *Amycolatopsis* strains TMB1 and TMB2 showed amplification good enough to allow for sequencing. Various changes in the conditions of the PCR reactions were attempted to allow amplification of the *gyrB* gene from the other two non-*Streptomyces* strains (*Amycolatopsis* strain UCZ5 and *Micromonospora* strain TY1), but with no success. The *gyrB* gene of strains TMB1 and TMB2 was sequenced and analysed using DNAMAN. The *gyrB* gene sequence similarity between these two strains was 99.93%. This high sequence similarity lends further supports to the belief that strains TMB1 and TMB2 belong to the same species. A *gyrB* gene phylogenetic tree (Fig 2.10) showed that strains TMB1 and TMB2 grouped together with a bootstrap value of 100%. Strains TMB1 and TMB2 clustered with *Amycolatopsis palatopharyngis* DSM 44832^{T} and *Amycolatopsis marina* NBRC 104263^{T}, but with low bootstrap support (46%). However, strains TMB1 and TMB2 formed part of a larger 12-strain cluster, containing *A. sacchari*, which was supported by a very high bootstrap value (96%). Based on the phylogenetic tree, the sequence similarities between strains TMB1 and TMB2 and the type strains of *A. sacchari*, *A. palatopharyngis* and *A. marina* were determined. The sequence similarity between TMB1 and *A. sacchari*, *A. palatopharyngis* and *A. marina* were 86.35%, 83.36% and 85.47%, respectively. The sequence similarities between TMB2 and *A. sacchari*, *A. palatopharyngis* and *A. marina* were 86.54%, 83.32% and 85.39%, respectively. The *gyrB* gene phylogenetic tree had a much higher resolution than seen in the 16S-rRNA gene tree (Fig 2.9), evident by the longer branch lengths. The topology of the *gyrB* gene tree was also more robust than that of the 16S-rRNA gene tree, as the number of bootstrap values greater than 90% increased from 12 to 19. The prominent differences seen between the two trees calls for further investigation of both these strains in order to determine whether they belong to a new species. A *gyrB* genetic distance of 0.02 is considered to be the threshold above which an *Amycolatopsis* isolate is considered likely to be a new species (Everest & Meyers, 2009). The results (appendix A) show that the genetic distance between TMB1 and TMB2 is zero (0) and that the genetic distances between strains TMB1, TMB2 and the other 35 *Amycolatopsis* strains is >0.02. This lends support to the idea that strains TMB1 and TMB2 are from the same, novel species.

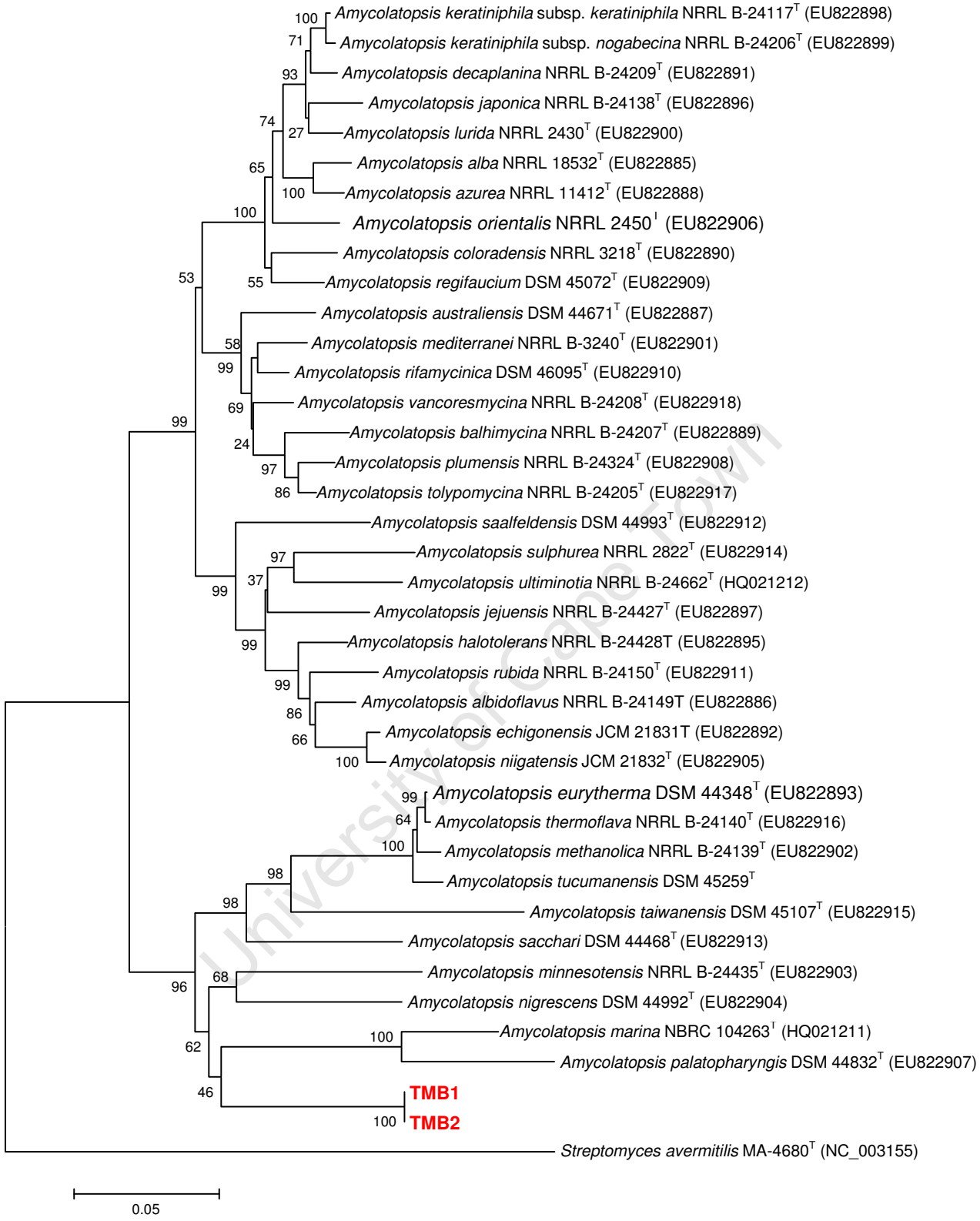

Fig 2.10 Unrooted *gyrB* gene neighbour-joining tree showing the phylogenetic relationships between *Amycolatopsis* strains TMB1and TMB2 and the members of *Amycolatopsis* that have validly published names and for which there are *gyrB* sequences available. The tree was constructed based on 1314nt of common sequence. *Amycolatopsis* strains TMB1 and TMB2 are shown in red. Numbers at the nodes are the percentage bootstrap values of 1000 replications. The scale bar represents 5 nucleotide substitutions per 100 nucleotides.

Table 2.6 Primer pairs used in the PCR amplification of the *gyrB* gene of *Amycolatopsis* strains TMB1 and TMB2

GyrB PCR primer pairs used
GgyrB-F1 & 7G-gyrB-R
GgyrB-F2 & KgyrB-R1892
7G-gyrB-F & GgyrB-R1
7G-gyrB-F & KgyrB-R

2.4.2.3.4 *Amycolatopsis* strain UCZ5

Amycolatopsis strain UCZ5 was isolated from the untreated Rhodes Memorial soil sample on a CZ plate. Strain UCZ5 showed white aerial mycelium, and produced no pigments. Strain UCZ5 had weak activity against *M. aurum A+* and no activity against *E. coli and S. aureus* (Appendix A). A GenBank BLAST search of the 1418-nt 16S-rRNA gene sequence showed that its closest evolutionary relative is *Amycolatopsis rifamycinica* strain DSM 46095^{T} with a sequence similarity of 99%. The neighbour joining tree (Fig 2.9) showed that strain UCZ5 grouped with the type strain of *Amycolatopsis balhimycina*, but with a very low bootstrap support (42%). The sequence similarities between strain UCZ5 and the type strains of *A. rifamycinica* and *A. balhimycina* are 99.15% and 99.08%, respectively. The whole cell hydrolysate contained *meso*-DAP, as well as arabinose and galactose. Strain UCZ5 was compared to strain TMB1 and the type strains of *A. balhimycina* and *A. rifamycinica* by using physiological tests and the ability to utilize sole carbon and nitrogen sources (Tables 2.4 & 2.5). There are five clear differences between strain UCZ5 and *A. balhimycina*, namely, growth at pH 9 and in the presence of 1% (w/v) NaCl and 3% (w/v) NaCl, as well as the ability to better utilise the nitrogen sources DL-α-amino-n-butyric acid and L-hydroxyproline. The differences between strain UCZ5 and *A. rifamycinica* are the ability to degrade L-tyrosine and allantoin; *A. rifamycinica* was also able to grow better at pH 9 than strain UCZ5 and at 45°C (strain UCZ5 did not grow at 45°C). *A. rifamycinica* was able to utilise salicin, while it also had a lesser ability to utilise α-amino-n-butyric acid, L-threonine and L-hydroxyproline. Due to the high 16S-rRNA gene sequence similarities, DNA-DNA hybridization will be needed to determine if strain UCZ5 belongs to a new species.

2.4.2.3.5 *Micromonospora* strain TY1

Micromonospora strain TY1 was isolated from the treated Rhodes Memorial soil sample on an ISP#2 plate. Strain TY1 showed no antibiotic activity and did not produce any pigments. During early growth of the isolate, it appeared orange, turning to black on sporulation. This is a common trait of this genus. Isolate TY1 showed antibiotic activity against any of the test bacteria (Appendix A).The whole-cell hydrolysate of strain TY1 contained *meso*-DAP and the sugars were arabinose and xylose. A GenBank

BLAST search of the 1413-nt 16S-rRNA gene sequence showed that its closest evolutionary relative is *Micromonospora endolithica* strain AA459^T with a sequence similarity of 99%. The neighbour joining tree (Fig 2.11) showed that strain TY1 grouped with the type strains of *Micromonospora chersina*, *Micromonospora coriariae* and *M. endolithica* with low bootstrap support (60%). The sequence similarities between strain TY1 and *M. chersina*, *M. endolithica* and *M. coriariae* are 99.43%, 99.65% and 98.58%, respectively. Physiological testing and the ability to utilise sole carbon and nitrogen sources was carried out on isolate TY1, as well as on *M. endolithica* AA459^T and *M. chersina* DSM 44151^T (Tables 2.7 and 2.8). Although there are several phenotypic differences between strain TY1 and the type strains of *M. endolithica* and *M. chersina*, the high 16S-rRNA gene sequence similarities between strain TY1 and these strains will require DNA-DNA hybridization to determine whether strain TY1 belongs to a new *Micromonospora* species.

This concludes the characterisation of the strains isolated by using conventional culture based techniques. Chapter 3 shows how metagenomic analysis was used as an investigative tool to assess the actinobacterial diversity of the same samples used for actinobacterial isolation described in Chapter 2.

Table 2.7 Physiological test results of *Micromonospora* strain TY1 and its closest relatives

Test	TY1	*M. endolithica* AA459^T	*M. chersina* DSM 44151^T
H_2S Production	+	-	+
Nitrate reduction	+	+	-
Adenine Deg.	-	-	-
Guanine Deg.	-	-	-
Hypoxanthine Deg.	-	-	-
Xanthine Deg.	-	-	-
Cellulose Deg.	-	-	-
Gelatin Deg.	+	weak	+
Starch Deg.	-	-	-
L-Tyrosine Deg.	-	+	-
Xylan Deg.	weak	weak	-
Casein Deg	+	+	+
Tween Deg.	+	weak	+
Urea Deg.	-	-	-
Allantoin Deg.	-	-	-
Aesculin Deg.	+	+	+
Arbutin Deg.	+	-	+
pH 4.3	-	-	-
pH 7	++	+	++
pH 9	++	++	+
0% NaCl	++	+	++
1% NaCl	+	+	+
3% NaCl	+	-	-
5% NaCl	-	-	-
7% NaCl	-	-	-
30°C	++	+	++
37°C	++	-	++
45°C	-	-	+
ISP#5	-	-	-
ISP#6	-	-	-
ISP#7	-	-	-
Pectin	-	-	-

Notes: -, no growth/degradation; weak+, growth between - & + (or a weak reaction); +, good growth/degradation; ++ very good growth; for ISP#5-7, + indicates melanin production and – indicates no production; Deg. = Degradation

Table 2.8 Sole carbon- and nitrogen-source utilization test results of *Micromonospora* strain TY1 and its closest relatives

Test	TY1	*M. endolithica* AA459^T	*M. chersina* DSM 44151^T
Carbon Sources			
D-Glucose	+	+	+
Negative Control	-	-	-
L-Arabinose	++	+	++
Sucrose	+	+	++
D(+) Xylose	+	-	++
D-Galactose	++	+	++
meso-Inositol	-	-	-
D(-) Mannitol	-	-	-
L-Rhamnose	-	-	-
Raffinose	-	+	+
Mannose	+	+	+
D(-) Fructose	++	+	++
Salicin	-	-	-
Nitrogen sources			
L-Asparagine	+	+	+
Negative Control	-	-	-
L-Histidine	-	+	-
DL-α-Amino-n-Butyric Acid	+	-	-
L-Cysteine	-	-	++
L-Hydroxyproline	-	-	-
L-Methionine	-	+	+
Potassium Nitrate	-	+	+
L-Phenylalanine	+	+	+
L-Serine	+	+	+
L-Threonine	+	+	+
L-Valine	+	-	+

Notes: -, no growth; weak+, growth between - & +; +, good growth; ++ very good growth

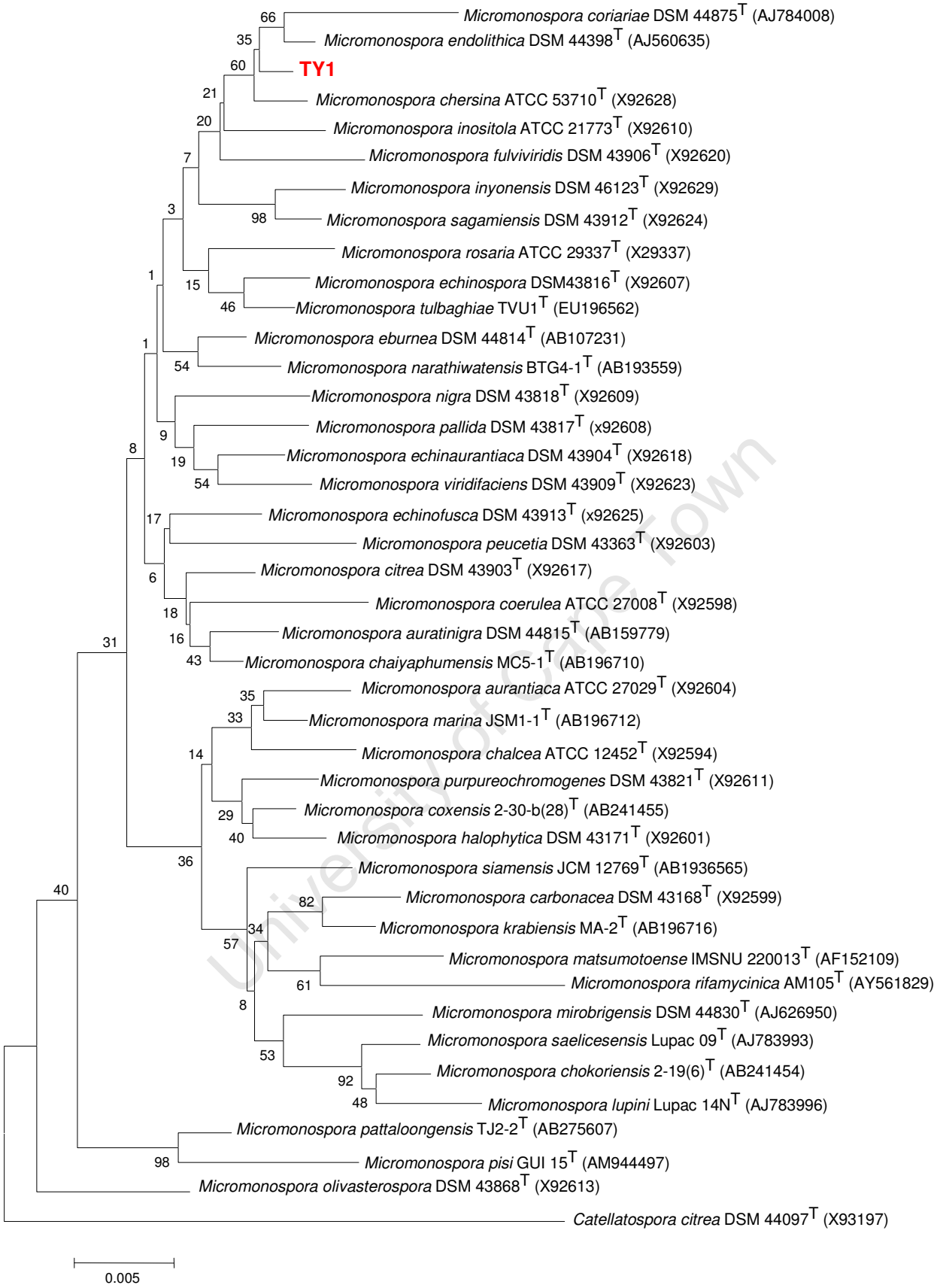

Fig 2.11 Unrooted 16S-rRNA gene neighbour-joining tree showing the phylogenetic relationships between *Micromonospora* strain TY1 and all the members of the genus that have validly published names. The tree was constructed based on 1417nt of common sequence. *Micromonospora* strain TY1 is shown in red. Numbers at the nodes are the percentage bootstrap values of 1000 replications. The scale bar represents 5 nucleotide substitutions per 1000 nucleotides.

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Chapter 3

Culture-independent analysis of the isolation samples

Chapter 3

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3.1 Abstract

A metagenomic study was done on all the samples used for actinobacterial isolation that were described in the Chapter 2. The results of the first metagenomics study done on the marine sand samples yielded a total of 384 clones, 133 from the SL sample, 115 from the RP sample and 136 from the OS sample. Of these clones, a small proportion from each sample was selected for sequencing based on the results of clone de-replication. Of the 16 SL clones selected, all were most similar to uncultured bacteria based on GenBank BLAST searches. The same was seen with the 21 RP clones and the 24 OS clones. Due to major contamination problems, the clones of the SS and DS samples from the UCT dam were lost, so identification of the microbial diversity in these samples could not be carried out.

A total of 120 clones were obtained from the Rhodes Memorial soil sample. Instead of subjecting these clones to de-replication, all were sequenced with the forward primer, Act-S20-F. The results showed that the majority of the clones (75.83%) were most closely related to uncultured bacteria. The remaining clones belonged to various families, namely, *Micrococcaceae, Nocardioidaceae, Sporichthyaceae, Pseudonocardiaceae, Nocardiaceae*, *Micromonosporaceae, Intrasporangiaceae, Streptomycetaceae* and *Geodermatophilaceae*. Within these families, the genera which were identified were *Actinophytocola*, *Actinoplanes*, *Arthrobacter*, *Blastococcus*, *Gordonia*, *Janibacter*, *Luedemannella*, *Micromonospora, Nocardioides*, *Pseudonocardia, Sporichthya* and *Streptomyces*. This contrasts with the actinobacterial strains isolated from the Rhodes Memorial soil sample (described in Chapter 2), which belonged to the genera *Streptomyces* (*Streptomycetaceae*), *Amycolatopsis* (*Pseudonocardiaceae*) and *Micromonospora* (*Micromonosporaceae*).

3.2 Introduction

The previous chapter gives a small glimpse of what is possible with the culture-based technique. There were incidences of duplicate isolation of strains and the majority of isolates found were from the genus *Streptomyces*. This genus has been very well screened and the possibility of isolating new bioactive secondary metabolites from the genus is thus lower than from other, less characterised genera. It has been shown that the best chance of finding new molecules is to try and isolate the rarer actinobacterial genera.

Many different methods are available to do this and metagenomics can be used as a tool to aid these various methods. This aspect of metagenomics will be illustrated in this chapter where metagenomic analysis is used in conjunction with the standard culture-based techniques seen in Chapter 2 to assess the actinobacterial diversity of the three sampling areas (sea sand, dam mud and terrestrial soil).

3.3 Materials and Methods

3.3.1 DNA extraction from soil, gel extraction and "touchdown" PCR

The ZR Soil Microbe DNA kit (Zymo Research, USA) was used to extract total microbial DNA from 0.25 g of sample soil (this was increased to 0.5 g of soil in the cases of all aquatic samples). The extraction protocol was followed with one change: to maximise the amount of extracted DNA, all of the supernatant was transferred and centrifuged through the IV spin filter (step 4), with all subsequent steps being adjusted accordingly. The DNA was eluted in 50 µl of elution buffer. After extraction, the eluted DNA was purified using the Wizard® DNA Clean-Up System (Promega, USA).

The isolated soil DNA was used as the template in a PCR reaction to amplify the 16S–rRNA genes in the extracted DNA, under the same conditions as previously described (section 2.3.4), but using 1.5 mM $MgCl_2$ rather than 2 mM. The PCR product of the 16S–rRNA gene amplification was then used as the template to amplify the actinobacterial 16S–rRNA genes using the actinobacterial specific primers Act-S20-F and Act-A19-R (identical to primers C-Act-0235-a-S-20-F and S-C-Act-0878-a-A-19-R of Stach *et al.*, 2003), which generate a ~640-nt product. This second, nested PCR was done with 1.5 mM $MgCl_2$ and all other components at the same concentrations as for 16S-rRNA gene amplification (section 2.3.4). The PCR programme used was the "touchdown" PCR protocol described by Stach *et al.* (2003). It was determined that 160 ng of template DNA gave the best amplification of product. The amplified actinobacterial 16S-rRNA gene DNA was then electrophoresed, excised from the gel and extracted using the BioSpin Gel Extraction Kit (BioFlux, Japan).

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3.3.2 Cloning

The purified DNA fragments from the actinobacterial 16S-rRNA gene PCR amplification from soil metagenomic DNA were ligated into the pGEM®-T Easy Vector (Promega, USA) using insert:vector ratios of 3:1 and 1:3, as per the pGEM®-T Easy Vector Systems protocol. Competent *Escherichia coli* DH5α cells were prepared by the $CaCl_2$ shock treatment method (Dagert & Ehrlich, 1979) and used for the transformations. Transformed cells were plated on Luria-Bertani agar (LA) supplemented with

ampicillin (100 µg/ml), IPTG (0.5 mM) and Xgal (80 µg/ml) and incubated for 16 hours at 37°C. White colonies were selected and sub-cultured onto fresh plates of the same LA/IPTG/Xgal medium and incubated for 16 hours at 37°C. This was done to confirm the blue-white selection result (i.e. that the selected colonies contained cloned DNA). The confirmed transformants were sub-cultured onto LA containing ampicillin (100 µg/ml) and allowed to grow at 37°C for 16 hours.

A colony PCR method, using the actinobacterial 16S-rRNA gene PCR conditions and protocol (section 2.3.4) was then performed to confirm the presence of inserts of the correct size. The KAPA2G Robust PCR kit (KAPABIOSYSTEMS, Cape Town, South Africa) was utilised for this PCR with buffer B of the system. All PCR products were electrophoresed as previously described (section 2.3.5).

3.3.3 Clone de-replication

The colony PCR products were used as substrates for digestion with endonucleases *Alu*I and *Rsa*I. The digestions were performed as previously described (section 2.3.5), with each reaction containing 2U of enzyme. All digests were visualised as previously described (section 2.3.5). After visualisation, each clone was placed into a group based on its *Alu*I digestion pattern (band patterns distinguished by eye). Once allocated to an *Alu*I digestion pattern, each clone was then digested with *Rsa*I to differentiate the clones within each *Alu*I group.

3.3.4 Sequencing and sequence analysis

The colony PCR reactions were used to sequence selected clones. The ~640-nt PCR products were purified using the MSB Spin PCRapace kit (Invitek, Germany). The DNA was then sequenced using the Act-S20-F primer. Sequencing results were analysed as described in section 2.3.6.

3.4 Results and discussion

3.4.1 Marine Soil Metagenomics study

The metagenome of the three marine samples was extracted separately and used to amplify actinobacterial DNA. A total of 133 confirmed clones was obtained from the shore-line sand sample (SL), 115 clones from the rock-pool sand sample (RP) and 136 clones from the ocean sand sample (OS). The SL sample was the first to undergo de-replication using the *Alu*I enzyme. After *Alu*I digestion, five distinct patterns were seen and the 133 clones were grouped accordingly. Each clone was then digested with *Rsa*I to distinguish between the clones within each *Alu*I pattern. A total of five different *Rsa*I patterns were seen. Sixteen (16) clones were chosen for sequencing based on the fact that they had distinct *Alu*I and *Rsa*I fragment patterns (i.e. they were most likely unique). The results of the sequence analysis can be seen in Table 3.1.

Although the sequenced clones represent a small sample of the total number of SL clones (12%), the results show that all of the top GenBank hits were to uncultured bacteria. This result provides a possible explanation for why no actinobacterial colonies were isolated in the culture-based techniques. What is also interesting to note is that, firstly, replicates were seen after sequencing, the four sets of replicates were: clones SL9 & SL27, clones SL11 & SL14 & SL124, clones SL76 & SL92 and clones SL20 & SL45. This suggests that the de-replication procedure was not perfect, yet still managed to minimise replicates. Secondly, it is interesting that non-actinobacterial 16S-rRNA genes were amplified with the actinobacterial specific primers. This phenomenon was also reported by Babalola *et al.* (2009) where *Planctomyces* and *Gemmatimonas* sequences were amplified with these primers. It must be noted that this is not a definitive reason why no actinobacteria were isolated from the aquatic samples. Since all sequenced clones were uncultured actinobacteria of some sort the real challenge would be to discover the medium/conditions that these bacteria require. This however could lead to a very lengthy study or in fact be a study all on its own. Based on the results of this metagenomic study informed the decision to abandon the investigation of the marine samples (i.e. the metagenomic study was used as a tool to evaluate whether it was worthwhile to continue the culture-based investigation of the marine samples).

Table 3.1 Results of the BLAST analysis of selected SL clones after de-replication

Representative clone	Top Blast Hit	Accession number	Maximum sequence similarity
SL Clone 8	Uncultured bacterium clone FCPP578	EF516742.1	74%
SL Clone 9	Uncultured Actinobacteria clone H3	GU591615.1	96%
SL Clone 11	Uncultured delta-proteobacterium clone PI r120c2	AY374745.1	77%
SL Clone 14	Uncultured delta-proteobacterium clone PI r120c2	AY374745.1	100%
SL Clone 17	Uncultured planctomycete clone Arabian sea cB11	EU478623.1	94%
SL Clone 20	Uncultured bacterium A7	FJ716841.1	93%
SL Clone 21	Uncultured bacterium P9X2b3A06	EU491094.1	93%
SL Clone 27	Uncultured actinobacterium clone H3	GU591615.1	99%
SL Clone 29	Uncultured bacterium partial clone GOM161	AM745145.1	95%
SL Clone 41	Uncultured actinobacterium clone SHAB560	GQ348658.1	96%
SL Clone 45	Uncultured bacterium clone A7	FJ716841.1	99%
SL Clone 76	Uncultured planctomycete clone Arabian sea	EU478623.1	95%
SL Clone 92	Uncultured planctomycete clone Arabian sea	EU478623.1	94%
SL Clone 104	Uncultured bacterium clone tet2ect2G4	EU290351.1	86%
SL Clone 107	Uncultured bacterium clone XCDL-20-WB44	FJ948257.1	94%
SL Clone 124	Uncultured delta-proteobacterium clone PI r120c2	AY374745.1	74%

A similar procedure was carried out with the RP marine clones. Similar digestion patterns were seen and the clones were allocated to six *Alu*I patterns and the same five *Rsa*I patterns seen for the SL clones. The 21 clones sent for sequencing were selected in the same manner as those for the SL clones. The results of the sequencing can be seen in Table 3.2. A very similar result was seen where all the top GenBank hits were to uncultured bacteria. There was an instance where a similarity was seen between the SL and RP clones: clone SL17 was the same as RP clones 25 & 29. It is expected that the RP samples might be similar to the OS samples considering that rock pools are constantly being flushed with sea water.

Table 3.2 Results of the BLAST analysis of selected RP clones after de-replication

Representative clone	Top Blast Hit	Accession number	Maximum sequence similarity
RP Clone 6	Uncultured bacterium clone EPR3968-O8a-Bc44	EU491711.1	96%
RP Clone 11	Uncultured bacterium clone GB7N87002DMXNQ	HM675698.1	79%
RP Clone 15	Uncultured planctomycete clone placlone27	HM369087.1	97%
RP Clone 25	Uncultured planctomycete clone ArabianSea_cB11	EU478623.1	94%
RP Clone 29	Uncultured planctomycete clone ArabianSea_cB11	EU478623.1	93%
RP Clone 31	Uncultured deep-sea bacterium clone Ulrdd_34	AM997498.1	90%
RP Clone 38	Uncultured bacterium clone CBM02B12	EF395711.1	94%
RP Clone 47	Uncultured bacterium clone AMSMV-10-B48	HQ588513.1	91%
RP Clone 51	Uncultured bacterium clone CBM02B12	EF395711.1	93%
RP Clone 53	Uncultured bacterium clone 193b2	EF459974.1	98%
RP Clone 57	Uncultured bacterium clone P0X4b3H02	EU491457.1	91%
RP Clone 65	Uncultured actinobacterium clone H54	GU591579.1	98%
RP Clone 70	Uncultured deep-sea bacterium clone Ucm1571	AM997294.1	92%
RP Clone 72	Uncultured bacterium clone A39	AJ966579.1	73%
RP Clone 79	Uncultured actinobacterium clone M4-38	EU682499.1	87%
RP Clone 82	Uncultured marine bacterium clone SiDSep07M20	GU326862.1	92%
RP Clone 91	Uncultured planctomycete clone JK238	DQ368325.1	96%
RP Clone 97	Uncultured actinobacterium clone MS-C124	FJ949412.1	94%
RP Clone 107	Uncultured actinobacterium clone H21	GU591598.1	93%
RP Clone 113	Uncultured bacterium clone bOHTK-60	FJ873305.1	91%
RP Clone 115	Uncultured planctomycete clone JK238	DQ368325.1	96%

Furthermore, identical hits were found amongst the RP clones, as was seen for the SL clones (Table 3.1). Only two examples were seen this time, namely, RP25 & RP29, RP38 & RP51 and RP91 & RP115. Most of the OS marine clones were contaminated by what appeared to be a mould during storage at 4 °C. Those clones which could be saved were subcultured onto new plates. A total of 24 clones was saved and

sent for sequencing. The results can be seen in Table 3.3. All of the top GenBank hits were to uncultured bacteria. More examples of replicates were seen in the OS samples as compared to the SL and RP samples. This could possibly be attributed to the fact that the de-replication procedure was not performed on this batch of clones, the six sets of replicates seen were: OS62 & OS78, OS64 & OS93, OS71 & OS79 & OS81, OS77 & OS98, OS85 & OS102 and OS90 & OS104.

When all samples were compared to each other there was not a single example where the same replicate was found in all three samples. However there were examples seen between two samples. One example was seen between the SL and OS samples, namely, clones SL9 and OS90 and OS104. Another one was seen between the SL sample and the RP sample, namely clones SL17 and RP25 and RP29. Three replicates were seen between the OS and RP samples. This is not surprising, as the rock pool areas are constantly being flushed by seawater. The replicate clones seen were OS64 & RP97, OS71 & RP53 and OS96 & RP79. These results show again that the de-replication method is not perfect, but does seem to minimise the number of replicates seen. The effectiveness of the de-replication method could possibly be improved by the use of a third restriction endonuclease.

3.4.2 Freshwater Soil Metagenomics study

A metagenomic study was started on both the DS and SS freshwater samples from the UCT dam. The procedure carried out was identical to that carried out on the marine samples. A total of 112 clones was obtained for the SS sample and 118 for the DS sample. Unfortunately, the Freshwater sample plates were contaminated by a similar mould to the OS clones during storage at 4 °C. No clones could be saved for further investigation and this part of the study was abandoned.

Table 3.3 Results of the BLAST analysis of the OS clones

Representative clone	Top Blast Hit	Accession number	Maximum sequence similarity
OS Clone 62	Uncultured actinobacterium clone MS-K14	FJ949208.1	96%
OS Clone 64	Uncultured actinobacterium clone MS-C124	FJ949412.1	93%
OS Clone 66	Uncultured bacterium clone XmE171	EF159904.1	95%
OS Clone 68	Uncultured bacterium clone SIMO-1891	AY711257.1	86%
OS Clone 71	Uncultured bacterium clone 193b2	EF459974.1	98%
OS Clone 72	Uncultured bacterium clone PropaneSIP6-6-47	GU584739.1	90%
OS Clone 73	Uncultured actinobacterium clone SAb11	AY124423.1	90%
OS Clone 76	Uncultured actinobacterium clone 117	FJ205298.1	99%
OS Clone 77	Uncultured bacterium clone TfC20L19	EU362289.1	99%
OS Clone 78	Uncultured actinobacterium clone MS-K14	FJ949208.1	97%
OS Clone 79	Uncultured bacterium clone 193b2	EF459974.1	99%
OS Clone 81	Uncultured bacterium clone 193b2	EF459974.1	99%
OS Clone 85	Uncultured bacterium clone P9X2b3A06	EU491094.1	96%
OS Clone 86	Uncultured forest soil bacterium clone DUNssu130	AY913337.1	98%
OS Clone 90	Uncultured actinobacterium clone H3	GU591615.1	99%
OS Clone 91	Uncultured bacterium clone Cm1-10	GQ246347.1	95%
OS Clone 93	Uncultured actinobacterium clone MS-C124	FJ949412.1	94%
OS Clone 96	Uncultured actinobacterium clone M4-38	EU682499.1	96%
OS Clone 98	Uncultured bacterium clone TfC20L19	EU362289.1	92%
OS Clone 101	Uncultured bacterium clone ncd864d08c1	HM298001.1	95%
OS Clone 102	Uncultured bacterium clone P9X2b3A06	EU491094.1	90%
OS Clone 104	Uncultured actinobacterium clone H3	GU591615.1	94%
OS Clone 106	Uncultured bacterium clone SIPt0-31	GU584860.1	85%
OS Clone 113	Uncultured bacterium clone Kazan-1B-44/BC19-1B-44	AY592121.1	94%

3.4.3 Rhodes Memorial Soil Metagenomic study

This sample provided actinomycete isolates, of which eight were chosen for further study (Chapter 2). This made the metagenomic study a lot more interesting, as a comparison could be made between the actinobacterial diversity detected by culture-based and culture-independent methods.

A total of 120 clones was obtained from the Rhodes Memorial soil sample. Instead of following the procedure carried out in the marine and freshwater metagenomics studies, clone de-replication was not carried out. Instead, all 120 clones were sequenced in order to assess the actinobacterial diversity (only 16% of all the marine clones were sequenced). The incidence of replication was very high in this sample. Of the 120 clones sequenced only 43 were unique clones, this means that 64% of the clones were duplicates. The incidence of replicates was much lower in the previous samples and is likely attributed to the de-replication process. This result shows that even though the de-replication process is imperfect, it is still very useful and should be employed and optimised. The results of the sequencing are represented in Fig 3.1, in which the pie chart shows the breakdown of actinobacterial families identified in the study. It is again clearly evident that the greatest proportion of the diversity was that of uncultured bacteria, which made up 75.83% of the clones sequenced. This clearly supports the idea stated in Chapter 1 that the majority of microorganisms present in soil are uncultured. Appendix B shows the complete list of clones sequenced along with the maximum similarities to (and accession numbers of) their top hits in GenBank. Among the clones most closely related to uncultured organisms, the top hits were to uncultured bacteria and uncultured actinobacteria, which is the trend also reported by Babalola *et al.* (2009). The rest of the uncultured clones were made up of *Gemmatimonadetes*, the 16S-rRNA genes of which are known to be amplified with the actinobacterial specific primers of Stach *et al*. (2003), *Acidimicrobidae*, *Actinopolymorpha* (a member of the *Nocardioidaceae* family with four described species) and lastly uncultured *Mycobacterium* species.

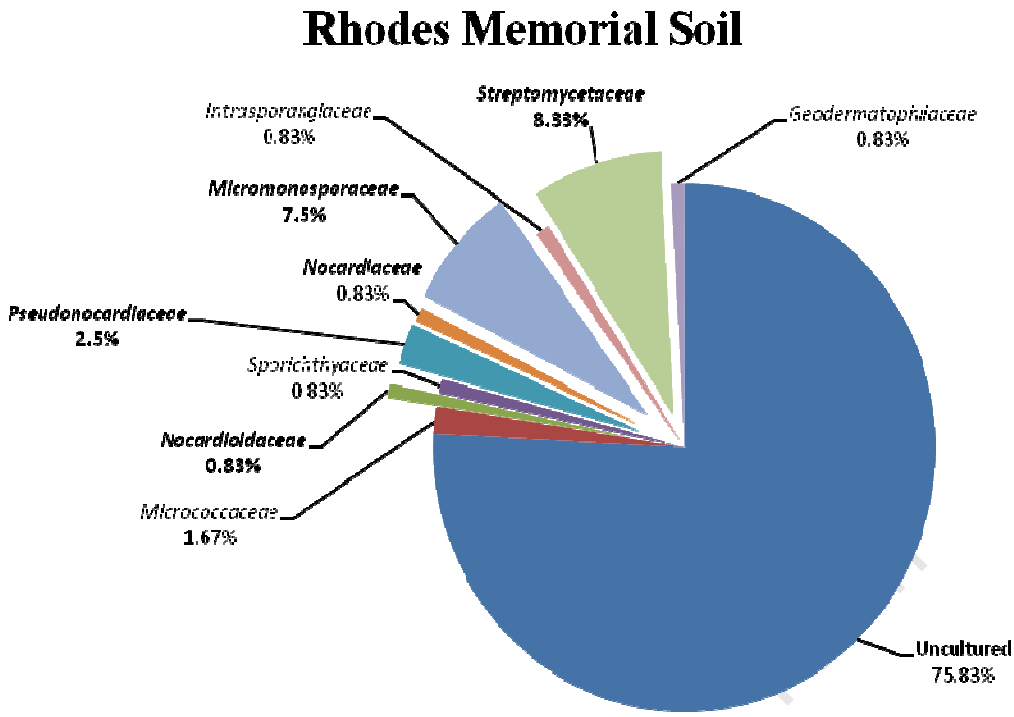

Fig 3.1 Pie chart showing the clone diversity found in the Rhodes Memorial soil as percentage compositions of all the clones found. Filamentous actinobacterial groups are shown in bold type and have been exploded out from the main body.

The second highest proportion of clones belonged to the family *Streptomycetaceae* with 8.33%. Streptomycetes are known to be abundant in soil samples (Seong *et al*, 2001). The third highest proportion of clones belonged to the family *Micromonosporaceae* with 7.5%. Interestingly, the genus *Luedemannella*, proposed by Ara and Kudo (2007), was identified and constituted 22% of the clones belonging to the *Micromonosporaceae* sequenced. This rare genus has only recently been described and has only two described species. Identifying *Luedemannella* shows the power of metagenomics, as it is unlikely to have been detected with conventional, culture-based methods.

A number of clones belonging to the genus *Micromonospora* were also seen; they constituted 66% of the *Micromonosporaceae* family and 5% of all the clones. Since this genus makes up such a small percentage of the total clones, it is not surprising that only one isolate from this genus was found using the culture-based method. The remaining seven families detected only had between one and three clones as representatives. One of the most interesting of these seven families is *Geodermatophilaceae* whose representative clone was shown to be most closely related to *Blastococcus jejuensis* (Lee, 2006) with a sequence similarity of 73%. There are only three species of *Blastococcus* with validly published names.

Another very interesting clone is that belonging to the family *Pseudonocardiaceae*. This clone was shown to belong to a very newly proposed genus, *Actinophytocola* (Indananda *et al.*, 2010), which has only one described species, *Actinophytocola oryzae*, and had a sequence similarity of 99%. This endophytic species was isolated from the roots of a Thai glutinous rice plant (Indananda *et al.*, 2010). The other two clones from the family *Pseudonocardiaceae* were most similar to strains from the genus *Pseudonocardia* and had sequence similarities of 98%. What is interesting is that, out of the 120 clones, not one was related to the genus *Amycolatopsis*, which belongs to the family *Pseudonocardiaceae*. This is surprising, since three isolates from this genus were described in Chapter 2. Although isolate UCZ15 was not fully studied, sequencing suggested it belonged to the genus *Nocardia*. However, no members of this genus were seen among the clones sequenced. Table 3.4 shows the families identified in the metagenomic study of the Rhodes Memorial soil sample along with their representative genera.

Table 3.4 Table showing the families and genera represented in Fig 3.1

Family	Genera/Genus
Micrococcaceae	*Arthrobacter*
Nocardioidaceae	*Nocardioides*
Sporichthyaceae	*Sporichthya*
Pseudonocardiaceae	*Pseudonocardia, Actinophytocola*
Nocardiaceae	*Gordonia*
Micromonosporaceae	*Micromonospora, Luedemannella, Actinoplanes*
Intrasporangiaceae	*Janibacter*
Streptomycetaceae	*Streptomyces*
Geodermatophilaceae	*Blastococcus*

The results presented in this study show that there is a great reservoir of untapped actinobacterial diversity in the soil. It also points to the potential of metagenomics as a tool to aid culture based techniques, for example, for targeting rare species. Metagenomics is also not perfect, as it is impossible for it to identify the entire actinobacterial diversity in a sample since there are just so many varieties of bacteria in any sample. For the Rhodes Memorial soil sample specifically, special methods, such as pre-treatment and growth conditions, could be employed to target the *Luedemannella*, *Actinophytocola* and *Blastococcus* species identified by the metagenomic analysis.

3.5 References

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Chapter 4

Conclusion

Chapter 4

Conclusion

Although the marine and freshwater samples yielded no actinobacterial isolates, the use of marine and freshwater sediment samples cannot be ignored as a potential source for novel species discovery. This is illustrated by the discovery of the obligate marine genus *Salinispora* (Bull & Stach, 2007), as well as by marine actinobacterial isolates that produce novel antibiotics, such as abyssomicin. The results of the metagenomic study of the marine sample showed that actinobacteria were present, although all the clones were most closely related to unculturable actinobacteria. Although only a small proportion (16%) of the marine-sample clones were sent for sequencing, the fact that all of these clones had no cultured relatives led to the decision to abandon actinobacterial isolation from this source, however future study on these samples cannot be entirely abandoned. The reason for failure can also be attributed to incorrect media. The bacteria actinomycetes present in the aquatic samples could require very specific media requirements. Using the water to make the media is a possible method to better simulate the environment the actinomycetes are used, this still however does not guarantee bacteria will be isolated.

Of the eight isolates from the Rhodes Memorial soil sample chosen for further study, four were shown to belong to the genus *Streptomyces*, three to the genus *Amycolatopsis* and one to the genus *Micromonospora*. The results clearly showed a skewed isolation pattern towards the genus *Streptomyces*, since more than 80% of the total of 52 isolates were identified as belonging to this genus. This pattern does not aid in the discovery of newer species as *Streptomyces* is already a well described genus and is easy to cultivate (Seong *et al*., 2001). This leads to a higher occurrence of re-isolation of strains belonging to known species. In this study, specifically, the four isolates belonging to the genus *Streptomyces* were UCZ4, UCZ14, TMC9 and TZC2, three of which (UCZ4, TMC9 and TCZ2) showed very high antibacterial activity against *M. aurum* A+. Strain UCZ14 was chosen since it was initially thought to be a non-*Streptomyces* species. More work needs to be done on these *Streptomyces* isolates to determine whether they belong to new species. This work will include further chemotaxonomic tests, such as determining phospholipid patterns and identification of the major menaquinones. Additional physiological tests should be carried out in parallel with the isolates' most closely related species to determine how many phenotypic differences there are. As the 16S-rRNA gene sequence similarities between each of the isolates and its closest phylogenetic relatives are high (>98.7%), DNA-DNA hybridisation will have to be carried out between each isolate and its relatives to determine whether the isolates belong to distinct genomic species.

Of greater interest were the four non-*Streptomyces* isolates, TMB1, TMB2, TY1 and UZC5. Strains TMB1, TMB2 and UCZ5 belong to the genus *Amycolatopsis* and strain TY1 to the genus *Micromonospora*. Strain TY1 was tested against is closest relatives by using different physiological tests and some differences were observed. The 16S-rRNA gene sequence similarities between strain TY1 and its closest relatives, *M. chersina*, *M. coriariae* and *M. endolithica*, were high (≥98%). Further phenotypic characterisation of these strains should be carried out, as suggested for the *Streptomyces* strains. DNA-DNA hybridization must also be done to determine whether these non-*Streptomyces* isolates belong to new species.

Isolate UCZ5 was one of the *Amycolatopsis* strains found. This isolate had high 16S-rRNA gene sequence similarities (≥98%) to the type strains of *A. rifamycinica* and *A. balhimycina*. Although differences were recorded in the ability to degrade certain substrates and in the ability to grow under different conditions, further phenotypic characterisation should be done. Isolate UCZ5 should be compared to more type strains of the genus *Amycolatopsis*, namely *Amycolatopsis kentuckyensis, Amycolatopsis mediterranei, Amycolatopsis pretoriensis, Amycolatopsis lexingtonensis, Amycolatopsis vancoresmycina*, *Amycolatopsis tolypomycina* and *Amycolatopsis plumensis* and the sequence of its *gyrB* gene should be determined. Furthermore, DNA-DNA hybridisation must also be performed to determine whether strain UCZ5 belongs to a distinct genomic species.

Of all the isolates, the most interesting are strains TMB1 and TMB2, which were shown to belong to the genus *Amycolatopsis*. Strains TMB1 and TMB2 showed physiological differences when compared to their closest relative, the type strain of *A. sacchari*, and also have a low 16S-rRNA gene sequence similarity to *A. sacchari* (97%). However, there was low bootstrap support in the 16S-rRNA gene neighbour-joining tree (Figure 2.9) for the association of these strains with *A. sacchari*. Although further investigation is required, based on the data obtained in this study, strains TMB1 and TMB2 are likely to belong to a new species of *Amycolatopsis*. Strains TMB1 and TMB2 are probably clones of the same strain, since they were isolated from the same plate, are morphologically identical and they could not be distinguished after conducting physiological tests (Tables 2.4 and 2.5).

The *gyrB* gene neighbour-joining tree (Figure 2.10) showed much better resolution (longer branch lengths) than the *Amycolatopsis* 16S-rRNA gene tree, but now strains TMB1 and TMB2 formed part of a larger cluster of 12 strains (including *A. sacchari*), which had very high bootstrap support (96%). When the genetic distances were calculated for the 35 *Amycolatopsis* type strains used in the *gyrB* gene neighbour-joining tree, strain TMB1 had a genetic distance of >0.02 against all the strains, strengthening the view that strains TMB1 and TMB2 belong to a new *Amycolatopsis* species. A genetic distance of

>0.02 is the threshold reported to indicate that an *Amycolatopsis* isolate is likely to belong to a distinct genomic species (Everest & Meyers, 2009). This genetic-distance analysis further suggested that strains TMB1 and TMB2 are clones of the same strain, as the *gyrB* genetic distance between them is zero.

The results of the metagenomic study of the marine samples suggest an explanation for the lack of actinobacterial isolates using culture based techniques (i.e. that there were no culturable actinobacteria in the marine samples). In this case, metagenomics is without a doubt a useful tool, as it was used to determine the merit of using the marine sample as a source for isolation and was the basis for deciding to abandon actinobacterial isolation from the this sample. The metagenomic study done on the Rhodes Memorial soil sample went into greater detail, as all the clones were sequenced. The results showed that some very interesting actinobacterial genera were present in the sample. These genera were *Luedemannella*, *Actinophytocola* and *Blastococcus*, all three of which have few species with validly-published names.

The detection of these rare genera illustrates the use of metagenomics as a tool to target specific genera or possibly species. In this sample, for example, the *Actinophytocola* clone would have been the most interesting genus to target, as there is only one described species. Since it is known that this genus is present in the soil sample, steps could be taken to maximise the chances of isolating it by manipulating the culture conditions. Another possibility is to narrow the search within the actinobacteria by using genus-specific primers, thereby targeting a specific genus of interest, such as the genera *Saccharomonospora* or *Amycolatopsis* (Salazar *et al.*, 2000; Tan *et al*., 2006). This can prove useful in establishing if the sample can yield interesting isolates before time is spent on investigating it. Metagenomics can also be used to screen for other targets by changing the primers used in the PCR. For example, by using antibiotic biosynthetic gene primers a snap-shot of the possible antibiotic synthesising genes present in the sample can be viewed.

Metagenomics on its own can only go so far, since not much can be done with the clones, since the insert is either just a portion of the 16S-rRNA or antibiotic synthetic gene. However, metagenomics can be used as a powerful tool in conjunction with culture-based techniques. Metagenomics has the potential to eliminate the randomness and luck-of-the-draw style isolating from culture based techniques and allow for some form of structure and direction. For these reasons, metagenomics has a definite future in studies directed toward new species and new antibiotic discovery. In conclusion the two methods employed in this study each have their weaknesses on their own, but together they become powerful tools to discover potentially novel antibiotics.

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APPENDICES

Appendix A

Antibiotic activity against *M.aurum* A+ of all isolates from the MS sample

Isolate	Antibiotic Activity (mm^2)			
	MB	**CZ**	**YEME**	**MC**
UY1	N/A	N/A	N/A	N/A
UY2	N/A	N/A	939.33	N/A
UY3	N/A	N/A	N/A	N/A
UCZ1	427.26	656.6	1809.56	537.21
UCZ2	N/A	N/A	N/A	N/A
UCZ3	740.3	1083.85	2309.1	656.59
UCZ4	N/A	N/A	5315.57	N/A
UCZ5	933.8	N/A	263.9	43.9
UCZ6	996.67	1193.02	3141.6	N/A
UCZ7	N/A	628.31	1929.72	73.23
UCZ8	395.84	1913.23	3298.67	1143.54
UCZ9	1231.5	867.08	1736.52	1039.08
UCZ10	628.32	1055.58	2174.77	1441.99
UCZ11	552.9	1055.6	3244.48	581.9
UCZ12	N/A	N/A	N/A	N/A
UCZ13	N/A	N/A	N/A	N/A
UCZ14	N/A	N/A	3141.6	N/A
UCZ15	N/A	N/A	N/A	N/A
UCZ16	N/A	N/A	N/A	N/A
UMC1	N/A	N/A	N/A	N/A
UMC2	921.3	760.3	2286.3	1143.5
UMC3	N/A	N/A	439.8	N/A
UMC4	N/A	N/A	N/A	N/A
UMC5	N/A	N/A	N/A	N/A
UMC6	N/A	N/A	3063.05	N/A
UMC7	N/A	N/A	N/A	N/A
UMC8	N/A	N/A	250.54	N/A
UMC9	141.37	219.91	82.47	301.59
UMC10	N/A	N/A	N/A	N/A

N/A – no activity

Antibiotic activity against *M.aurum* A+ of all isolates from the MS sample

Isolate	Antibiotic Activity (mm^2)			
	MB	**CZ**	**YEME**	**MC**
TY1	N/A	N/A	N/A	N/A
TY2	2325.56	1206.40	5360.34	2277.65
TY3	1388.58	263.90	2714.34	3462.03
TY4	1642.27	1884.95	5256.67	3784.83
TY5	3798.20	1899.90	5315.57	2591.90
TCZ1	1526.81	267.82	1178.10	559.99
TCZ2	N/A	691.15	5315.57	2095.44
TCZ4	1244.00	204.20	115.50	608.70
TCZ8	307.88	577.28	628.32	N/A
TCZ9	N/A	1178.10	N/A	596.90
TMC2	N/A	N/A	58.90	N/A
TMC3	N/A	N/A	1868.50	N/A
TMC4	N/A	N/A	N/A	N/A
TMC5	2799.16	341.64	1884.95	5332.07
TMC6	N/A	49.48	N/A	N/A
TMC7	N/A	N/A	N/A	N/A
TMC8	1401.93	412.30	2028.70	5346.90
TMC9	1583.36	1511.89	5360.34	5382.33
TMC10	N/A	N/A	N/A	N/A
TMC11	N/A	N/A	N/A	N/A
TMC12	219.90	N/A	2748.90	N/A
TMB1	N/A	N/A	N/A	N/A
TMB2	N/A	N/A	N/A	N/A

N/A – no activity

Antibiotic activity against *S.aureus* of the 8 chosen isolates from the MS sample

Isolate	Antibiotic Activity (mm^2)			
	MB	**CZ**	**YEME**	**MC**
TMB1	N/A	N/A	N/A	N/A
TMB2	N/A	N/A	N/A	N/A
UCZ5	285.9	134.3	N/A	N/A
TY1	N/A	N/A	N/A	N/A
UCZ14	N/A	N/A	671.5	104.5
UCZ4	N/A	N/A	N/A	N/A
TMC9	N/A	138.23	341.6	2969
TCZ2	109.9	162.5	285.1	2073

N/A – no activity

Appendix B

Amycolatopsis gyrB **genetic distance matrix for isolates TMB1 and TMB2**

[1] A. *alba*
[2] A. *albidoflavus*
[3] A. *australiensis*
[4] A. *azurea*
[5] A. *balhimycina*
[6] A. *coloradensis*
[7] A. *decaplanina*
[8] A. *echigonensis*
[9] A. *Eurytherma*
[10] A. *halotolerans*
[11] A. *japonica*
[12] A. *jejuensis*
[13] A. *keratiniphila* subsp. *keartiniphila*
[14] A. *keratiniphila* subsp. *nogabecina*
[15] A. *lurida*
[16] A. *marina*
[17] A. *mediterranei*
[18] A. *methanolica*
[19] A. *minnesotensis*
[20] A. *nigrescens*
[21] A. *niigatensis*
[22] A. *orientalis*
[23] A. *palatopharyngis*
[24] A. *plumensis*
[25] A. *regifaucium*
[26] A. *rifamycinica*
[27] A. *rubida*
[28] A. *saalfeldensis*
[29] A. *sacchari*
[30] A. *sulphurea*
[31] A. *taiwanensis*
[32] A. *thermoflava*
[33] A. *tolypomycina*
[34] A. *tucumumanensis*
[35] A. *ultiminotia*
[36] A. *vancoresmycina*
[37] S. *avermitilis*
[38] TMB1
[39] TMB2

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
[1]																																								
[2]	0.103																																						
[3]	0.087	0.100																																					
[4]	0.024	0.102	0.087																																				
[5]	0.104	0.099	0.062	0.103																																			
[6]	0.054	0.102	0.087	0.052	0.103																																		
[7]	0.038	0.104	0.085	0.038	0.103	0.051																																	
[8]	0.111	0.037	0.110	0.109	0.101	0.108	0.111																																
[9]	0.180	0.178	0.157	0.181	0.174	0.183	0.178	0.180																															
[10]	0.101	0.033	0.100	0.100	0.094	0.102	0.099	0.052	0.181																														
[11]	0.054	0.103	0.088	0.052	0.098	0.057	0.030	0.110	0.178	0.102																													
[12]	0.113	0.062	0.104	0.116	0.092	0.116	0.107	0.071	0.185	0.065	0.108																												
[13]	0.038	0.104	0.086	0.037	0.101	0.047	0.016	0.110	0.171	0.097	0.030	0.111																											
[14]	0.040	0.106	0.087	0.040	0.102	0.050	0.018	0.112	0.171	0.098	0.030	0.113	0.005																										
[15]	0.045	0.106	0.087	0.041	0.104	0.051	0.025	0.115	0.175	0.106	0.030	0.114	0.020	0.021																									
[16]	0.208	0.202	0.199	0.206	0.193	0.198	0.201	0.211	0.180	0.208	0.194	0.212	0.194	0.193	0.199																								
[17]	0.089	0.100	0.046	0.086	0.046	0.086	0.087	0.110	0.170	0.092	0.081	0.096	0.087	0.087	0.088	0.192																							
[18]	0.185	0.186	0.159	0.184	0.180	0.184	0.180	0.188	0.011	0.190	0.181	0.192	0.174	0.175	0.177	0.185	0.176																						
[19]	0.189	0.179	0.163	0.186	0.159	0.180	0.176	0.173	0.157	0.180	0.175	0.181	0.176	0.178	0.174	0.198	0.167	0.158																					
[20]	0.172	0.171	0.154	0.173	0.153	0.169	0.171	0.181	0.159	0.169	0.167	0.188	0.167	0.166	0.173	0.163	0.161	0.160	0.121																				
[21]	0.114	0.038	0.111	0.111	0.102	0.110	0.114	0.011	0.181	0.052	0.115	0.073	0.111	0.115	0.116	0.210	0.114	0.189	0.172	0.176																			
[22]	0.051	0.104	0.093	0.045	0.099	0.050	0.047	0.111	0.175	0.103	0.051	0.111	0.044	0.046	0.048	0.195	0.081	0.177	0.177	0.166	0.114																		
[23]	0.223	0.232	0.215	0.217	0.217	0.219	0.217	0.234	0.200	0.234	0.212	0.234	0.212	0.215	0.217	0.086	0.210	0.199	0.198	0.171	0.232	0.210																	
[24]	0.098	0.089	0.059	0.097	0.036	0.093	0.095	0.103	0.168	0.087	0.089	0.093	0.097	0.098	0.095	0.196	0.050	0.175	0.155	0.150	0.106	0.092	0.218																
[25]	0.053	0.095	0.080	0.047	0.090	0.041	0.048	0.104	0.173	0.090	0.051	0.108	0.048	0.050	0.046	0.194	0.067	0.175	0.179	0.167	0.106	0.042	0.214	0.080															
[26]	0.080	0.093	0.044	0.075	0.048	0.079	0.075	0.102	0.164	0.087	0.075	0.090	0.076	0.077	0.080	0.189	0.028	0.171	0.168	0.161	0.105	0.075	0.213	0.044	0.067														
[27]	0.117	0.038	0.106	0.111	0.110	0.108	0.111	0.049	0.188	0.043	0.114	0.073	0.112	0.114	0.119	0.213	0.104	0.195	0.171	0.173	0.050	0.113	0.237	0.098	0.105	0.102													
[28]	0.119	0.088	0.094	0.120	0.097	0.107	0.109	0.103	0.175	0.087	0.112	0.094	0.109	0.111	0.112	0.197	0.094	0.177	0.177	0.160	0.102	0.106	0.222	0.102	0.103	0.094	0.089												
[29]	0.170	0.172	0.161	0.167	0.165	0.169	0.159	0.172	0.120	0.166	0.161	0.166	0.157	0.159	0.160	0.179	0.168	0.119	0.141	0.170	0.174	0.163	0.190	0.160	0.159	0.159	0.180	0.168										
[30]	0.133	0.087	0.118	0.132	0.112	0.130	0.122	0.099	0.189	0.082	0.127	0.085	0.123	0.122	0.129	0.221	0.115	0.195	0.188	0.176	0.098	0.129	0.237	0.111	0.119	0.105	0.091	0.103	0.190									
[31]	0.219	0.204	0.195	0.221	0.220	0.215	0.209	0.212	0.130	0.212	0.214	0.209	0.212	0.211	0.215	0.216	0.210	0.132	0.178	0.175	0.209	0.218	0.220	0.207	0.212	0.208	0.210	0.204	0.147	0.212								
[32]	0.181	0.179	0.158	0.182	0.176	0.184	0.179	0.181	0.002	0.182	0.179	0.187	0.172	0.172	0.175	0.181	0.171	0.013	0.158	0.160	0.182	0.175	0.201	0.170	0.173	0.165	0.189	0.177	0.121	0.191	0.132							
[33]	0.089	0.085	0.053	0.090	0.037	0.083	0.087	0.093	0.169	0.078	0.082	0.082	0.089	0.090	0.089	0.193	0.050	0.176	0.154	0.152	0.096	0.087	0.217	0.019	0.074	0.042	0.088	0.095	0.158	0.101	0.205	0.171						
[34]	0.185	0.183	0.161	0.185	0.180	0.186	0.181	0.185	0.016	0.186	0.187	0.188	0.177	0.177	0.181	0.187	0.175	0.019	0.156	0.163	0.186	0.179	0.211	0.177	0.176	0.173	0.191	0.184	0.122	0.198	0.130	0.018	0.177					
[35]	0.125	0.078	0.106	0.127	0.105	0.120	0.124	0.086	0.198	0.076	0.126	0.082	0.122	0.124	0.121	0.218	0.100	0.208	0.194	0.193	0.089	0.127	0.242	0.106	0.115	0.100	0.082	0.102	0.192	0.079	0.220	0.199	0.091	0.205				
[36]	0.080	0.087	0.043	0.080	0.046	0.081	0.081	0.093	0.155	0.081	0.079	0.086	0.081	0.080	0.085	0.180	0.030	0.163	0.161	0.155	0.095	0.080	0.208	0.042	0.067	0.029	0.098	0.091	0.159	0.105	0.201	0.156	0.038	0.162	0.097			
[37]	0.305	0.309	0.298	0.300	0.310	0.298	0.304	0.305	0.325	0.312	0.296	0.304	0.298	0.297	0.296	0.322	0.296	0.329	0.328	0.322	0.309	0.303	0.350	0.303	0.293	0.289	0.309	0.323	0.331	0.326	0.375	0.324	0.295	0.331	0.337	0.282		
[38]	0.180	0.163	0.164	0.180	0.169	0.174	0.172	0.177	0.151	0.165	0.175	0.169	0.171	0.172	0.172	0.156	0.159	0.158	0.143	0.122	0.175	0.173	0.178	0.164	0.169	0.152	0.170	0.162	0.147	0.176	0.161	0.151	0.163	0.157	0.172	0.152	0.326	
[39]	0.180	0.163	0.164	0.180	0.169	0.174	0.172	0.177	0.151	0.165	0.175	0.169	0.171	0.172	0.172	0.156	0.159	0.158	0.143	0.122	0.175	0.173	0.178	0.164	0.169	0.152	0.170	0.162	0.147	0.176	0.161	0.151	0.163	0.157	0.172	0.152	0.326	0.000

Appendix C

Top hit of BLAST search of the 120 clones isolated from the Rhodes Memorial sample

Representative Clone	Top Blast Hit	Accession number	Maximum sequence similarity
MS clone 1	Uncultured bacterium clone ncd242c07c1	HM269030.1	99%
MS clone 2	Uncultured bacterium clone FCPN751	EF516421.1	99%
MS clone 3	Uncultured bacterium clone ncd325f02c1	HM317673.1	99%
MS clone 4	Uncultured bacterium clone FFCH11334	EU132705.1	99%
MS clone 5	Uncultured actinobacterium clone D-16S-121	GU552225.1	97%
MS clone 6	Uncultured bacterium clone ncd258a11c1	HM263183.1	99%
MS clone 7	Uncultured bacterium clone FFCH11334	EU132705.1	99%
MS clone 8	Uncultured bacterium clone FFCH12153	EU132751.1	99%
MS clone 9	Uncultured bacterium clone ncd262b04c1	HM270195.1	99%
MS clone 10	Uncultured Chloroflexi bacterium clone S1-56	HQ674892.1	93%
MS clone 11	Uncultured Gemmatimonadales clone GASP-KA1S2_E02	EU297050.1	97%
MS clone 12	Uncultured actinobacterium clone EB1017	AY395336.1	100%
MS clone 13	Uncultured actinobacterium clone D-16S-121	GU552225.1	97%
MS clone 14	Uncultured Gemmatimonas sp. clone PE19.5.1	GU047659.1	99%
MS clone 15	Micromonosporaceae bacterium NBRC 104874	FM208261.1	99%
MS clone 16	Uncultured bacterium clone FFCH11334	EU132705.1	99%
MS clone 17	Arthrobacter sp. CTLB06	GU300616.1	99%
MS clone 18	Uncultured bacterium clone FCPN751	EF516421.1	99%
MS clone 19	Uncultured Gemmatimonas sp. clone PE19.5.1	GU047659.1	99%
MS clone 20	Uncultured actinobacterium clone OH16a/16	GQ203398.1	99%
MS clone 21	Nocardioides sp. GT-L17	GQ355282.1	98%
MS clone 22	Uncultured Gemmatimonadetes bacterium	FN394977.1	98%
MS clone 23	Uncultured bacterium clone AA094	GQ859711.1	96%
MS clone 24	Micromonosporaceae bacterium NBRC 104874	FM208261.1	98%
MS clone 25	Uncultured actinobacterium clone GASP-WC1S1_F01	EF074420.1	99%
MS clone 26	Uncultured bacterium clone FCPP755	EF516186.1	97%
MS clone 27	Uncultured Actinobacteria bacterium clone AKYG770	AY922063.1	97%
MS clone 28	Uncultured bacterium clone FCPT588	EF516495.1	98%
MS clone 29	Uncultured bacterium clone FFCH11334	EU132705.1	99%
MS clone 30	Uncultured bacterium clone FFCH12153	EU132751.1	99%

Top hit of BLAST search of the 120 clones isolated from the Rhodes Memorial sample

Representative Clone	Top Blast Hit	Accession number	Maximum sequence similarity
MS clone 31	Uncultured bacterium clone Elev_16S_1407	EF020016.1	99%
MS clone 32	Actinoplanes sp. BMG5730	HM216931.1	97%
MS clone 33	Uncultured actinobacterium clone OH16a/16	GQ203398.1	99%
MS clone 34	Sporichthya polymorpha strain IFO 12702	NR_024727.1	99%
MS clone 35	Uncultured Gemmatimonadetes bacterium clone FAC37	DQ451476.1	98%
MS clone 36	Uncultured bacterium clone FFCH12550	EU132773.1	94%
MS clone 37	Uncultured Gemmatimonadetes clone GASP-MB1W1_E03	EF664770.1	98%
MS clone 38	Streptomyces sp. zx-10-18	HQ611065.1	96%
MS clone 39	Uncultured bacterium clone ncd1007d06c1	HM341135.1	100%
MS clone 40	Uncultured actinobacterium clone NPK-46	EF135031.1	98%
MS clone 41	Uncultured bacterium clone Elev_16S_1407	EF020016.1	99%
MS clone 42	Arthrobacter pascens strain X2	HQ530516.1	99%
MS clone 43	Streptomyces alanosinicus	HQ426712.1	96%
MS clone 44	Uncultured bacterium clone ncd1007d06c1	HM341135.1	99%
MS clone 45	Micromonosporaceae bacterium NBRC 104875	FM208262.1	98%
MS clone 46	Uncultured bacterium clone Elev_16S_1158	EF019814.1	96%
MS clone 47	Uncultured bacterium clone FCPN544	EF516384.1	99%
MS clone 48	Uncultured bacterium clone C17.11WL	AF432618.1	99%
MS clone 49	Uncultured organism clone ctg_CGOGA19	DQ395955.1	98%
MS clone 50	Uncultured bacterium clone FCPN544	EF516384.1	99%
MS clone 51	Streptomyces alanosinicus	HQ426712.1	96%
MS clone 52	Uncultured bacterium clone ncd545c02c1	HM277796.1	99%
MS clone 53	Uncultured actinobacterium clone Amb_16S_471	EF018137.1	99%
MS clone 54	Streptomyces costaricanus	FN796808.1	99%
MS clone 55	Uncultured actinobacterium clone Amb_16S_471	EF018137.1	98%
MS clone 56	Uncultured actinobacterium clone Elev_16S_1626	EF020178.1	98%
MS clone 57	Uncultured actinobacterium clone EB1077	AY395396.1	98%
MS clone 58	Uncultured Gemmatimonadetes clone GASP-KB1W2_G12	EU297797.1	98%
MS clone 59	Uncultured bacterium clone AR242	GQ860235.1	70%
MS clone 60	Uncultured Gemmatimonadetes bacterium clone A6YL03RM	FJ570452.1	99%

Top hit of BLAST search of the 120 clones isolated from the Rhodes Memorial sample

Representative Clone	Top Blast Hit	Accession number	Maximum sequence similarity
MS clone 61	Gordonia sp. 13679F	EU741242.1	74%
MS clone 62	Uncultured Actinopolymorpha sp. clone 1.15	GQ183148.1	97%
MS clone 63	Uncultured bacterium clone Elev_16S_1407	EF020016.1	99%
MS clone 64	Uncultured bacterium clone Elev_16S_1270	EF019903.1	99%
MS clone 65	Micromonosporaceae bacterium NBRC 104874	FM208261.1	99%
MS clone 66	Uncultured actinobacterium clone Amb_16S_471	EF018137.1	98%
MS clone 67	Uncultured bacterium clone FFCH13867	EU132841.1	100%
MS clone 68	Uncultured actinobacterium clone NPK-46	EF135031.1	98%
MS clone 69	Uncultured bacterium clone P5-mS41-67	GU573997.1	99%
MS clone 70	Streptomyces costaricanus	FN796808.1	99%
MS clone 71	Uncultured bacterium clone ncd241h08c1	HM269010.1	96%
MS clone 72	Janibacter sp. 0705C10-1	HM222661.1	99%
MS clone 73	Uncultured bacterium clone Elev_16S_1407	EF020016.1	99%
MS clone 74	Uncultured Gemmatimonadales clone GASP-KA1S2_E02	EU297050.1	93%
MS clone 75	Uncultured actinobacterium clone Amb_16S_471	EF018137.1	99%
MS clone 76	Uncultured actinobacterium clone OH16a/16	GQ203398.1	99%
MS clone 77	Uncultured Acidimicrobidae bacterium	AM935602.1	99%
MS clone 78	Uncultured bacterium clone FFCH12550	EU132773.1	94%
MS clone 79	Uncultured bacterium clone ncd258a11c1	HM263183.1	99%
MS clone 80	Uncultured actinobacterium clone TG_FD0.2_AC31	GU798071.1	99%
MS clone 81	Streptomyces corchorusii strain NF0919	HM989898.1	99%
MS clone 82	Uncultured bacterium clone FCPT588	EF516495.1	98%
MS clone 83	Streptomyces costaricanus	FN796808.1	99%
MS clone 84	Luedemannella sp. 119-1-07	GU929200.1	94%
MS clone 85	Streptomyces sp. MM322-72F1	AB609053.1	99%
MS clone 86	Micromonosporaceae bacterium NBRC 104874	FM208261.1	99%
MS clone 87	Uncultured Gemmatimonadetes bacterium clone FAC37	DQ451476.1	98%
MS clone 88	Uncultured Gemmatimonadetes bacterium clone FAC37	DQ451476.1	98%
MS clone 89	Luedemannella sp. 119-1-07	GU929200.1	94%
MS clone 90	Uncultured actinobacterium clone NPK-46	EF135031.1	98%

Top hit of BLAST search of the 120 clones isolated from the Rhodes Memorial sample

Representative Clone	Top Blast Hit	Accession number	Maximum sequence similarity
MS clone 91	Uncultured Acidimicrobidae bacterium	AM935602.1	98%
MS clone 92	Uncultured Gemmatimonadales bacterium clone GASP-KA1S2_E02	EU297050.1	97%
MS clone 93	Uncultured bacterium clone HDB_SIPP420	HM187223.1	98%
MS clone 94	Uncultured bacterium clone ncd257c01c1	HM263169.1	99%
MS clone 95	Uncultured actinobacterium clone A19YD13RM	FJ568399.1	100%
MS clone 96	Uncultured actinobacterium clone Amb_16S_471	EF018137.1	99%
MS clone 97	Streptomyces griseoruber strain A316	HQ335354.1	99%
MS clone 98	Uncultured actinobacterium clone TG_FD0.2_AC31	GU798071.1	99%
MS clone 99	Pseudonocardia spinosispora strain JLM090630-03	GU318369.1	98%
MS clone 100	Uncultured bacterium clone ncd242c07c1	HM269030.1	99%
MS clone 101	Uncultured actinobacterium clone OaN_F11 16S	FJ661788.1	99%
MS clone 102	Uncultured bacterium clone PAS2_F10	DQ830577.1	98%
MS clone 103	Uncultured bacterium clone FFCH11334	EU132705.1	99%
MS clone 104	Uncultured actinobacterium clone D-16S-121 16S	GU552225.1	97%
MS clone 105	Micromonosporaceae bacterium NBRC 104874	FM208261.1	99%
MS clone 106	Uncultured Gemmatimonadetes clone GASP-MB1W1_E03	EF664770.1	98%
MS clone 107	Uncultured Gemmatimonas sp. clone PE19.5.1 16S	GU047659.1	99%
MS clone 108	Uncultured actinobacterium clone EB1017	AY395336.1	100%
MS clone 109	Blastococcus jejuensis strain KST3-10	DQ200983.1	73%
MS clone 110	Uncultured bacterium clone Elev_16S_1407	EF020016.1	99%
MS clone 111	Uncultured bacterium clone Elev_16S_1270	EF019903.1	99%
MS clone 112	Uncultured Mycobacterium sp. clone act-66 16S	FJ655977.1	96%
MS clone 113	Streptomyces griseoruber strain A316	HQ335354.1	99%
MS clone 114	Uncultured actinobacterium clone TG_FD0.2_AC31	GU798071.1	99%
MS clone 115	Pseudonocardia spinosispora strain JLM090630-03	GU318369.1	98%
MS clone 116	Uncultured bacterium clone ncd242c07c1	HM269030.1	99%
MS clone 117	Actinophytocola sp. QAIII60	EU274339.2	99%
MS clone 118	Uncultured bacterium clone PAS2_F10	DQ830577.1	98%
MS clone 119	Uncultured bacterium clone FFCH11334	EU132705.1	99%
MS clone 120	Uncultured actinobacterium clone D-16S-121 16S	GU552225.1	97%