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**ISOLATION AND CHARACTERISATION OF INDIGENOUS
ACTINOBACTERIA FROM DIVERSE SOUTH AFRICAN
ENVIRONMENTS**

By

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Thesis presented for the Master of Science Degree in Molecular and Cell Biology in the Department of Molecular and Cell Biology, Faculty of Science, University of Cape Town, South Africa.

August 2010

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Abstract

One soil sample and various indigenous plant and seaweed samples were used to isolate actinobacteria, with a particular focus on isolating rarer actinobacteria (non-*Streptomyces* species). A total of 169 putative actinobacterial strains was isolated, of which 42 were selected, based on their morphology, for further identification using a rapid molecular identification method and/or 16S rRNA gene sequence analysis. This includes seven strains isolated from various plant and seaweed samples. A total of 28 non-*Streptomyces* species was identified, with 23 being isolated from the soil sample, four isolated as plant endophytes (strains SM3BL1, YPC1, YPC2 and YPL1), and one isolated as a seaweed epiphyte (Y2UE1). Two *Streptomyces* species were isolated as endophytes (with strain SC1 isolated from a seaweed sample, and YMH1 isolated a plant species). Forty-two strains, including all non-*Streptomyces* species, were screened for their antibacterial activity against *Mycobacterium aurum* A+. Six strains showing promising antibacterial activity were selected for antibiotic extraction. The strains were also investigated for their antibiotic biosynthetic potential by PCR screening for the genes for ansamycin, glycopeptide and Type II (aromatic) polyketide antibiotics. Amplification of the AHBA synthase gene (ansamycin biosynthesis) was achieved for strains SE22 and YPC1. Strains SE22 and YMH1 were positive for the presence of the KS_{α} - KS_{β} gene pair, with strain SE22 potentially producing a Type II (aromatic) polyketide. The gene product of isolate YMH1 was identified as a spore pigment gene. Antibiotic extraction was successful for strains SE22 and YM55, with numerous active compounds isolated from strain SE22, and one active compound isolated from strain YM55.

Fifteen strains were selected for full characterisation based on their isolation source, their identification to non-*Streptomyces* genera and/or their antibacterial activity. These included four *Micromonospora* strains, three *Kribbella* strains, three *Streptomyces* strains, one *Actinomadura* strain, one *Kineococcus* strain, one *Nocardia* strain, one *Nonomuraea* strain and one *Verrucosispora* strain. Two previously isolated *Microbispora* strains were also characterised. The 17 strains were subjected to 16S rRNA gene sequence analysis to determine their closest phylogenetic relatives. The use of *gyrB* gene based phylogeny was also investigated for the two *Microbispora* isolates, resulting in a more stable phylogenetic tree. However, differences observed between the 16S rRNA and *gyrB* gene tree topologies suggest that horizontal gene transfer has occurred within the genus. The 17 isolates were distinguished from their closest phylogenetic

neighbours through morphological and physiological characteristics. It is likely that the majority of the isolates are novel, although 16 isolates will require DNA-DNA hybridisation studies to determine if they are new species. *Actinomadura* strain YPC2 may be proposed as a novel species without the need for DNA-DNA hybridisation.

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Abbreviations

ANI	Average Nucleotide Identity
ARDRA	Amplified Ribosomal DNA Restriction Analysis
DAP	Diaminopimelic acid
DDH	DNA-DNA Hybridisation
HIV	Human Immunodeficiency Virus
ISP	International <i>Streptomyces</i> Project
MC	Modified Czapek solution
MDR	Multi-Drug Resistant
MLSA	Multilocus Sequence Analysis
MRSA	Methicillin Resistant <i>Staphylococcus aureus</i>
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD	Optical Density
R _f	Retention Factor
SC	Starch-Casein agar
SE	Soil Extract agar
TB	Tuberculosis
TLC	Thin Layer Chromatography
TMNP	Table Mountain National Park
v/v	Volume to Volume
VRSA	Vancomycin Resistant <i>Staphylococcus aureus</i>
w/v	Weight per Volume
XDR	Extensively Drug Resistant
YEME	Yeast Extract Malt Extract

Chapter 1

General Introduction

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Chapter 1

General Introduction

Filamentous actinobacteria are known as some of the most widely distributed bacteria found in soil environments (Cook & Meyers 2003, Waksman 1950). The filamentous actinobacteria, also commonly known as actinomycetes, are classified as Gram-positive prokaryotes that form branching filaments called hyphae, which may form a stable mycelium, or fragment into rods or cocci (Lechevalier & Lechevalier 1981). These mycelia are produced in a diverse range of colours and may produce asexual spores, called conidiospores when present at the end of filaments, or sporangiospores when present in a sporangium (Prescott *et al.* 2005b). The spores of the filamentous actinobacteria usually develop in response to a lack of nutrients in their environment, and are very tolerant of desiccation (Prescott *et al.* 2005b). Actinobacteria are primarily aerobic and are predominantly saprophytic in nature (Margarvey *et al.* 2004) and, as such, are degraders of organic matter, helping to maintain environmental stability through the recycling of nutrients (Babalola *et al.* 2009, Waksman 1950).

The filamentous actinobacteria belong to the order *Actinomycetales*, which is one of nine orders with validly-published names in the class *Actinobacteria* (the other orders are *Acidimicrobiales*, *Bifidobacteriales*, *Coriobacteriales*, *Euzebyales*, *Nitriliruptorales*, *Rubrobacterales*, *Solirubrobacterales* and *Thermoleophilales*) (Euzéby 2010). The intraclass relatedness is indicated for five of the nine classes in Figure 1.1. The filamentous actinobacteria are characterised by a high G+C content in their genomic DNA. Most of the actinobacterial members that have been discovered are from soil environments, however these diverse bacteria have also been isolated from numerous other environments. These include, fresh water and marine environments, in association with plants and marine sponges as endophytes (Gandhimathi *et al.* 2008), as endosymbionts within termites (Kurtböke & French 2007), and isolated as causative agents of diseases in both plants and animals (Kaltenpoth 2009, Kunoh 2002).

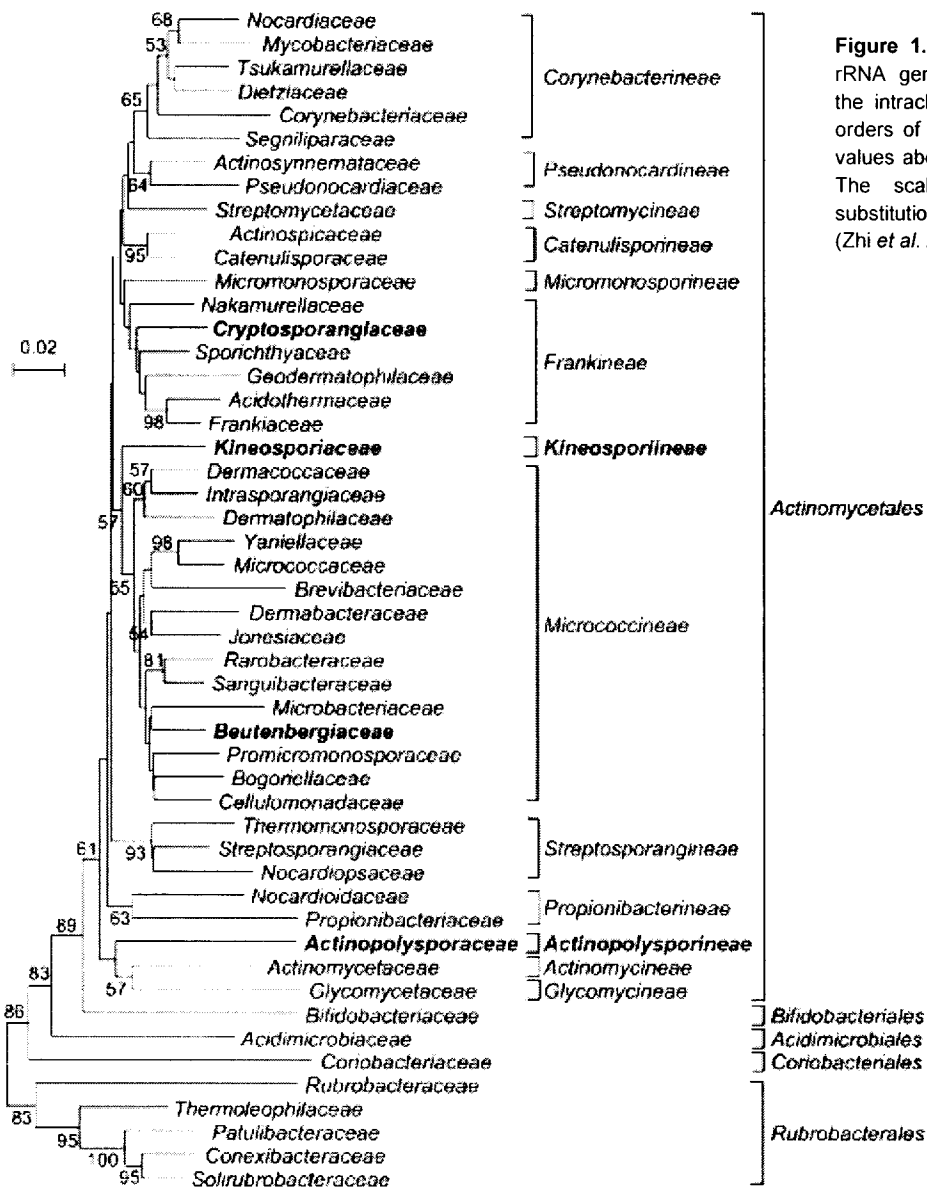


Figure 1.1 Phylogenetic tree based on 16S rRNA gene sequence comparisons showing the intraclass relatedness of five of the nine orders of the class *Actinobacteria*. Bootstrap values above 50% are indicated at the nodes. The scale bar represents 2 nucleotide substitutions per 100 nucleotides of sequence. (Zhi *et al.* 2009)

Initially, actinobacteria were thought to be a separate group of organisms between the fungi, due to their filamentous characteristics, in part, and the bacteria (Lechevalier & Lechevalier 1981). This is evident in the naming of some of the first described actinobacteria, such as *Actinomyces bovis* and *Mycobacterium*, with *Actinomyces* meaning “ray fungus” and *Mycobacterium* meaning “fungus bacterium” in Greek (Hopwood 2007). With time, and the discovery of antibiotics from other bacterial and fungal studies, the actinobacteria, particularly the *Streptomyces* species (Hopwood 2007) were shown to be a very valuable source of secondary metabolites. These special bacteria are now regarded as the most

biotechnologically valuable prokaryotes which, to date, are producers of approximately half of the secondary metabolites that have been discovered (Lam 2006).

1.1 Actinobacterial Taxonomy

Bacterial taxonomy may be defined as the science of classifying organisms into groups called taxa, based on their overall similarity in characteristics (Prescott *et al.* 2005a). Classification of bacterial species is important as it enables the organisation of large amounts of knowledge, enabling researchers to make sense of the diversity of bacteria. This organisation allows for efficient communication between researchers, enables accurate identification and allows for predictions based on existing classifications (Prescott *et al.* 2005a). In modern times, bacterial taxonomy may be described as polyphasic taxonomy (as demonstrated in Figure 1.2), where phenotypic and genotypic (including phylogenetic) methods are commonly used in the classification of bacterial species (Vandamme *et al.* 1996).

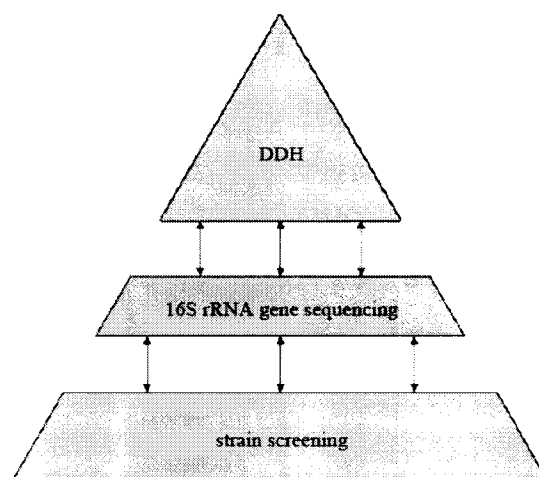


Figure 1.2 Diagrammatic representation of polyphasic taxonomy. Strain isolation and selection is followed by 16S rRNA gene sequence analysis, and if necessary, DNA-DNA hybridization (DDH) is performed on selected strains in order to establish their novelty (Gevers *et al.* 2006).

1.1.1 Phenotypic Methods

1.1.1.1 Morphological and Physiological Properties

Initially, studies on taxonomy relied heavily on classical morphological, physiological and biochemical characteristics (i.e. phenetic traits) (Anderson & Wellington 2001, Vandamme *et al.* 1996). Morphological characteristics were obtained from the gross colony observations and from the cellular level (Vandamme *et al.* 1996). For the actinobacteria, these characteristics are based on standard cultivation media prescribed by the International *Streptomyces* Project (ISP media). Some of these characteristics include shape and form of colonies, colour of vegetative and aerial mycelium and spores, spore chain morphology, spore surface ornamentation and spore motility, as well as the production and colour of pigments. Spore details are determined from electron microscopic observations. Physiological and biochemical characteristics are directly linked to the activities of microbial transport proteins and enzymes, and indirectly to their microbial genome (Prescott *et al.* 2005a). Specifically, some characteristics include the ability of species to grow at different temperatures, NaCl concentrations, pH values and in the presence of inhibitory compounds, as well as utilisation of sole carbon and nitrogen sources and degradation of compounds. Phenotypic characteristics in general need to be determined with standardised methods to enable reproducibility between different laboratories (Vandamme *et al.* 1996).

1.1.1.2 Chemotaxonomy

The development of quicker, more reliable and sensitive methods (including spectroscopy, chromatography and electrophoresis) allowed for the development of chemotaxonomic characterisation techniques to be included in the phenotypic methods of characterising bacterial species (Anderson & Wellington 2001). These characterisation techniques rely on various chemical constituents within bacterial cells that aid in the identification of prokaryotes, particularly when other phenetic characterisation methods fail to provide a satisfactory classification (Schleifer 2009). Some important chemotaxonomic characteristics indicated in Figure 1.3 for actinobacterial classification include cell wall type, whole cell sugar patterns,

phospholipid patterns and menaquinone analysis as well as membrane fatty acids (Labeda 1987).

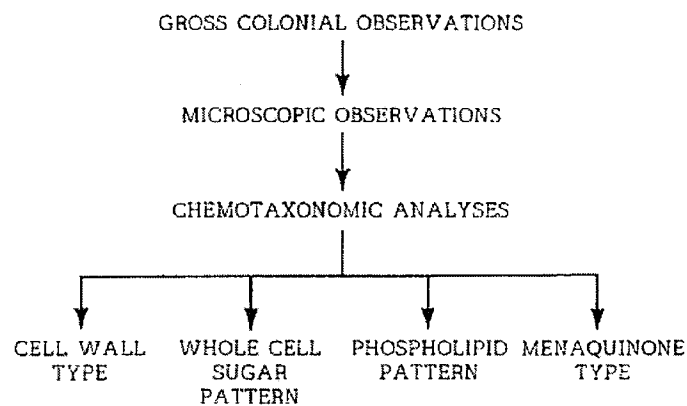


Figure 1.3 Flow chart of partial phenotypic identification of actinobacteria (Labeda 1987).

For actinobacterial species, the most common cell wall types consist of peptidoglycan with diaminopimelic acid (DAP) as the diagnostic diamino acid (Lechevalier & Lechevalier 1981). There are three isomeric forms of DAP, namely LL-DAP, *meso*-DAP (DL-DAP) and DD-DAP. The LL- and *meso*-isomers of DAP may be separated by paper chromatography to aid in their identification, however the DD-isomer cannot readily be separated from *meso*-DAP and has no taxonomic value. Actinobacteria are identified as having either a type I (LL-DAP with glycine), II (*meso*-DAP with glycine), III (*meso*-DAP) or IV (*meso*-DAP with arabinose and galactose) cell wall (Lechevalier & Lechevalier 1981). The diagnostic diamino acid in actinobacterial peptidoglycan can also be L-lysine (e.g. *Couchioplanes*), 2,4-diaminobutyric acid (e.g. *Leifsonia*), ornithine (e.g. *Microbacterium*) or serine (e.g. *Serinicoccus*). Some genera have two diagnostic diamino acids, e.g. *Actinoallomurus* (*meso*-DAP and D- & L-lysine) and *Serinicoccus* (ornithine and serine).

The whole cell sugar patterns of actinobacteria are also useful in their description. There are five recognised whole cell sugar patterns that help to discriminate between actinobacteria containing *meso*-DAP. These five patterns contain the following sugars: A – arabinose and

galactose, B – madurose, C – no diagnostic sugar, D – arabinose and xylose and E –rhamnose. (Busse *et al.* 1996). Whole cell sugar patterns, combined with the different cell wall chemotypes, help to confirm the placing of actinobacteria within the genera determined by analysis of their 16S-rRNA genes.

Bacterial membranes are composed mainly of phospholipids. There are currently five phospholipid patterns observed in actinobacteria based on characteristic phospholipids. These are: PI – no characteristic phospholipid (but the phospholipids often include phosphatidylglycerol), PII – phosphatidylethanolamine, PIII – phosphatidylcholine, PIV – phosphatidylethanolamine and glucosamine-containing phospholipids of unknown structure, and PV – glucosamine-containing phospholipids only (Labeda 1987). The phospholipid patterns aid in the confirmation of assignment of actinobacteria to specific genera, however, determination of phospholipid patterns can be quite complicated as there are limited commercial standards (Labeda 1987, Busse *et al.* 1996) and the chromatograms are not perfectly reproducible.

A few actinobacteria with a type-IV cell wall also contain mycolic acids, specifically in some coryneform genera (Busse *et al.* 1996). The actinobacteria that contain mycolic acids may be separated from other mycolic-acid containing bacteria according to the molecular weights of the mycolic acids (Lechevalier & Lechevalier 1981).

Filamentous actinobacteria, as Gram positive bacteria, possess only menaquinones in their cytoplasmic membranes (as part of the electron transport chain). Taxonomically, the isoprenoid side chain length, as well as the number of saturated units of the side chains of menaquinones, are valuable and allow for a more detailed differentiation (Busse *et al.* 1996, Alderson *et al.* 1985). Different genera may be distinguished by the different lengths of the isoprenoid side chains and degrees of saturation of those side chains.

1.1.2 Genotypic Methods

The genotypic and phylogenetic methods used in actinobacterial taxonomy are derived from the nucleic acids present in the cell, namely the DNA and RNA molecules (Vandamme *et al.* 1996). These genotypic methods arose due to great technological progress, and comparisons involving nucleic acids are believed to provide the most accurate and true reflection of species relatedness. Some important classic genotypic (and phylogenetic) methods used in actinobacterial taxonomy include: the determination of the DNA base composition, 16S rRNA gene sequence comparisons and DNA-DNA hybridisation studies (Vandamme *et al.* 1996, Busse *et al.* 1996, Schleifer 2009, Coenye *et al.* 2005).

1.1.2.1 DNA Base Composition

DNA base compositions involve determining the moles percent guanine plus cytosine (G+C) in the bacterial genome. The G+C content of well defined species do not vary by more than 3%; those of a well-defined genus do not vary by more than 10% (Vandamme *et al.* 1996). The G+C content may be used to confirm taxonomic assignments of species to a specific genus arrived at through other characterisation methods.

1.1.2.2 DNA-DNA Hybridisation Studies

DNA-DNA hybridisation (DDH) studies allow for definitive allocation of new strains to the species level. Two strains belonging to the same genomic species share DNA-relatedness values (determined by DDH) of greater than 70%, with a melting temperature difference that is less than 5°C (Wayne *et al.* 1987). This method has become the “gold standard” for species descriptions, however there are a number of drawbacks with DDH studies. For one, only very closely related species can be distinguished. Another drawback is that the methods are very complex, time-consuming and expensive for those researchers that do not have the facilities readily available to them. Finally, comparative databases cannot be established and DDH studies are not applicable when studying non-cultivable bacteria (Schleifer 2009, Richter & Roselló-Móra 2009). It has often been suggested that other reliable methods are needed to

replace DDH. These methods should allow for the creation of databases that may be accessed for comparative studies by other researchers (Richter & Roselló-Móra 2009).

1.1.2.3 16S rRNA gene sequence analysis

A great deal of hope was placed in the use of 16S rRNA gene sequence analysis (Richter & Roselló-Móra 2009), which is probably the most commonly used phylogenetic method today for initial bacterial species identification (Busse *et al.* 1996). The advent of polymerase chain reaction technologies have allowed for great progress in phylogenetic studies, enabling researchers to amplify genes of interest from bacterial genomes relatively quickly. The advantages of 16S rRNA gene studies are that the genes are ubiquitous, highly conserved, functionally constant and less subject to horizontal gene transfer than other genes (Schleifer 2009, Vandamme *et al.* 1996). 16S rRNA gene sequences can be obtained by any lab with a PCR machine and are relatively cheap to determine when compared to DDH. Comparisons involving the 16S rRNA gene can also be performed with presently non-cultivable prokaryotes, allowing for a greater understanding of previously unknown bacterial diversity. In essence, scientists can identify bacteria to a particular genus using 16S rRNA gene sequence analysis with great certainty, and may even be able to determine whether a strain is a new species – if the sequence identity is <97%. This threshold value has been shown to correlate well with DDH similarity values of less than 70% for novel species definitions (Stackebrandt & Goebel 1994). A great advantage with using 16S rRNA gene sequence comparisons is that the determined sequences may be readily stored in databases, allowing for comparisons to be made between researchers in different parts of the world. Due to the conserved nature of the gene, however, species circumscription cannot be achieved where sequence identity levels between two strains are greater than the prescribed threshold. Species circumscription will then rely on the presence of numerous phenotypic differences as well as DDH studies.

1.1.2.4 Alternative methods

Two noteworthy alternative methods to the “gold standards” of DDH and 16S rRNA gene sequence analysis are phylogenetic analysis with house keeping genes (combined with multi-

locus sequence analysis), as well as the average nucleotide identity (ANI) between two genomes (Schleifer 2009, Richter & Roselló-Móra 2009).

Multi-locus sequence analysis (MLSA) is performed using concatenated sequences of multiple house-keeping genes that are essential to bacterial survival. These house-keeping genes evolve faster than 16S rRNA genes, hence allowing for potentially better resolution at the species level in cases where 16S rRNA gene sequence analysis does not allow resolution (i.e. the sequence similarity is >97%) (Schleifer 2009). The use of multiple genes is an advantage as concatenated sequences provide more nucleotide positions for comparison than single genes do and may buffer the effects of horizontal gene transfer of any one of the genes used. It is suggested that the genes selected should meet certain criteria, including being widely distributed among bacterial genomes and being conserved enough and long enough to provide useful relationship analyses (Coenye *et al.* 2005). This method can be seen as an answer to concerns of single gene trees not adequately reflecting phylogenetic relationships due, in part, to the effects of horizontal gene transfer (Coenye *et al.* 2005). An advantage over DDH studies is the fact that MLSA sequences can be stored in databases (Schleifer 2009). However, MLSA also has certain drawbacks, for example, primers for gene amplifications are not universal and designing primers is often difficult. Furthermore, studies cannot be applied to non-cultivable bacteria (unlike 16S rRNA gene sequencing) (Schleifer 2009).

The average nucleotide identity (ANI) involves the pairwise comparison between all conserved genes within any two bacterial genomes (Schleifer 2009, Richter & Roselló-Móra 2009, Roselló-Móra 2005). It has been shown that ANI values of 95% correlate with DDH similarity values of 70% (Schleifer 2009), and values over 95% correlate with 98.5% or higher 16S rRNA gene sequence similarity. This method is believed to be the most likely to be a substitute for DDH in the current genomics era (Schleifer 2009, Richter & Roselló-Móra 2009). A main advantage ANI has over DDH is that ANI can allow for the creation of cumulative databases (Roselló-Móra 2005). This method is obviously dependent on fully sequenced genomes of bacteria, thus may have limited use until a database of the majority of bacterial type strains is created. In the mean time, it has been demonstrated that partial sequencing (as little as 20%) of bacterial genomes can be used to achieve reliable ANI results (Richter & Roselló-Móra 2009).

1.2 Actinobacterial Genera

1.2.1 Suborder *Corynebacterineae*

The genus *Nocardia*

The genus *Nocardia* was proposed by Trevisan in 1889 (Goodfellow & Lechevalier 1989). *Nocardia* species belong to the *Nocardiaceae* family. The nocardiae characteristically have aerial and substrate mycelia that fragment into bacilliary and coccoid elements (Goodfellow & Minnikin 1981). Currently there are over 75 species with validly-published names in the genus *Nocardia*, with *Nocardia asteroides* as the type species (Euzéby 2010). This genus was previously over speciated due to limited morphological characterisation of species, resulting in its troubled taxonomic past (Zhang & Goodfellow 2004). Cell pigmentation varies within the genus, from off-white to gray, yellow, orange, pink, purple or brown in color. *Nocardia* species typically have *meso*-DAP with arabinose and galactose as characteristic sugars (cell wall chemotype IV and whole cell sugar pattern type A) (Lechevalier & Lechevalier 1981). The *Nocardia* phospholipid type is PII, with MK-8(H₄) or MK-9(H₂) as the predominant menaquinone (Goodfellow & Lechevalier 1989). *Nocardia* are well known human and animal pathogens, being the causative agents of nocardiosis (including pulmonary and systemic infections) and actinomycetoma (Goodfellow & Minnikin 1981). *Nocardia* species have been found in very diverse environments from soil, water and sewerage to their association with insects and plants (Goodfellow & Lechevalier 1989). Species within this genus also play an important role in organic matter turnover and some have been shown to produce industrially valuable secondary metabolites such as muraceins (Zhang *et al.* 2004, Bush *et al.* 1984), as well as antibiotic compounds such as nocardicin A (Aoki *et al.* 1976).

1.2.2 Suborder *Kineosporiineae*

The genus *Kineococcus*

The genus *Kineococcus* was proposed in 1993 by Yokota *et al.* Bacterial cells of this genus are typically coccoid in nature and may occur in pairs, tetrads or clusters (Yokota *et al.* 1993).

Colonies of *Kineococcus* species are either orange or cream coloured. The type species of the genus is *Kineococcus aurantiacus*. Currently there are only six species with validly-published names in the *Kineococcus* genus (Euzéby 2010), which was initially placed in the *Pseudonocardiaceae* family (Phillips *et al.* 2002). However, it was recently accommodated in the newly proposed family, *Kineosporiaceae* (Zhi *et al.* 2009). Chemotaxonomically, the genus has *meso*-DAP as the characteristic cell wall diamino acid (type III), with arabinose and galactose as the characteristic sugars (whole cell sugar pattern type A). *Kineococcus* species have the phospholipid type PI, with MK-9(H₂) as the predominant menaquinone (Lee 2006). Interestingly, *Kineococcus radiotolerans* was shown to be resistant to γ -radiation doses of up to 3.5 kGy.

1.2.3 Suborder *Micromonosporineae*

The genus *Micromonospora*

The genus *Micromonospora* is considered to encompass one of the larger culturable groups of actinobacteria from soil (Lazzarini *et al.* 2000). *Micromonospora* species are widely distributed in the environment, ranging from soil to marine sediments to their association with plants (Hirsch & Valdés 2010). The genus was described by Ørskov in 1923 (Kawamoto 1989). Currently there are 42 species in the genus *Micromonospora*, with *Micromonospora chalcea* as the type species (Euzéby 2010). The genus *Micromonospora* belongs to the family *Micromonosporaceae*. Colonies are characteristically light orange, orange, red, brown, blue-green or purple in colour, becoming brown-black, green-black or black upon sporulation and eventually mucoid in consistency upon maturation (Kawamoto 1989). No aerial mycelium is produced and single non-motile spores are characteristic of the genus. The spore surface ornamentation is generally blunt-spiny in nature and offers no diagnostic value for species differentiation. Chemotaxonomically, *Micromonospora* species contain *meso*-DAP and/or its 3-hydroxy derivative and glycine (cell wall type II), and xylose and arabinose are the characteristic sugars (whole cell sugar pattern D) (Kawamoto 1989). The characteristic phospholipid pattern of the genus *Micromonospora* is type PII with MK-9(H₄), MK-10(H₄), MK-10(H₆) or MK-12(H₆) as the major menaquinone (Lechevalier & Lechevalier 1981). The genus is biotechnologically a very valuable source of secondary metabolites including antibiotics such as aminoglycosides

(gentamicins), macrolides (mycinamicins) and polysaccharide antibiotics (everninomycin) (Lazzarini *et al.* 2000), as well as anti-tumour (tetrocarcin) and anthracycline antibiotics (Hirsch & Valdés 2010).

The genus *Verrucosispora*

The genus *Verrucosispora* was proposed by Rheims *et al.* in 1998 and belongs to the *Micromonosporaceae* family. Currently there are only two species with validly-published names, namely *Verrucosispora gifhornensis* (the type strain) and *Verrucosispora lutea* (Euzéby 2010), with the description of *Verrucosispora sediminis* currently *in press*. Colonies of *Verrucosispora* species are typically orange in colour, however they may become brownish upon maturation (Rheims *et al.* 1998). *Verrucosispora* species have meso-DAP with glycine in the cell wall (chemotype II). The characteristic whole cell sugars are mannose and xylose. Arabinose (which is a characteristic sugar of *Micromonospora* species) is absent. The phospholipid pattern is type II, with MK-9(H₄) as the major menaquinone (Rheims *et al.* 1998). The spores of *Verrucosispora* species are characteristically single and may be smooth or warty, changing to hairy upon maturation (Liao *et al.* 2009). It has been demonstrated that species of the *Verrucosispora* genus may be useful secondary metabolite producers, in particular production of antibiotics (such as abyssomicin C) (Baltz 2008).

1.2.4 Suborder *Propionibacterineae*

The genus *Kribbella*

The genus *Kribbella* was described in 1999 by Park *et al.* This genus contains 16 species with validly-published names with *Kribbella flavida* as the type species (Euzéby 2010). The *Kribbella* genus belongs to the *Nocardioideae* family. Typically *Kribbella* species' colonies are pasty with lichenous shapes. The kribbellae are described as nocardioform in nature (as the mycelium generally fragments into rod to coccoid-shaped elements), and are generally Gram-variable (Park *et al.* 1999). The genus typically has LL-DAP with glycine as its characteristic cell wall chemotype (type II), with no characteristic whole cell sugar pattern (type C). The predominant menaquinone is MK-9(H₄), with PIII as the phospholipid pattern (Park *et al.* 1999).

Some *Kribbella* species have been shown to produce antibiotics (such as the antitumour antibiotic, sandramycin, produced by *Kribbella sandramycini*). *Kribbella* species have been isolated mainly from soil environments, as well as from plant associations (such as *Kribbella lupini* from root nodules) (Trujillo *et al.* 2006).

1.2.5 Suborder Streptomycineae

The genus *Streptomyces*

Streptomyces is not only the most prolific genus found in any habitat, but *Streptomyces* species are also the most frequently isolated. Consequently, they are the most well studied and best characterised of the actinobacteria (Alam *et al.* 2010). The *Streptomyces* genus was proposed by Waksman and Henrici in 1943 (Williams *et al.* 1989). There are currently over 500 species with validly-published names in the *Streptomyces* genus, with *Streptomyces albus* as the type species (Euzéby 2010). The streptomycetes belong to the *Streptomycetaceae* family. Colonies of *Streptomyces* species are leathery, lichenoid or butyrous, with powdery, velvety, floccose or granular aerial mycelium (Williams *et al.* 1989). The mycelia are generally produced in a wide variety of colours and species may also produce characteristic diffusible pigments. The spore mass colour of *Streptomyces* initially played an important role in streptomycete taxonomy, resulting in species assignment to one of seven colour series, namely red, yellow, green, blue, gray, violet and white. Other phenotypically important traits used in streptomycete characterisation include colour of diffusible pigments, spore chain morphology and spore surface ornamentation. Spore chain morphology is divided into three categories, namely *Spirales*, *Retinaculiaperti* and *Rectiflexibiles*, and the spore surface ornamentation is smooth, warty, spiny, hairy or rugose (wrinkled). The *Streptomyces* species contain LL-DAP (cell wall type I) with no characteristic diagnostic sugars. The phospholipid pattern is PII, with MK9-(H₆) or MK9-(H₈) as the predominant menaquinone (Williams *et al.* 1989). *Streptomyces* species are found in diverse habitats, ranging from terrestrial to aquatic, to their association with plants as endophytes. Streptomycetes are well known as producers of biotechnologically useful secondary metabolites such as antibiotics, antiparasitic agents, herbicides, and anti-tumour drugs (Alam *et al.* 2010). Some species in this genus have been shown to cause disease in plants, such as potato scab (e.g. *Streptomyces scabies* and *Streptomyces acidiscabies*), as well as in humans, for example causing actinomycetoma (e.g. *Streptomyces somaliensis* and

Streptomyces sudanensis). *Streptomyces* strains are responsible for the smell of moist soil due to their production of volatile substances including geosmin (Prescott *et al.* 2005b).

1.2.6 Suborder Streptosporangineae

The genus *Microbispora*

The genus *Microbispora* was proposed in 1957 by Nonomura & Ohara (Nonomura 1989). This genus is considered to be a common actinobacterium found in the soil environment. The *Microbispora* genus was so named due to the species' characteristic production of longitudinal paired spores, either on sporophores or directly on aerial hyphae. The genus *Microbispora* belongs to the *Streptosporangiaceae* family. Currently there are five species with validly published names within this genus, with two subspecies (*Microbispora amethystogenes*, *Microbispora corallina*, *Microbispora mesophila*, *Microbispora rosea* subsp. *rosea*, *Microbispora rosea* subsp. *aerata* and *Microbispora siamensis*). Species within this genus were divided into mesophilic and thermophilic members, with *Microbispora rosea* as the type species (Euzéby 2010). The ten *Microbispora* species that were initially described were combined into the two subspecies, *M. rosea* subsp. *rosea* and *M. rosea* subsp. *aerata* (Miyadoh *et al.* 1990). The name *M. amethystogenes*, considered a synonym of *M. rosea* subsp. *rosea* by Miyadoh *et al.* (1990) has been revived (Boondaeng *et al.* 2009). Morphologically, colonies of *Microbispora* species vary in colour from white to pink to yellowish brown and orange. Chemotaxonomically, *Microbispora* species contain meso-DAP (wall chemotype III) with madurose as the diagnostic sugar (B type whole cell sugar pattern) (Rao *et al.* 1987). *Microbispora* species' phospholipid pattern is of type PIV with partially saturated menaquinones and MK-9(H₄) as a predominant isoprenologue (Nonomura 1989). Production of the pigment iodinin is characteristic of a number of *Microbispora* species. Some *Microbispora* species have been shown to produce antimicrobial compounds, such as microbisporicin, which is one of the most potent lantibiotics discovered, as well as other useful bioactive microbial metabolites (Castiglione *et al.* 2008, Bérdy 2005).

The genus *Actinomadura*

The genus *Actinomadura* was proposed by Lechevalier and Lechevalier in 1970 (Meyer 1989), and belongs to the *Thermomonosporaceae* family. Currently there are 44 species with validly-published names in this genus. The type strain is *Actinomadura madurae*, a well known human pathogen (Euzéby 2010, Williams & Wellington 1981). *Actinomadura* colonies are typically cartilaginous or leathery in appearance (Meyer 1989). Mature aerial mycelium forms short or long chains of arthrospores that are either hooked, straight or form irregular spirals. The spores of *Actinomadura* are formed in diverse colours from white to gray, yellow, pink, blue, green or violet, and spore surfaces are generally smooth or warty in appearance. This genus contains species with *meso*-DAP (cell wall chemotype III) and madurose as the characteristic sugar (whole cell sugar pattern B). The phospholipid pattern is PI or PIV (Williams & Wellington 1981), with MK-9(H₄) and MK9-(H₆) as the predominant menaquinones. As mentioned, some *actinomadurae* have been shown to be pathogenic, causing human actinomycetoma such as “Madura foot”. Other species have been shown to produce over 250 antibiotics of diverse structural classes (Cook *et al.* 2005, Badji *et al.* 2006, Lazzarini *et al.* 2000). Despite their isolation from clinical specimens (Yassin *et al.* 2010), *Actinomadura* species are widespread in soil environments. *Actinomadura flavalba* was isolated recently as an endophyte from the leaves of *Maytenus austroyunnanensis* (Qin *et al.* 2009b).

The genus *Nonomuraea*

The genus *Nonomuraea* was proposed in 1998 by Zhang *et al.* Currently there are 23 species with validly-published names in the genus, with *Nonomuraea pusilla* as the type species (Euzéby 2010). The genus *Nonomuraea* belongs to the *Streptosporangiaceae* family. Species typically have *meso*-DAP as the cell wall diamino acid (type III), and madurose as the characteristic whole cell sugar (type B) (Quintana *et al.* 2003). *Nonomuraea* species have the PIV phospholipid pattern (Quintana *et al.* 2003), with MK-9(H₄) as the predominant menaquinone. Some *Nonomuraea* species have been shown to be valuable producers of antibiotics and anti-tumour agents (Qin *et al.* 2009a, Gunnarsson *et al.* 2003). *Nonomuraeae* have been isolated mainly from soil environments, but some have also been isolated as endophytes from various plant species (Qin *et al.* 2009a, Li *et al.* 2010).

1.3 Actinobacterial Secondary Metabolites

Due to their excellent track record as antibiotic producers, actinobacteria, particularly the *Streptomyces* species, have been the focus of extensive drug screening programs since the 1940s, resulting in the discovery of most of the important antibacterial compounds in the early decades that followed (Bérdy 2005, Peláez 2006). In fact, between 70% and 80% of the antibiotics discovered in the 1950s and 1960s (what is referred to as the “Golden Age” of antibiotic discovery) were from the *Streptomyces* species, with increasing contributions from the rare actinobacteria in the 1970s and 1980s (as demonstrated in Figure 1.4, Bérdy 2005).

Further research involving the actinobacteria resulted in an increased discovery of other useful secondary metabolites, such as antitumour and immunosuppressive agents, antifungals and enzymes (Lam 2006). A great number of antibiotics currently used today in the pharmaceutical industry are derivatives of natural products from actinobacteria, such as vancomycin and erythromycin (Baltz 2008, Peláez 2006). In recent years, however, the rate at which novel antibiotics were being discovered from terrestrial actinobacteria began to dwindle, and the rate at which known compounds were being re-isolated began to increase (Lam 2006). Natural product discovery was also in the process of being replaced by two new technologies in the industry, namely combinatorial chemistry and high-throughput screening (Peláez 2006). However, these technologies struggled to provide comparable numbers of new molecules, as well as complex and effective synthetic antibacterial compounds, resulting in a renewed interest in the natural products produced by microbes in general (Peláez 2006, Baltz 2008).

New strategies are currently being developed in order to overcome the issue of re-isolation of known compounds (Williams 2008). Two such strategies may include bioprospecting previously under-explored niches for novel actinobacteria, as well as developing novel isolation procedures for the rarer actinobacteria. Some previously under-explored niches, for example, include marine environments as well as isolations from actinobacterial associations with plants as endophytes. Novel isolation procedures using novel selective isolation media for specific genera, such as *Amycolatopsis*, have been developed (Tan *et al.* 2006). It is expected that through isolations of novel actinobacteria, resulting in an increased taxonomic diversity, the

discovery of new bioactive molecules will also be achieved (Williams 2008, Lazzarini *et al.* 2000).

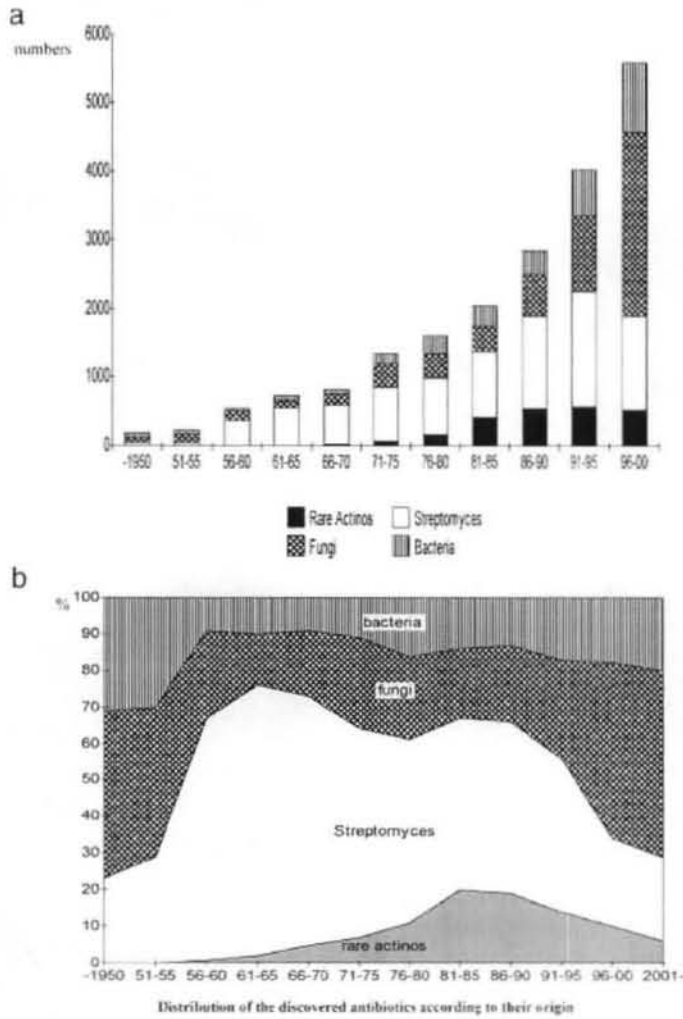


Figure 1.4 Distribution of antibiotics discovered between the 1950s and 2000, with *Streptomyces* and the rare actinobacteria being the main producers of antibiotics between the 1950s and 1980s and early 1990s (Bérdy 2005).

1.3.1 Antibiotic Resistance and Tuberculosis

Novel antibiotics are constantly needed due to an increase in bacterial antibiotic resistance. Antibiotic resistance may be defined as the ability of a microorganism to withstand exposure to one or multiple antibiotics, or classes of antibiotics that are commonly used to treat the diseases or infections that they cause. These once easy to control infections have now become a threat as patients' symptoms no longer respond to conventional treatment, resulting in an increase in time of infection with more complications as well as an increased risk of death.

The problem of antibiotic resistance began soon after the introduction of antibiotics into health care systems around the world (Levy & Marshall 2004). Incorrect use and over prescription of antibiotics has resulted in the selection of antibiotic resistant bacterial strains. Only those bacteria that per-chance develop or gain some sort of mechanism that enable them to survive exposure to antibiotics are able to survive antibiotic treatments. Specifically, bacteria may be intrinsically resistant due to their chromosomal make up, or acquire antibiotic resistance due to horizontal gene transfer or random genomic mutations (Mulvey & Simor 2009). The resistance mechanisms work in a number of ways which may: chemically alter or destroy the antibiotics with specific enzymes (Walsh & Fischbach 2009), alter the site to which the antibiotics bind, or decrease access of the antibiotic to the active site (this can be achieved by either changing the structure of proteins found in the outer membrane resulting in a changed permeability, or evolving efflux pumps that pump out the antibiotics before they are able to bind to their targets (Mulvey & Simor 2009)).

Three major threats to public health due to antibiotic-resistant pathogens include: methicillin resistant *Staphylococcus aureus* (MRSA), multidrug-resistant or pandrug-resistant Gram negative bacteria, and multidrug-resistant and extensively drug resistant *Mycobacterium tuberculosis* (Fischbach & Walsh 2009).

MRSA has the ability to resist antibiotics in the beta-lactam class (Fischbach & Walsh 2009). It is estimated that MRSA causes approximately 19000 deaths in the United States every year.

These infections are probably one of the main causes of hospital acquired infections (Levy 1998), with a high mortality rate. Currently, treatments involve the use of vancomycin, the “antibiotic of last resort” (Fischbach & Walsh 2009), however emergence of vancomycin resistant *Staphylococcus aureus* (VRSA) and vancomycin resistant enterococci are a major concern, as it seems that infections caused by these strains are becoming more and more untreatable.

Another major threat includes that of the multi-drug and pandrug-resistant Gram negative bacteria. Infections caused by these bacteria are less prevalent than those caused by MRSA, however they have an alarming antibiotic resistance profile (Fischbach & Walsh 2009, Walsh & Fischbach 2009). Some Gram negative pathogens showing such resistance include *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. These pathogens cause a variety of illnesses including food poisoning, pneumonia, meningitis and bacteraemia (Walsh & Fischbach 2009). The problem is that these Gram negative pathogens have been shown to be resistant to some or all of the antibiotic classes used in their treatment (Fischbach & Walsh 2009). One major difficulty is that, due to the outer membrane, many antibiotics are prevented from entering the Gram-negative cell.

M. tuberculosis, the causative agent of tuberculosis (TB), has become a major and difficult problem to deal with, especially in developing countries. In fact, TB is so prevalent that it can be said that every second, a new person is infected with the tuberculosis bacillus (Singh 2004). Around 2 million people die each year due to TB infections, and it is estimated that around one third of the world’s population, who do not show any symptoms of TB, are currently infected with the bacillus (Meya & McAdam 2007). Around 9 million people will develop TB symptoms every year around the world (Loddenkemper & Hauer 2010), and it is estimated by the World Health Organization that between 2000 and 2020, close to a billion people in the world will be infected with *M. tuberculosis*, with 200 million people developing symptoms of TB, and approximately 35 million people dying from the disease (Pieters 2005). The treatment for standard non-resistant tuberculosis involves the use of a combination of four first-line drugs (rifampicin, isoniazid, pyrazinamide and ethambutol or streptomycin) (Loddenkemper & Hauer 2010). Treatment lasts for 6 months.

Despite being a treatable disease (Jain & Dixit 2008), TB has become increasingly difficult to cure due to the rise in multi-drug resistant tuberculosis (MDR-TB). MDR-TB may be defined as being resistant to two or more of the first line drugs used in the treatment of tuberculosis, particularly isoniazid and rifampicin, the most effective first-line drugs (Heller *et al.* 2010). It is estimated that more than 4% of the global TB burden is due to MDR-TB (Andrews *et al.* 2007). Drug resistance is believed to have arisen due to inadequate compliance with treatment regimens, which is believed to have resulted in the selection of mutated strains that became resistant to the treatments (Zager & McNeerney 2008, Singh 2004). Patients that develop MDR-TB are treated with a selection of six main classes of second-line drugs (aminoglycosides, polypeptides, fluoroquinolones, thioamides, cycloserine and *para*-aminosalicylic acid) (Loddenkemper & Hauer 2010, Jain & Mondal 2008), which are more toxic to the patients (Wells *et al.* 2007). The treatment for MDR-TB is generally administered over 24 months, and is significantly more expensive than conventional treatment for non-resistant tuberculosis. The World Health Organization estimated that sub-Saharan Africa accounts for 14% of the global MDR-TB burden, with 2.4% pertaining to persons between 15-49 years of age in South Africa (Wells *et al.* 2007).

Due to the prolonged treatment required for MDR-TB (six months), patient adherence has become a major problem (Pepper *et al.* 2007). It is believed that this broad lack of compliance, along with general mismanagement of tuberculosis cases can be linked to the origins of extensively drug resistant tuberculosis (XDR-TB) (Jain & Dixit 2008). Extensively drug-resistant tuberculosis is defined as being resistant to not only the first-line drugs isoniazid and rifampicin, but also to fluoroquinolones and any of the three injectable second-line drugs capreomycin, kanamycin and amikacin (Jain & Dixit 2008). This form of tuberculosis has seriously limited treatment options, depending on a number of factors, including the extent of drug resistance and the patient's immune system. XDR-TB has been reported worldwide, and the disease is almost untreatable (Zager & McNeerney 2008) having a high mortality rate, particularly in patients co-infected with HIV (Jassal & Bishai 2009).

The rapid spread of TB, particularly XDR-TB, among HIV infected individuals is a major concern, especially because of the rapid spread of the disease and rapid death (Jain & Mondal 2008). South Africa has one of the highest populations in the world of individuals that are

infected with HIV (Andrews *et al.* 2007), where approximately 20% of the adult population is affected. In 2005 it was reported that South Africa had the second highest number of TB cases in Africa, and the seventh highest in the world, with 58% of HIV patients co-infected with TB (Andrews *et al.* 2007). In 2002, approximately 40% of patients with MDR-TB were co-infected with HIV (Wells *et al.* 2007). Tuberculosis is the leading cause of death in individuals that are HIV positive. HIV and TB co-infections seem to fuel each other, with an increase in the risk of developing active tuberculosis, while HIV progression is accelerated by tuberculosis infection (Pepper *et al.* 2007). Interestingly, it seems that most cases of XDR-TB have appeared among HIV positive individuals, which is not surprisingly associated with high mortality rates (Jain & Mondal 2008). This serious problem of HIV and TB co-infection needs urgent attention, requiring improved management programs and novel antibiotics, amongst others.

The problems faced with increasing drug resistance highlight the need for novel antibiotics that may be used to treat these serious diseases. It is important that ongoing research focus on the discovery of novel antibiotics. This may be achieved through screening for unknown species of actinobacteria that may produce novel antibiotics. It is hoped that studies that focus on previously unexplored environments will yield unique actinobacterial strains that produce novel compounds (Bérdy 2005).

1.4 Aims of This Study

The main aims of this project were to isolate non-*Streptomyces* actinobacterial species (rarer actinobacteria) from terrestrial plants, seaweed and soil environments, to screen the isolates for their antibacterial activity against *Mycobacterium aurum* A+, and to characterise the isolates to determine whether they represent novel species.

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Chapter 2

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Chapter 2

Isolation of Actinobacteria, Preliminary Identification and Antibacterial Activity

2.1 Introduction

Actinobacteria are probably the most important producers of antibiotics (Lam 2006, Lazzarini *et al.* 2000) with a number of classes of antibiotics having been isolated from them. These include aminoglycosides (e.g. streptomycin, kanamycin, gentamicin), ansamycins (e.g. rifampin), anthracyclines (e.g. doxorubicin), β -lactams, glycopeptides (e.g. vancomycin), macrolides (e.g. erythromycin, tylosin), nucleosides (e.g. tubercidin, tunicamycin), peptides (e.g. capreomycin, tuberactinomycin) and tetracyclines (Champness 2000). The rise in drug resistance among common disease-causing pathogens, combined with the decrease in the rate of discovery of new compounds (Margarvey *et al.* 2004), has caused a renaissance in the interest of discovering novel antibacterials (Bascom-Slack *et al.* 2009).

2.1.1 Soil environments for the isolation of actinobacteria

Of course, the microbial diversity in terrestrial environments has proven to be extraordinary, with a great number of actinobacteria having been isolated from soil environments. This is clearly seen in the efforts invested over the past 50 years in isolating and researching actinobacteria from soil environments, combined with drug screening programs (Lam 2006). Although the soil environments have proven to be one of the richest and most diverse environments for the isolation of actinobacteria, there has been an increased rate of re-isolation of known actinobacterial species. This is particularly true for strains of easily isolated genera such as *Streptomyces*, which is the most well studied genus of the actinobacteria, and is also particularly common in soil. In order to obtain novel strains of actinobacteria from commonly explored soil environments, and thus potentially isolate novel secondary metabolites, novel methods, including novel isolation procedures and media, are required (Tan *et al.* 2006). These new methods may also help to isolate novel actinobacteria from other unexplored environments. This is particularly relevant when, realistically, only a very small percentage of the terrestrial

environment has been sampled (Baltz 2008). The focus of research may also be placed on isolation of strains of the non-*Streptomyces* genera that may produce novel antibiotics. Some important non-*Streptomyces* antibiotic producers are some of the *Micromonospora* and *Amycolatopsis* species, such as *Micromonospora purpurea* and *Amycolatopsis mediterranei*, which produce gentamicin and rifamycins, respectively, some of the most successful antibacterials found on the market (Lazzarini *et al.* 2000).

2.1.2 Alternative environments for the isolation of actinobacteria

Importance must also be placed on isolating potentially novel species from previously under-explored environments. It is hoped that actinobacteria isolated from such sources will possess unique metabolic pathways that will lead to the isolation of novel metabolites.

2.1.2.1 Plants and endophytes

One often overlooked biological niche is that of bacterial endophytes in numerous plants around the world (Bascom-Slack *et al.* 2009). It is believed that the relationships between plants and endophytes began hundreds of millions of year ago, when plants first appeared on earth. This has allowed for endophytes to adapt to their ecological niche over time and, as such, have developed ways of contributing to the relationship (Strobel 2003). Bacterial endophytes may be defined as "bacteria that colonize the internal tissue of the plant showing no external sign of infection or negative effect on their host" (Ryan *et al.* 2007). These endophytic bacteria have been isolated from a range of plant species, including both monocotyledonous and dicotyledonous plants (Ryan *et al.* 2007). They colonize intracellular spaces and may be found in all plant compartments, including roots, stems, leaves and even seeds. With almost 300 000 plant species on the planet, and given the belief that each individual plant is host to one or more endophytes, there exist great opportunities for the discovery of potentially novel bacterial strains (Ryan *et al.* 2007) and, indirectly, novel secondary metabolites. In fact, a study by Schulz *et al.* (2002) on endophytic fungi showed that just over half of the biologically active secondary metabolites isolated were previously unknown. The same could be true for endophytic actinobacteria. The endophytic actinobacteria are believed to play important roles in plant development and growth through nutrient assimilation, as well as by conferring disease resistance, particularly through the production of active secondary metabolites (Kunoh 2002). In

return, endophytes get protection as well as nutrition from the host plant (Hasegawa *et al.* 2006).

A number of plants are commonly used in the treatment of medical conditions (Strobel 2003), and it has been suggested that their medicinal properties may actually be related more to the endophytes residing in them than to their own biochemistry (Strobel *et al.* 2004). For example, Aboriginal groups in Australia commonly use the snakevine plant (*Kennedia nigricans*) for the treatment of skin infections. An endophyte, *Streptomyces* sp. strain NRRL 30562, was isolated from this plant and was shown to produce a number of novel peptide antibiotics, named munumbicins, that have broad-spectrum activity against a number of human and plant pathogenic bacteria and fungi, as well as a *Plasmodium* sp., a malarial parasite (Strobel 2003). The munumbicins have been shown to have antibacterial activity against Gram positive bacteria, including *M. tuberculosis* (Strobel *et al.* 2004). A number of other antibiotic-producing endophytes have been isolated from various plant sources (Kunoh 2002).

Currently, the isolation of endophytic actinobacteria seems to be of particular interest to researchers, with various rarer genera also being isolated. Some recently described endophytic bacteria include *Actinomadura flavalba*, *Dietzia schimae*, *Dietzia cercidiphylli*, *Glycomyces scopariae*, *Glycomyces mayteni*, *Pseudonocardia endophytica* and *Saccharopolyspora gloriosae* (Qin *et al.* 2009a, Li *et al.* 2008, Qin *et al.* 2009b, Chen *et al.* 2009, Qin *et al.* 2010). These actinobacterial endophytes, like others, are isolated from various plant sections, namely the roots, stems and leaves. There are also a number of descriptions of endophytic actinobacteria currently *In Press* at the International Journal of Systematic and Evolutionary Microbiology (IJSEM), including '*Actinoallomurus oryzae*', '*Amycolatopsis samaneae*', '*Nocardia callitridis*', '*Nocardioides caricicola*', '*Nonomuraea endophytica*', '*Pseudonocardia adelaidensis*', '*Pseudonocardia eucalypti*', '*Pseudonocardia tropica*' and '*Streptomyces lacticiproducens*', as well as species belonging to two new genera, '*Jishengella endophytica*' and '*Phytohabitans suffuscus*'. (<http://ijs.sgmjournals.org/papbyrecent.dtl> - accessed July 2010). This indicates that there exists great diversity in relation to the actinobacteria and their association with plants, a previously underexplored niche, which may result in the discovery of novel secondary metabolites. An advantage of the discovery of potentially novel endophytic actinobacteria is that

any antibiotics discovered from these endophytes may have a reduced risk of toxicity to eukaryotes, as a result of their association with plants (Bascom-Slack *et al.* 2009).

2.1.2.2 Marine environments

Another rich and diverse source of actinobacteria is the under-explored marine environment - the oceans that cover over 70% of the world's surface and their diverse ecosystems (Lam 2006, Bull & Stach 2007). Marine environments may be extreme, varying from cold temperatures to high pressure and high salinity. As a result, it is believed that marine actinobacteria may possess different metabolisms to their terrestrial counterparts, hence possibly producing novel metabolites (Lam 2006). In fact, recent work has focused on the isolation of marine actinobacteria, with diverse taxa being isolated from the various marine environments (Baltz 2008) from sediments, deep sea floors, coral reefs and plants, to name a few (Lam 2006). Some of these marine isolates have been shown to produce novel compounds, including antibiotics (such as abyssomicin C from a novel *Verrucosispora* strain) and antitumour agents (Baltz 2008, Williams 2008). With the unique chemical structures that may be isolated, marine actinobacteria can potentially provide fresh therapeutic leads (Williams 2008).

2.1.3 Aims of this study

The aim of this part of the study was to isolate actinobacteria from soil, plant and seaweed samples, with particular focus on the rarer (non-*Streptomyces*) species, and to test them for antibacterial activity against *M. aurum* A+. Various plants, mostly indigenous, were selected from the Table Mountain National Park Reserve, as well as various species of seaweed from the coast of Simon's Town, Cape Town, South Africa, in an attempt to discover potentially novel endophytic actinobacteria and novel secondary metabolites. The isolation media used are commonly utilised in actinobacterial research (e.g. International *Streptomyces* Project (ISP) Medium 2), the *Amycolatopsis* selective medium SM3 (Tan *et al.* 2006), as well as media that more closely mimic the environments from which actinobacteria were to be isolated (i.e. starch-casein agar and ISP 2 + 2% NaCl for seaweed samples). Finally a selected number of isolates were screened for their antibiotic biosynthetic potential and antibiotics were extracted.

2.2 Materials and Methods

2.2.1 Isolation sources, pretreatment and culture conditions

2.2.1.1 Soil sample

A soil sample was obtained from a hill near the dam at the University of Cape Town, South Africa, at a depth of 10 cm, and placed into a sterile plastic tube. The soil pH of 8.3 was determined by suspending a small amount of soil in water, shaking the sample and then measuring the pH of the water.

A petri dish full of soil was subjected to dry heat pre-treatment at 60°C for 1 hour (Levy 1998), while the remainder of the soil remained untreated. A sterile pestle and mortar were used to finely grind the treated and untreated soil samples, after which 0.1 g of soil was added to 1 ml sterile water, vortexed for 1 minute and allowed to settle. Both soil samples (treated and untreated) were serially diluted in a 10-fold series to 10⁻⁵, and 100 µl of each dilution was plated onto the isolation media.

The isolation media used in the isolation of actinobacteria from soil were ISP 2 (YEME) (Shirling & Gottlieb 1966), *Amycolatopsis* selective medium SM3 (Tan *et al.* 2006) and SE (soil extract agar at pH 8 - based on the pH of the soil sample) (Hamaki *et al.* 2005). Soil extract agar was made using soil extract prepared from 250 g soil mixed with 500 ml of 50 mM NaOH, according to the methods described by Hamaki *et al.* (2005). All media were prepared using reagent grade chemicals and distilled water, and sterilized by autoclaving at 121°C for 20 minutes (unless otherwise stated). All isolation media were supplemented with cycloheximide (50 µg/ml) to inhibit the growth of fungi, and nalidixic acid (10 µg/ml) to inhibit the growth of Gram negative bacteria. All plates were incubated at 30°C. Colonies were subcultured weekly using sterile toothpicks for a total of 3 weeks (to allow for the growth of rarer actinobacteria), onto their corresponding media without antibiotics. Isolates from SE media were further subcultured onto YEME plates. Colony selection was based on morphological characteristics that corresponded to that of actinobacterial species. Isolates were named according to the media they were initially

isolated from and the colony number (i.e. YM – YEME agar, SE – soil extract agar, SM3 - SM3 medium). All isolates were grown in their respective YEME or SM3 broths for 3-14 days for further research.

2.2.1.2 Plant and seaweed samples

A total of 16 plant samples was used for the isolation of actinobacteria between February and June 2008 (Table 2.1). Two plant samples were obtained from the Kirstenbosch Botanical Garden in Cape Town, South Africa, with three samples being obtained from a private garden in the suburb of Plumstead, Cape Town. The remaining plant samples were obtained from the contour path above Rhodes Memorial on Devil's Peak in the Table Mountain National Park (TMNP), Cape Town.

Table 2.1 Plant species used in this study

Plant species	Source	Date
<i>Aloe africana</i>	Plumstead Garden	February 2008
<i>Bolusanthus speciosus</i>	Plumstead Garden	February 2008
<i>Carpobrotus murii</i>	Plumstead Garden	February 2008
<i>Chironia baccifera</i>	Devil's Peak, TMNP	June 2008
<i>Chrysanthemoides monilifera</i>	Devil's Peak, TMNP	June 2008
<i>Cliffortia ruscifolia</i>	Devil's Peak, TMNP	June 2008
<i>Leucadendron argenteum</i>	Devil's Peak, TMNP	June 2008
<i>Metalasia muricata</i>	Devil's Peak, TMNP	June 2008
<i>Muraltia heisteria</i>	Devil's Peak, TMNP	June 2008
<i>Olea europea</i> subsp. <i>africana</i>	Devil's Peak, TMNP	June 2008
<i>Pelargonium cucullatum</i>	Devil's Peak, TMNP	June 2008
<i>Peucedanum galbanum</i>	Devil's Peak, TMNP	June 2008
<i>Podocarpus latifolius</i>	Devil's Peak, TMNP	June 2008
<i>Rhus lucida</i>	Devil's Peak, TMNP	June 2008
<i>Sutherlandia frutescens</i>	Kirstenbosch Botanical Garden	February 2008
<i>Tetradenia riparia</i>	Kirstenbosch Botanical Garden	February 2008

A total of seven seaweed samples was also used for the isolation of actinobacteria (Table 2.2). One sample was obtained from a rocky portion along the road's edge in Simon's Town, Cape Town, with the remaining 6 samples being obtained from Kalk Bay's rocky shore in Cape Town. All samples were obtained in March 2008. Samples were collected in sterile jars containing sea water.

Table 2.2 Seaweed species used in this study

Seaweed species	Source
<i>Aeodes orbitosa</i>	Kalk Bay
<i>Ulva</i> sp.	Kalk Bay
<i>Bifurcariopsis capensis</i>	Kalk Bay
<i>Splachnidium rugosum</i>	Kalk Bay
<i>Ecklonia maxima</i>	Simon's Town
<i>Ecklonia radiata</i>	Kalk Bay
<i>Porphyra capensis</i>	Kalk Bay

The surfaces of the plant and seaweed samples were sterilized after approximately 18 hours storage in the fridge at 4°C. Leaves obtained from the various plant species and sections of seaweed samples were separately surface sterilized in 70% ethanol for 1 minute, soaked in 1% NaOCl for 3 minutes and rinsed twice with sterile distilled water. The surface-sterilized leaves and seaweed samples, as well as samples of unsterilized seaweeds, were roughly cut into 1cm pieces and placed into sterile universal containers containing 10 ml sterile quarter-strength phosphate buffer (full strength phosphate buffer: 5.62 g K₂HPO₄, 2.13 g KH₂PO₄, 1 litre distilled water, pH 7, autoclaved) (Demain & Davies 1999) and vortexed. The universals containing plant leaves were then incubated at 30°C for 30 minutes to 1 hour, with shaking, whilst those containing seaweed samples were incubated at room temperature (approximately 20°C) with shaking for 30 minutes to 1 hour.

After incubation, both the plant and seaweed (sterilized and unsterilized) buffer extracts were serially diluted in a ten-fold series, of which 100 µl of each dilution was spread onto isolation media. The media were YEME and SM3 (supplemented with cycloheximide and nalidixic acid as for the soil sample above) for the plant isolates, while the seaweed samples were plated onto YEME supplemented with 2% NaCl and on starch-casein (SC) agar plates (10 g soluble starch, 1 g casein dissolved in 3 ml 1M NaOH, 0.5 g K₂HPO₄, 20 g NaCl, 20 g, 1 litre distilled water, pH 7.5, autoclaved). The plates were incubated at room temperature (for seaweed samples) or 30°C (for plant samples) for 3-4 weeks, with weekly observation for growth and subculturing of actinobacteria-like colonies. Isolates were subcultured onto their respective media without antibiotics, with isolates from YEME + 2% NaCl and SC plates being further subcultured onto

YEME plates. Seaweed isolates were named according to the media they were isolated on and the colony number (i.e. Y2 – YEME + 2% NaCl, SC – starch-casein medium). Plant isolates were named according to the medium they were isolated on, the abbreviated name of the plant species from which they were isolated, and the colony number (i.e. SM3xy and Yxy, where xy represents the abbreviated name of the plant species).

2.2.2 DNA extraction

All isolates were grown up from a loopful of cells, obtained from subcultured plates, in 10 ml broths of either YEME or SM3 (depending on their isolation media) at 30°C with shaking, except for the seaweed isolates that were grown at room temperature. Isolates were allowed to grow until an adequate amount of cell mass was observed, varying from 3 to 14 days. Gram stains were performed as per standard procedure to ensure that broths were not contaminated. The genomic DNA was extracted by the method of Wang *et al.* (1996) with modifications including the addition of 0.2 mg/ml proteinase K and cell lysis with 20mg/ml lysozyme for a period of greater than 16 hours.

2.2.2.1 PCR amplification of 16S rRNA genes

The amplification of the 16S rRNA gene sequences of isolates was performed in 50 µl reaction volumes containing the following: 2 µl template DNA (200-500 ng/µl), 0.5 U *Taq* polymerase (Super-Therm, JMR Holdings, USA), 2 mM or 4 mM MgCl₂, 150 µM of each dNTP and 0.5 µM of each primer. The F1 and R5 primers used were described by Cook & Meyers (2003). The PCR reactions were performed in a Techne TC-512 thermal cycler. The PCR cycling program was as follows: initial denaturation for 2 minutes at 96°C followed by; 30 cycles of denaturation for 45 seconds at 96°C, annealing for 30 seconds at 56°C and extension for 2 minutes at 72°C; with a final extension for 5 minutes at 72°C.

All PCR products were electrophoresed on 0.8% agarose gels containing 0.8 µg/ml ethidium bromide, along with a molecular mass marker (*Pst*I digest of phage λ DNA). All gels were visualised on a GelDoc XR system at 254 nm (Bio-Rad Laboratories Inc.).

2.2.3 Amplified ribosomal DNA restriction analysis

Isolated actinobacteria were identified to the genus level (or to a small group of genera) using the rapid identification method involving the digestion of amplified 16S-rRNA gene fragments with single restriction endonucleases (Cook & Meyers, 2003). Digestions were performed separately in 20 µl reaction volumes with specific endonucleases and appropriate restriction buffers. All initial digestions involved *Mbol* (*Sau3AI* isoschizomer) and then *Vspl* (*AsnI* isoschizomer). The remaining digestions were performed using various other enzymes, depending on the *Mbol* and *Vspl* digestion patterns, as outlined in Cook & Meyers (2003). These included *HindIII*, *KpnI*, *PaeI* (*SphI* isoschizomer), *PspI*, *PstI*, *SalI*, *Scal* and *Eco105I* (*SnaBI* isoschizomer). The total volume of each digestion (containing 2 µl tracking dye) was electrophoresed on a 1.5% agarose gel (with 0.8 µg/ml ethidium bromide) with a molecular size marker (λ *PstI*) and visualised on the GelDoc XR system.

2.2.4 Determination of antibacterial activity

A total of 42 selected actinobacterial isolates were tested for their antibacterial activity against *M. aurum* A+. These included all the non-*Streptomyces* isolates identified by the rapid identification method of Cook & Meyers (2003) and/or 16S rRNA gene sequence analysis (covered in Chapter 3), as well as 14 *Streptomyces* species. Twelve isolates, selected based on the strength of their antibacterial activity against *M. aurum* A+ and their genus assignment, were further tested for their activity against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 (both standard antibiotic-susceptibility test strains).

Actinobacterial isolates were stab-inoculated, using sterile toothpicks, onto YEME, SM3, MC (Modified Czapek solution (Nonomura & Ohara 1971)) and Middlebrook 7H9 (containing 10 mM glucose, albumin-dextrose-catalase supplement omitted) agar plates, for each test bacterium. Plates were incubated at 30°C for 9 days to allow for the production of antibiotics by the isolates. The diameters of the colonies were then recorded and the colony area was determined.

The test bacteria (*M. aurum*, *E. coli* and *S. aureus*) were inoculated into 10 ml Luria-Bertani broth (Sambrook *et al.* 1989) and incubated for between 16 to 20 hours at 37°C with shaking. Gram stains were performed to confirm the purity of the cultures and the optical density of each culture at 600 nm was recorded (Beckman DU530 UV Spectrophotometer). To ensure comparability between tests, the volume (in microlitres) of each test bacterium to be added to tubes containing 6ml sloppy Luria-Bertani agar (0.7% agar; Sambrook *et al.* 1989) was calculated using the formula $160 = OD_{600} \times \mu\text{l}$ for *M. aurum* and *S. aureus*, and $4 = OD_{600} \times \mu\text{l}$ for *E. coli*, where OD_{600} was the optical density of the culture. The inoculated sloppy Luria-Bertani agar samples were overlaid onto stab-inoculated actinobacterial plates, allowing the agar to spread over the plate and settle around the edges of the colonies. Plates were incubated at 37°C (for approximately 18 hours for *E. coli* and *S. aureus*, and for 2 days for *M. aurum*). The areas of inhibition (mm^2) were calculated as the difference between the area of the zone of inhibition and the area of each colony, as a measure of the degree of antibacterial activity. The arbitrary assignment of the strength of the antibacterial activity of isolates is represented in the Table 2.3. Strains displaying strong to very strong activity were re-tested individually to determine a more accurate zone of inhibition. Six isolates were chosen for further antibiotic work based on their strength of inhibition and their genus assignment.

Table 2.3 Strength of antibacterial activity

Antibacterial Activity	Zone Area (mm^2)
Very weak (VW)	<100
Weak (W)	100 – 1000
Moderate (M)	1001 – 2000
Strong (S)	2001 – 3000
Very strong (VS)	>3000

2.2.5 Antibiotic biosynthetic potential

All isolates showing moderate to strong antibacterial activity were screened for the presence of antibiotic biosynthetic genes by PCR. Genomic DNA, extracted as described in section 2.2.2, was used in the PCR experiments involving specific primers that amplify genes involved in the

production of antibiotics (Table 2.4). For ansamycin antibiotics, ANSA-F and ANSA-R primers were used to amplify a fragment of the 3-amino-5-hydroxy-benzoic acid (AHBA) synthase gene that is essential for ansamycin biosynthesis (Wood *et al.* 2007). The presence of genes for Type II (aromatic) polyketide biosynthesis was determined using ARO-PKS-F and ARO-PKS-R primers that amplify a section of the KS_{α} - KS_{β} polyketide synthase tandem gene pair. Finally, Foxy and Roxy primers were used in the amplification of the *oxyB* gene that plays an essential role in glycopeptide synthesis (Wood *et al.* 2007). PCR conditions for all antibiotic-gene primers were as for 16S-rRNA gene amplification, but with annealing temperatures of 60°C for *oxyB* gene amplification and 64°C for the KS_{α} - KS_{β} gene pair amplification (Wood *et al.* 2007). PCR products were electrophoresed and visualized on 1.5% agarose gels as described above with appropriate controls.

Table 2.4 List of antibiotic PCR primers used (Wood *et al.* 2007)

Primer	Sequence	Product
ANSA-F	5' – CCS GCS TTC ACS TTC ATC TC – 3'	AHBA synthase
ANSA-R	5' – AIS YGG AIC ATI GCC ATG TAG – 3'	AHBA synthase
ARO-PKS-F	5' – GGC AGC GGI TTC GGC GGI TTC CAG – 3'	KS_{α} - KS_{β}
ARO-PKS-R	5' – CGI TGT TIA CIG CGT AGA ACC AGG CG – 3'	KS_{α} - KS_{β}
Foxy	5' – CTG GTC GGC AAC CTG ATG GAC – 3'	<i>oxyB</i>
Roxy	5' – CAG GTA CCG GAT CAG CTC GTC – 3'	<i>oxyB</i>
I = Inosine; S = C or G; Y = C or T		

2.2.6 DNA sequencing and phylogenetic analysis of antibiotic biosynthetic genes

PCR products of three selected isolates were purified using the Invitex MSB® Spin PCRapace (Germany) PCR purification kit and sequencing was performed as a service by MacroGen Incorporated (Seoul, Korea) using the dye termination method.

All gene sequences were edited in Chromas (Version 2.01, Technelysium, 1999) and analysed using DNAMAN (version 4.13, Lynnon Biosoft, 1999). Consensus sequences were subjected to Basic Local Alignment Search Tool (BLAST) comparisons with other sequences within the

GenBank database, using the BLASTn search tool (Altschul *et al.* 1997). The neighbour-joining method (Saitou & Nei 1987) was used in the construction of phylogenetic trees for antibiotic biosynthetic genes, using MEGA (version 4, Tamura *et al.* 2007).

2.2.7 Antibiotic extraction

Six isolates that showed good antibacterial activity were inoculated into 10ml broths of either YEME or SM3 (in some cases both) and incubated at 30°C with shaking for 3-5 days. Gram stains were performed to ensure cultures were not contaminated. The culture broths were used to inoculate 100 ml broths of the respective media and grown for 9 days at 30°C with shaking. The cultures were filtered through Perco size 1x6 coffee filters to separate the cell mass from the culture broth. The culture filtrate (approximately 100 ml) was separated into 4 equal volumes of approximately 25 ml each in 250 ml bottles. The separate culture filtrates were extracted with equal volumes of organic solvents (hexane, chloroform and ethyl acetate, in order of increasing polarity), to allow for the extraction of extracellular antibiotics, by shaking for 2 hours on a rotary shaker at room temperature followed by storage at 4°C. Solvent extracts were removed using a pipette and allowed to evaporate in a fume hood in beakers. Each evaporated sample was concentrated 50-fold by dissolution in 0.5 ml of its extraction solvent. The fourth sample of culture filtrate was freeze dried, concentrated to 50X in water and tested to assess the original amount of extracellular antibiotic activity. The cell mass was dried and extracted with methanol for over 16 hours at room temperature in a sterile universal, to determine if the antibiotic(s) produced is/are intracellular. All extracts were stored in 1.5 ml plastic microfuge tubes at -20°C.

2.2.8 Thin layer chromatography and bioautography

A total of 10 µl of each concentrated sample was spotted on silica (Silica gel 60 F₂₅₄) thin layer chromatography (TLC) plates (Merck). *M. aurum* A+ was heavily inoculated in 10 ml sterile Luria-Bertani broth and grown for between 16 to 20 hours at 37°C with shaking. The culture was Gram stained to confirm that it was not contaminated and diluted with sterile Luria-Bertani broth to an approximate OD₆₀₀ of 0.5 in order to standardise the amount of culture inoculated onto each TLC plate. The diluted *M. aurum* culture was applied with sterile cotton wool to the TLC plates, which were transferred to a plastic container containing moistened paper towels

and incubated for between 16 to 20 hours at 37°C to allow for the interaction of any antibiotics with *M. aurum*. After incubation, the plates were dabbed with 0.25% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) diluted with phosphate saline buffer (1.78 g Na₂HPO₄, 8.50 g NaCl, 1 l distilled water, pH 7.3), and re-incubated at 37°C for approximately 2 hours. MTT turns from yellow to blue/purple when reduced to its formazan derivative by actively respiring bacteria. Clear zones on the blue/purple TLC plate after incubation with MTT indicate where there are antibiotics present.

Samples that were found to contain antibiotics (clear zones) were subjected to thin layer chromatography, where they were each spotted as above on 12 cm long silica TLC plates, with 1.5 cm wide lanes for each sample. The chromatograms were developed with various solvent systems in glass beakers covered with tin foil. The chromatography was stopped when the solvent front was 1 cm from the top of the plate. The solvents were evaporated in a fume hood and active compounds were detected by bioautography as described above. Each extract was subjected to TLC using different solvents to obtain adequate resolution of the active compound(s).

2.3 Results and Discussion

A total of 169 putative actinobacterial strains was isolated. Forty-two isolates that displayed interesting and characteristic actinobacterial morphologies were selected for further identification. All 42 isolates, including both *Streptomyces* and non-*Streptomyces* strains, were screened for their antibiotic activity against *M. aurum* A+.

2.3.1 Isolations

Of the 169 isolates obtained from soil, plant and seaweed samples (Table 2.5), a total of 162 isolates (95.85%) were obtained from the soil sample, with half of the soil isolates being isolated

on YEME (50%), followed by those isolated on SM3 (30.86%) and finally those isolated on SE plates (19.14%). Five potential actinobacterial species were isolated from plants (on YEME and SM3 plates), with two others being isolated from seaweed samples (on YEME+2% NaCl and SC plates). The plant and seaweed species from which apparent actinobacterial isolates were obtained are listed in Table 2.5. The inclusion of antibiotics in all of the isolation media, as well as the soil pretreatment method, helped to reduce the number of fungi and other non-actinobacterial isolates. This was evident when comparing plates that had pretreated samples as opposed to those that did not, which were often overgrown by fungi.

Table 2.5 List of the number of isolates from each source

Source	Number of isolates
Soil	162
Plant	5
<i>Bolusanthus speciosus</i>	1
<i>Muraltia heisteria</i>	1
<i>Pelargonium cucullatum</i>	2
<i>Podocarpus latifolius</i>	1
Seaweed	2
<i>Ecklonia maxima</i>	1
<i>Bifurcariopsis capensis</i>	1

2.3.2 Amplified ribosomal DNA restriction analysis

Amplified ribosomal DNA restriction analysis (ARDRA) has proven to be useful in quickly differentiating between bacterial genera. This is very helpful particularly when traditional methods such as chemotaxonomic tests, which are laborious and time consuming, often do not identify an isolate to a single genus. Similarly, 16S rRNA gene sequence analysis can be costly with limited availability of sequencing facilities, as is the case in developing countries such as South Africa. The method developed by Cook & Meyers (2003) allows for the rapid initial identification of non-*Streptomyces* species, which allows the focus to be placed on these species that may produce novel antibiotics (Cook & Meyers 2003).

Of the 169 isolates, 42 isolates were initially selected for further identification and were subjected to antibacterial testing. The majority of the selected soil isolates were chosen based

on their morphology, with particular focus on isolates that displayed non-*Streptomyces* characteristics. The remaining soil isolates displayed morphologies typical of the streptomycetes. All the plant and seaweed isolates were chosen for further identification, using the rapid identification method and/or 16S rRNA gene sequence analysis (Chapter 3). A total of 39 of the isolates were subjected to identification using the rapid method of Cook & Meyers (2003).

Initial restriction endonuclease digests involved the use of *Mbol* (*Sau3AI* isoschizomer) for the 39 isolates. The *Mbol* digestions yielded bands smaller than 750 bp in size for 13 of the isolates (~33.33%), which placed them in Group 1 of Cook & Meyers (2003). The remaining 26 isolates were distributed between the other two groups, strongly suggesting that two thirds of the 39 isolates would be assigned to non-*Streptomyces* genera.

A total of 35 isolates that could be further identified to a genus/small group of genera using the rapid identification method (Cook & Meyers 2003) are shown in Table 2.6. Isolates that could not be assigned to a single genus using this method were identified, together with the seven remaining isolates (that were not identified using the Cook & Meyers (2003) method), through 16S rRNA gene sequence analysis (Chapter 3). These were two plant isolates (SM3BL1 and YPL1, identified as *Micromonospora* and *Kribbella* strains, respectively), two seaweed isolates (SC1 and Y2UE1, identified as *Streptomyces* and *Kineococcus* strains, respectively) and three soil isolates (YM53 and YM55, both identified as *Kribbella* strains and YM63, identified as a *Streptomyces* strain). Overall, a final total of 28 non-*Streptomyces* isolates were identified.

It may be assumed that the eight isolates that were identified to belong to either the genus *Streptomyces* or *Sporichthya*, most likely belong to the *Streptomyces* genus due to the fact that the genus *Sporichthya* is extremely rare and does not have typical *Streptomyces* morphology (Cook & Meyers 2003). A total of six isolates could be definitively identified to a single genus (*Streptomyces*, *Nonomuraea* and *Nocardia* species), with the remaining isolates identified to a group of genera. These results proved useful and allowed an emphasis to be placed on the identification of the majority of non-*Streptomyces* species, as well as testing for their potential to produce antibiotics.

Table 2.6 Identification of isolates to a genus/group of genera

Genus/Group of Genera	Group (G) and Table (T)	Isolates
<i>Streptomyces</i>	G1, T1	SM346, YM18, YM46, YMH1
<i>Streptomyces</i> or <i>Sporichthya</i>	G1, T1	SE7, SE22, SM39, SM313, SM315, SM328, SM335, YM61
<i>Nonomuraea</i>	G1, T1	SM343
<i>Actinoplanes</i> , <i>Micromonospora</i> , <i>Couchioplanes</i> , <i>Pilimelia</i> , <i>Spirilliplanes</i> , <i>Verrucosipora</i> or <i>Virgisporangium</i>	G3, T2	SE6, SE18, SE17, SE27, SM336, SM338, SM342, SM349, SM354, SM357, YM20, YM48, YM49, YM51, YM52, YM57, YM76, YM78, YM80, YPC1
<i>Nocardia</i>	G3, T4	SM331
<i>Actinocorallia</i> , <i>Actinomadura</i> , <i>Saccharothrix</i> or <i>Spirillospora</i>	G3, T5	YPC2

2.3.3 Antibacterial testing

Antibacterial testing was performed on the 42 selected isolates that were stab inoculated into YEME, SM3, Middlebrook 7H9 and MC (Modified Czapek) media. YEME and SM3 were commonly used in the maintenance and subculturing of actinobacterial isolates, with Middlebrook 7H9 and MC as defined media commonly used in the testing of antibiotic production in our lab. As the focus of this study was to isolate rarer non-*Streptomyces* actinobacteria, the non-*Streptomyces* isolates were also the focus of the antibacterial screening, together with the inclusion of selected *Streptomyces* strains. The *Streptomyces* strains were selected based on different colony morphologies, to increase the likelihood that they were different species.

For the *M. aurum* A+ overlay results, a total of five of the *Streptomyces* strains were selected, which showed antibacterial activity with zones of inhibition greater than 1000 mm². Similarly, seven non-*Streptomyces* strains showed zones of inhibition of approximately 1000 mm² or greater. The non-pathogenic, fast-growing *M. aurum* A+ was used as the test bacterium as it displays a similar antibiotic-susceptibility profile to the slow-growing, pathogenic *M. tuberculosis* (Chung *et al.* 1995). The zones of inhibition of the isolates, and relative degree of activity can be seen in Table 2.7. Most of the antibacterial activity was seen on YEME and SM3 plates.

Only two isolates (YM63 and SE22, both identified as *Streptomyces* species) showed antibacterial activity when grown on Middlebrook 7H9 and MC, with the greatest overall, very strong antibacterial activity shown on Middlebrook 7H9 by isolate SE22 (4778 mm²).

Table 2.7 Zones of inhibition of isolates against *M. aurum* on selected media (non-*Streptomyces* indicated in orange)

Isolate	YEME Zone of inhibition (mm ²)	Relative degree of activity	7H9 Zone of inhibition (mm ²)	Relative degree of activity	MC Zone of inhibition (mm ²)	Relative degree of activity	SM3 Zone of inhibition (mm ²)	Relative degree of activity
YM46	548	Weak	0	-	0	-	1913	Moderate
YM53	0	-	0	-	0	-	2777	Strong
YM55	2211	Strong	0	-	0	-	3918	Very strong
YM63	0	-	1781	Moderate	1508	Moderate	1760	Moderate
YMH1	1102	Moderate	0	-	0	-	1461	Moderate
YPC1	1230	Moderate	0	-	0	-	0	-
YPL1	2579	Strong	0	-	0	-	3393	Very strong
SC1	1696	Moderate	0	-	0	-	104	Weak
SE17	980	Weak	0	-	0	-	0	-
SE22	3019	Very strong	4778	Very strong	2640	Strong	3631	Very strong
SM3BL1	989	Weak	0	-	0	-	679	Weak
SM338	990	Weak	0	-	0	-	985	Weak

The 12 isolates in Table 2.7 were subjected to further testing for their antibacterial activity against *S. aureus* (Gram positive) and *E. coli* (Gram negative) to determine the specificity of the antibiotics produced. None of the isolates showed antibacterial activity against *E. coli*, suggesting that the antibiotics produced are specific for Gram positives. Only strains SE22 and YMH1 showed antibacterial activity against *S. aureus*, with SE22 showing very strong activity on all media tested, and YMH1 showing activity only on YEME, designated as strong (Table 2.8).

Table 2.8 Zones of inhibition of isolates against *S. aureus* on selected media

Isolate	YEME (mm ²)	Degree of activity	7H9 (mm ²)	Degree of activity	MC (mm ²)	Degree of activity	7H9 (mm ²)	Degree of activity
YMH1	2350	Strong	0	None	0	None	0	None
SE22	5000+	Very strong	4000	Very strong	3217	Very strong	5000+	Very strong

2.3.4 Antibiotic biosynthetic potential

PCR screening of isolates with antibiotic biosynthetic primers is useful in identifying the antibiotic-producing potential of an isolate as well as potentially identifying the class/classes of antibiotics produced by a particular isolate. This may allow for the application of the correct extraction and purification methods for that particular type/class of antibiotic (Wood *et al.* 2007).

The 12 isolates were all screened for the presence of ansamycin, glycopeptide and aromatic polyketide antibiotic genes (Table 2.9).

Table 2.9 Detection of antibiotic biosynthetic genes (non-*Streptomyces* in orange)

Isolate	ANSA	ARO-PKS	oxyB
SC1	-	+	-
SE17	+	+	-
SE22	+	-	-
SM3BL1	+	-	-
SM338	+	+	-
YM46	-	+	-
YM63	-	+	-
YM53	-	-	-
YM55	-	-	-
YPL1	-	-	-
YMH1	-	+	-
YPC1	+	+	-

ANSA, AHBA synthase gene; **ARO-PKS**, KS_{α} - KS_{β} gene pair; **oxyB**, *oxyB* gene

Five isolates showed a positive result when screened for the AHBA synthase gene (Table 2.9; Figure 2.1 which indicates a positive band for three of the five isolates showing a positive result), with seven isolates being positive for the KS_{α} - KS_{β} polyketide synthase gene pair (Table 2.9). The product of the AHBA synthase gene is important in ansamycin biosynthesis, while the KS_{α} - KS_{β} gene products are involved in the elongation of aromatic polyketides. It is important to note that the ARO-PKS primers may also amplify fragments of the KS_{α} - KS_{β} gene pair involved in the synthesis of spore pigments (Wood *et al.* 2007).

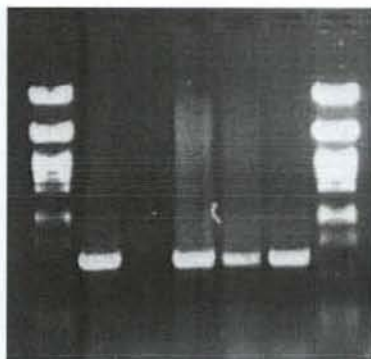


Figure 2.1 Agarose gel photograph indicating a positive band of 491 bp for the AHBA synthase gene. Lane 1, λ *Pst*I DNA marker (kb); lane 2, *Amycolatopsis* strain JS72 (positive control); lane 3, isolate YM46; lane 4, isolate SM3BL1; lane 5, isolate SM338; lane 6, isolate YPC1; lane 7, λ *Pst*I DNA marker (kb).

No amplification was achieved with the Foxy/Roxy primers used to amplify genes important in glycopeptide antibiotic biosynthesis, suggesting that glycopeptides are not produced by these strains. It must be noted, however, that a negative result may be attributed to false negatives, whereby the biosynthetic genes may be present, but may not be amplified in any of the PCR screening tests. This may be attributed to a lack of primer binding, which can arise due to variations in primer target sequences (Wood *et al.* 2007).

Three isolates (YM53, YM55 and YPL1, which are all *Kribbella* strains – see section 3.3) did not yield any PCR products with the selected primers, which may indicate one of two things. Firstly, the theory of “false negatives” may be applied. Alternatively, the antibiotics produced by these isolates may belong to other classes that were not screened for in this study, and may even belong to novel structural classes. These isolates and their antibiotics are worth pursuing as they may be the most interesting (Wood *et al.* 2007).

A total of six isolates was selected for further analysis. These were the strongest overall antibiotic producer, SE22, two endophytes YPC1 and YMH1, as well as YM53, YM55 and YPL1 that showed strong to very strong activity only against *M. aurum* A+, indicating the potential specificity of the antibiotics produced to mycobacteria.

The antibiotic biosynthetic genes obtained for three of the six isolates, SE22, YMH1 and YPC1, were sequenced and subjected to phylogenetic analysis. The relatedness of the antibiotic biosynthetic sequences amplified from SE22 and YPC1 to known ansamycin and aromatic polyketide producers is demonstrated in the phylogenetic trees (Figures 2.2 and 2.3). The ANSA gene products of SE22 and YPC1 group with known producers of ansamycin antibiotics, as confirmed by the PCR amplified gene product. This suggests that the isolates SE22 and YPC1 may have the potential to produce ansamycin-type antibiotics. The gene product of the ARO-PKS primers of isolate YPC1 groups with known antibiotic producers. However, the YMH1 gene product grouped with the spore pigment genes of *Streptomyces avermitilis* and *Streptomyces collinus*, indicating that the primers amplified genes for a spore pigment rather than an aromatic polyketide antibiotic. One must note that the presence of these genes does not necessarily guarantee that the isolates do, in fact, produce these classes of antibiotics, as there is no indication that the genes are actually expressed *in vivo*, nor that the full set of biosynthetic genes required to produce the particular class of antibiotic is present (Wood *et al.* 2007).

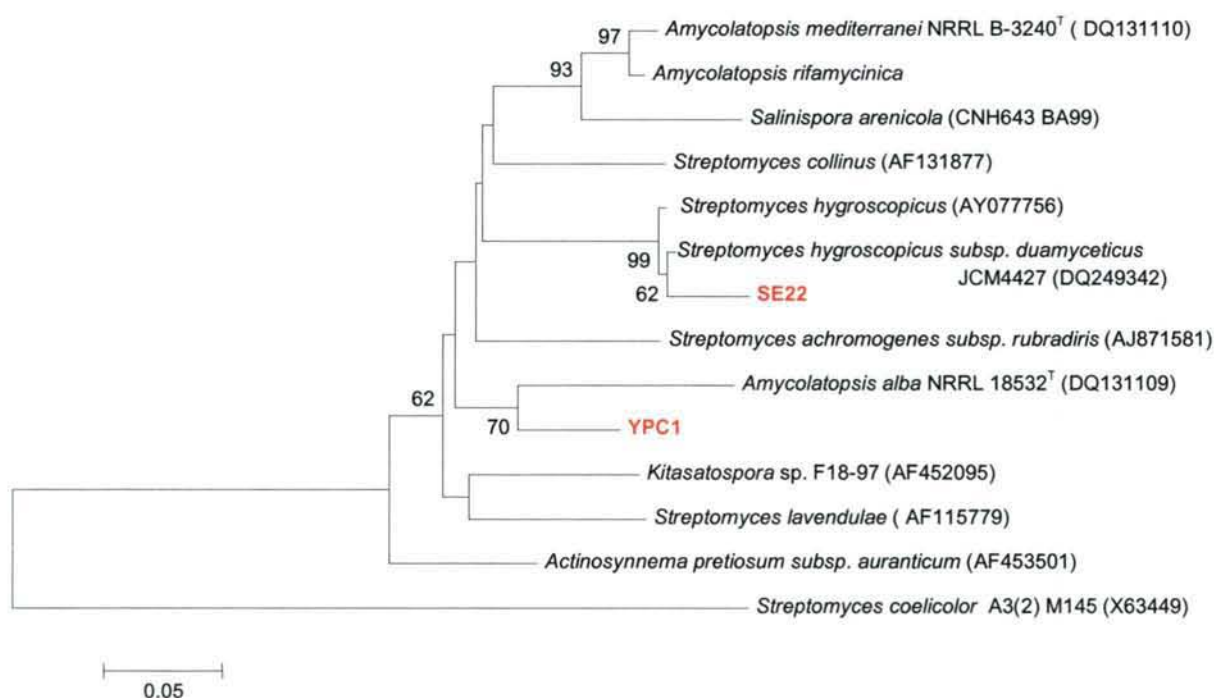


Figure 2.2 Neighbour-joining phylogenetic tree constructed from 507 bp of AHBA synthase gene sequences with species known to produce ansamycins. Isolates from this study are indicated in red. Bootstrap values above 40% are indicated at the nodes, calculated from 1000 resampled datasets. The bar represents 5 nucleotide substitutions per 100 nucleotides.

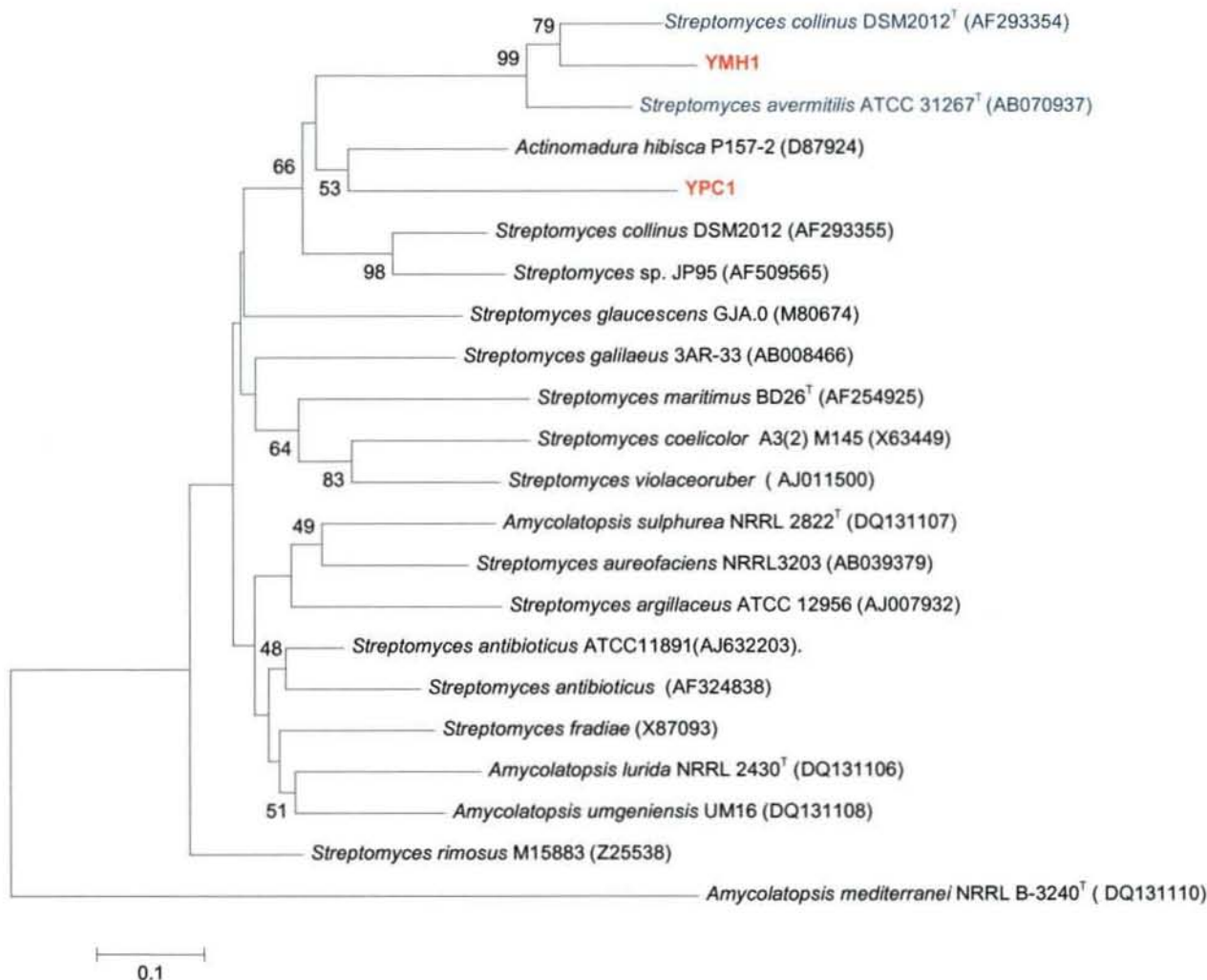


Figure 2.3 Neighbour-joining phylogenetic tree of strains containing the KS_{α} : KS_{β} polyketide synthase gene fragment. The tree was constructed with 313 bp of sequence. Species containing the spore pigment gene fragment are indicated in blue. Isolates from this study are indicated in red. Bootstrap values above 40% are indicated at the nodes, calculated from 1000 resampled datasets. The scale represents 10 nucleotide substitutions per 100 nucleotides.

2.3.5 Antibiotic extraction

Isolates SE22, YPC1, YMH1, YM53, YM55 and YPL1 were subjected to the antibiotic extraction methods described after growth in SM3, YEME or both (determined from the overlay experiments – section 2.3.3). Both the culture filtrate as well as the cell mass of each isolate were tested in order to ascertain whether the antibiotics are found within the cells, or are excreted into the surrounding medium. After extraction with the specified solvents, only isolates

SE22 (grown in YEME) and YM55 (grown in SM3) showed active spots on a *M. aurum* A+ bioautography plate (Figure 2.4). Isolate SE22 showed active spots for all the solvents used (including methanol used for the cell mass) as well as in the freeze dried sample. This indicates the likelihood of multiple active compounds being produced by isolate SE22, which are found both within the cells as well as excreted into the surrounding medium. Isolate YM55 only showed an active spot when the culture filtrate was extracted with ethyl acetate. This indicates that the antibiotic(s) produced is/are excreted into the cell broth.

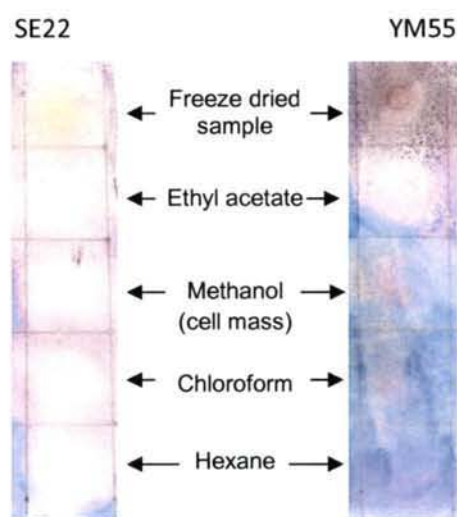


Figure 2.4 Bioautography results showing active spots against *M. aurum* A+ of isolates SE22 and YM55 culture filtrates extracted with the indicated solvents.

The concentrated ethyl acetate, chloroform, hexane and methanol extracts of strain SE22, as well as the concentrated ethyl acetate extract of strain YM55 were subjected to TLC with various solvent systems, to help determine the system to use for antibiotic purification by silica-gel column chromatography. The optimal solvent system should show an R_f value of about 0.5 for the active spot, allowing the separation of the spot from many other compounds present in the extract.

For isolate SE22, all extracts were subjected to the same solvent systems. This helps to identify potentially identical antibiotics that may have been extracted in all the solvents. The results of chromatography with two solvent systems, namely 80% (v/v) chloroform : 20% (v/v) acetone, and 70% (v/v) chloroform : 30% (v/v) acetone, showed that a number of antibiotics are produced by isolate SE22. A difficulty was presented by the number of antibiotics present in all the solvent extracts, as can be seen by the "merging" of active spots (Figure 2.5). A minimum of four antibiotics is produced by isolate SE22. Multiple solvent systems could not separate the antibiotics.

For isolate YM55, the optimal solvent system determined was that of 70% (v/v) chloroform : 30% (v/v) acetone, as can be seen in Figure 2.5. This system revealed one active spot with an R_f value of approximately 0.7. This indicates that the antibiotic identified by the active spot is moderately non-polar. Attempts to lower the R_f value to 0.5 could not be performed as the sample lost activity after a few days of storage at -20 °C.

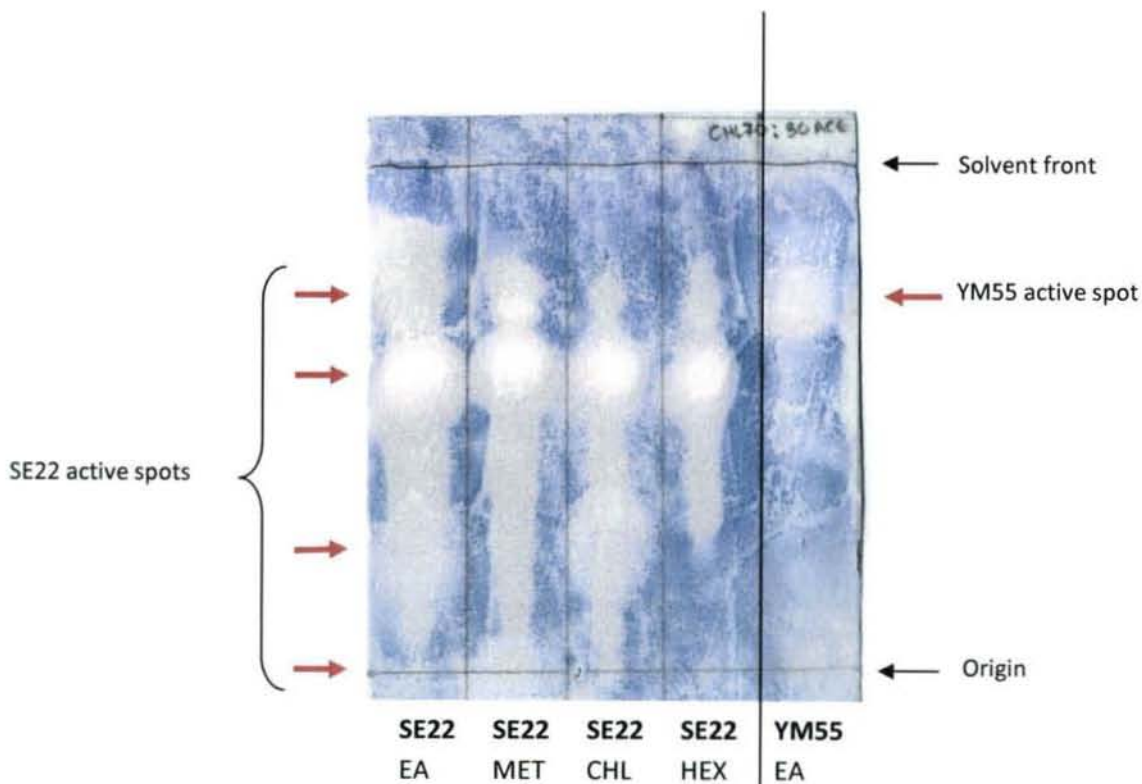


Figure 2.5 TLC bioautography results for solvent extracts (EA – ethyl acetate, MET – methanol (cell mass), CHL – chloroform, HEX – hexane) of isolates SE22 and YM55 (indicated in bold) showing the migration of the active spots followed by testing against *M. aurum* A+. Red arrows indicate the positions of various active spots on the TLC plate. The solvent system used was 70 chloroform : 30 acetone.

The lack of detectable antibiotic activity from isolates YM53, YPL1, YPC1 and YMH1 extracted in various solvents, as well as in the culture filtrate may be attributed to a lack of antibiotic production in liquid culture. If antibiotics were produced in the liquid media, they may have been unstable at low temperatures, with the freeze-drying of the sample resulting in a loss of activity of the antibiotic(s) produced.

2.4 Conclusion

A total of 28 non-*Streptomyces* actinobacteria was isolated from the soil, plant and seaweed samples. The non-*Streptomyces* are often considered to be rarer amongst the actinobacteria, hence a focus was placed on further characterising some of these isolates in the hope that they are more likely to be novel. The majority of the isolates was obtained from the soil sample on YEME and SM3 media, indicating the ease of isolating actinobacteria from soil environments. The five endophytic isolates together with the two seaweed isolates are of particular interest, as it is hypothesised that these actinobacteria have an increased potential of being novel due to their unusual isolation sources.

A selected number of the non-*Streptomyces* species showed good antibiotic activity against *M. aurum* A+, together with selected *Streptomyces* strains. All the antibiotic producing isolates were tested for their antibiotic biosynthetic potential (Table 2.9), with only 3 isolates (YM53, YM55 and YPL1 – identified as *Kribbella* species in Chapter 3.3) failing to amplify gene products with any of the primers used. Isolates SE17, SE22 (*Streptomyces* species), SM3BL1, SM338 and YPC1 were positive for the AHBA synthase gene, while isolates SC1, YM46, YM63, YMH1 (*Streptomyces* species), SE17, SM338 and YPC1 were positive for the KS_{α} - KS_{β} gene pair. Six isolates were selected for further antibiotic work, including YPC1, YMH1 and SE22, as well as the three *Kribbella* isolates that showed strong to very strong antibiotic activity against *M. aurum* A+, but from which no antibiotic biosynthetic gene products could be amplified. Phylogenetic analyses of the antibiotic biosynthetic genes of YPC1 and SE22 showed their grouping with

other known antibiotic producers, however, isolate YMH1 was shown to contain a spore pigment gene, as is evident from the grouping in Figure 2.2.

Antibiotic extraction was successful for two of six isolates, SE22 and YM55, however more work is required to determine the best solvent systems for antibiotic separation of isolate SE22. Attempts could be made to partially purify the antibiotic produced by isolate YM55 with the determined solvent system, on a silica column.

The results obtained in this part of the study influenced the selection of the number of isolates which were subjected to characterisation studies via a polyphasic approach, as can be seen in chapter 3.

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Chapter 3

Characterisation of Isolated Actinobacteria

3.1 Introduction

In this study, the identification of isolates was achieved by a polyphasic approach. The identification of selected actinobacterial strains to the genus level was first achieved through the analysis of their 16S rRNA gene sequences. Phylogenetic trees were constructed based on nearly complete 16S rRNA gene sequences, resulting in the identification of each isolate's closest phylogenetic neighbours. Determined phenetic characteristics (morphological, physiological and chemotaxonomic) were then used to allow for the differentiation of the isolated strains from their closest phylogenetic neighbours, aiding in determining whether they belong to novel species.

A 16S rRNA gene sequence similarity of less than 97% was suggested to comfortably distinguish between bacterial species (Stackebrandt & Goebel 1994). However, this threshold value was recently adjusted to 98.7% 16S rRNA gene sequence similarity (Stackebrandt & Ebers 2006). In essence, sequence similarities of greater than 98.7% require the differentiation between species to be demonstrated through DNA-DNA hybridisation (DDH) studies. As such, strains showing less than 70% DNA relatedness by DDH analysis (*and* which exhibit phenotypic differences) belong to distinct genomic species (Wayne *et al.* 1987).

A potential molecular alternative to the DDH studies required to distinguish closely related species, is the sequencing of house-keeping genes, which commonly code for proteins that are essential for metabolism. An advantage to using house-keeping genes is that their sequences change faster than the highly conserved 16S-rRNA gene, but slowly enough to allow for their use in phylogenetic studies. One such gene, the *gyrB* gene, has been used for phylogenetic

studies of a number of actinobacterial genera, including *Amycolatopsis*, *Gordonia*, *Kribbella*, *Micromonospora*, *Nocardiopsis*, and *Nocardia* (Everest & Meyers 2009, Le Roes *et al.* 2008, Shen *et al.* 2006, Kirby *et al.* 2010, Kasai *et al.* 2000, Yang *et al.* 2008, Takeda *et al.* 2010). The *gyrB* gene encodes the β -subunit of DNA gyrase, which regulates double stranded DNA supercoiling, and has been proposed to provide greater resolution and clarity in distinguishing between closely related strains than the 16S rRNA gene (Yamamoto & Harayama 1995). A study by Kasai *et al.* (2000) showed that *gyrB* based phylogeny is consistent with DDH studies, and as such may be a useful alternative to DDH.

The aim of this part of the study was to identify 15 selected actinobacterial isolates and determine if they belong to new species. Two previously isolated *Microbispora* strains, SM359 and TJ5, were included in the study. Additionally, *gyrB* gene phylogeny was used to aid in determining whether isolates SM359 and TJ5 represent new species in the genus *Microbispora*.

3.2 Materials and Methods

The selection of the 15 isolates used in this study was based on a combination of three factors, namely, the source of their isolation, their preliminary identification to non-*Streptomyces* genera and their antibiotic activity. All isolates obtained from plant or seaweed samples were subjected to further study. The isolate showing the greatest antibiotic activity against *M. aurum* A+ (strain SE22, preliminarily identified as a streptomycete) was also included.

3.2.1 Morphological and physiological testing

The identification of selected actinobacterial isolates was achieved through direct observation of the colour of aerial and substrate mycelia on inorganic salts-starch (ISP 4) agar (or, if growth was not sufficient on ISP 4 plates, on YEME or SM3 plates), and the production of diffusible pigments on glycerol-asparagine (ISP 5) agar incubated at 30°C for 14 days (Shirling & Gottlieb 1966). Spore chain morphology and spore surface ornamentation were determined by scanning electron microscopy of growth from 14 day old ISP 4 plates, unless otherwise indicated. The production of melanin was observed on peptone yeast extract iron agar (ISP 6) and tyrosine

agar (ISP 7) plates incubated at 30°C for 4 days (Locci 1989). ISP media were prepared as described by Shirling & Gottlieb (1966).

Selected physiological tests were performed on all isolates. These included: degradation of adenine, allantoin, casein (autoclaved at 121°C for 10 minutes), gelatin, guanine, hypoxanthine, starch, L-tyrosine, urea (Gordon *et al.* 1974), xanthine and xylan; hydrolysis of aesculin, arbutin and pectin (from citrus fruit rind); nitrate reduction and production of H₂S; growth at 20°C, 30°C, 37°C and 45°C, growth at pH 4.3, pH 5, pH 7 and pH 9, and growth in the presence of 2%, 3%, 4%, 5%, 7% and 10% (w/v) NaCl as recommended by Locci (1989). All physiological test plates were incubated for the specified number of days at 30°C (Shirling & Gottlieb 1966), except for plates testing for growth at 20°C, 37°C and 45°C.

All sole carbon source utilisation was determined at a final carbon source concentration of 1% (w/v), except for sodium acetate, sodium butyrate, sodium citrate and sodium succinate which were determined at a final concentration of 0.1% (v/w), as recommended by Shirling & Gottlieb (1966). All sole nitrogen source utilisation was determined at a final nitrogen source concentration of 0.1% (w/v) as recommended by Locci (1989). All carbon and nitrogen sources were filter sterilized before being added to the basal media.

Morphological and physiological tests (excluding sole carbon and nitrogen source utilisation) were performed for all species of the genus *Microbispora*, excluding *Microbispora siamensis*, grown under the same conditions. Tests were carried out concurrently with *Microbispora* isolates SM359 and TJ5 (isolated in 2007 by the author and Ms Tsungai Jongwe, respectively, as part of their B.Sc. (Honours) research projects) to allow the data to be compared. The *Microbispora* species studied are indicated in Table 3.1.

Table 3.1 Strain numbers and sources of *Microbispora* reference species used in this study

Strain	Source
<i>Microbispora corallina</i> DSM 44682 ^T	DSMZ
<i>Microbispora mesophila</i> NRRL B-16986 ^T	USDA ARS Culture Collection
<i>Microbispora rosea</i> subsp. <i>aerata</i> DSM 43176 ^T	DSMZ
<i>Microbispora rosea</i> subsp. <i>aerata</i> DSM 43166 (previously <i>Microbispora thermodiastatica</i>)	DSMZ
<i>Microbispora rosea</i> subsp. <i>aerata</i> DSM 43840 (previously <i>Microbispora thermorosea</i>)	DSMZ
<i>Microbispora amethystogenes</i> DSM 43164 ^T	DSMZ
<i>Microbispora rosea</i> subsp. <i>rosea</i> NRRL D-2634 (previously <i>Microbispora chromogenes</i>)	USDA ARS Culture Collection
<i>Microbispora rosea</i> subsp. <i>rosea</i> NRRL B-2630 (previously <i>Microbispora diastatica</i>)	USDA ARS Culture Collection
<i>Microbispora rosea</i> subsp. <i>rosea</i> JCM 8971 (previously <i>Microbispora indica</i>)	JCM
<i>Microbispora rosea</i> subsp. <i>rosea</i> JCM 8972 (previously <i>Microbispora karnatakensis</i>)	JCM
<i>Microbispora rosea</i> subsp. <i>rosea</i> NRRL B-2629 (previously <i>Microbispora parva</i>)	USDA ARS Culture Collection
<i>Microbispora rosea</i> subsp. <i>rosea</i> NRRL B-2632 ^T	USDA ARS Culture Collection

DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen, **JCM** - Japan Collection of Microorganisms, **USDA ARS** – U.S. Department of Agriculture Agricultural Research Service.

3.2.2 Chemotaxonomy

Whole cell sugar patterns and cell wall chemotypes were determined by the methods of Hasegawa *et al.* (1983), with the exception that freeze dried cells were used instead of colonies from plates (10 mg for DAP isomer determination, 100 mg for whole cell sugar patterns). A solvent system of ethyl acetate:pyridine:distilled water (100:35:25, v/v) was used for whole cell sugar pattern analysis.

3.2.3 PCR amplification and DNA sequencing

Genomic DNA was extracted from the selected isolates using the method described by Wang *et al.* (1996) – see section 2.2.2.

3.2.3.1 16S rRNA gene sequencing

The amplification of the 16S rRNA gene sequences of isolates and the electrophoresis of the PCR products were as described in section 2.2.2.1. PCR products were purified using the Invitex MSB® Spin PCRapace PCR purification kit (Germany) and sequencing was performed as a service by Macrogen Incorporated (Seoul, Korea) and the University of Stellenbosch (South Africa) sequencing unit using the dye termination method. Sequencing was performed using the F1, R1, F3, R3, F5 and R5 universal bacterial 16S rRNA gene primers. At least two overlapping sequences for each part of the gene were used to allow for the confirmation of each almost full length 16S rRNA gene sequence. All gene sequences were edited in Chromas (Version 2.01, Technelysium, 1999) and analysed using DNAMAN (version 4.13, Lynnon Biosoft, 1999).

3.2.3.2 *gyrB* gene sequencing

PCR amplification of the DNA-gyrase subunit B gene (*gyrB*) was achieved for *Microbispora* strains SM359 and TJ5.

The amplification of the *gyrB* gene sequences was performed in 50 µl reaction volumes containing the following: 2 µl template DNA (400-500 ng/µl), 0.5 U *Taq* polymerase (Super-Therm, JMR Holdings, USA), 4 mM MgCl₂, 150 µM of each dNTP and 0.5 µM of each primer. The PCR reactions were performed in a Techne TC-512 thermal cycler. The PCR cycling program was as follows: initial denaturation for 2 minutes at 96°C followed by: 30 cycles of denaturation for 45 seconds at 96°C, annealing for 30 seconds at 56°C and extension for 2 minutes at 72°C; with a final extension for 5 minutes at 72°C. PCR products were electrophoresed on 0.8% agarose gels containing 0.8 µg/ml ethidium bromide, along with a molecular mass marker (*Pst*I digest of phage λ DNA), and visualised on a GelDoc XR system at 254 nm (Bio-Rad Laboratories Inc.). PCR products were purified and sequenced as mentioned above. Sequencing was performed using the primers listed in Table 3.2.

Table 3.2 List of primers used to amplify the *gyrB* gene of *Microbispora* strains SM359 and TJ5

Primer	Sequence	Reference
GgyrB-F1	5' – CAR GAR ATG GCN TTC YTS AAC AAG – 3'	Le Roes <i>et al.</i> 2008
GgyrB-F2	5' – CAG TTC GAG GGH CAG ACS AAG AC – 3'	Le Roes <i>et al.</i> 2008
GgyrB-R1	5' – GTT CCA YTG CAT SGC SAB CTC - 3'	Le Roes <i>et al.</i> 2008
7G-gyrB-F	5' – GTI CGY AWV CGI CCS GGH ATG TAC – 3'	Le Roes <i>et al.</i> 2008
7G-gyrB-R	5' – CCG TCV ACR TCR GCR TCS GCC ATS – 3'	Everest & Meyers 2009
KgyrB-R1892	5' – CCS AGR CCC TTG WAG CGC TGG – 3'	Kirby <i>et al.</i> 2010
I = Inosine; S= C or G; Y= C or T; W = A or T; H = A, C or T, V = A, C or G; B = C, G or T		

3.2.4 Phylogenetic analysis

Consensus 16S rRNA and *gyrB* gene sequences were subjected to Basic Local Alignment Search Tool (BLAST) comparisons with other sequences in the GenBank database, using the BLASTn search tool (Altschul *et al.* 1997). The neighbour-joining method (Saitou & Nei 1987) was used in the construction of phylogenetic trees using MEGA (version 4.0, Tamura *et al.* 2007) from genetic-distance matrices calculated using the Kimura 2-parameter model. Phylogenetic trees of the 16S rRNA gene sequences were constructed with the 30 most similar sequences of species with validly published names (Euzéby 2010) for the *Actinomadura*, *Micromonospora*, *Nocardia* and *Streptomyces* isolates. All species with validly published names in the genera *Kineococcus* (including the *Kineosporia* species), *Kribbella*, *Microbispora* (including species previously known by different names) and *Nonomuraea* were used in constructing phylogenetic trees. The phylogenetic tree for *Verrucosipora* strain YM76 was constructed with representative members, including all type species, of all the genera in the family *Micromonosporaceae*.

3.3 Results and Discussion

A total of 15 isolates was selected for identification to the genus level and phenetic characterisation. Two *Microbispora* isolates (TJ5 and SM359) obtained as part of two B.Sc. (Honours) research projects in 2007 were also included in this study. The 15 isolates were identified to the genus level using BLAST analysis against the GenBank database (see Table 3.3). Physiological characteristics for all strains are indicated in Table 3.4 and sole carbon and nitrogen source utilisation are indicated in Table 3.5.

Table 3.3 Identification of isolates to the genus level

Isolate	Genus
YM53, YM55, YPL1	<i>Kribbella</i>
SM3BL1, SM338, YM48, YPC1	<i>Micromonospora</i>
YMH1, SE22, SC1	<i>Streptomyces</i>
SM359, TJ5	<i>Microbispora</i>
SM331	<i>Nocardia</i>
YPC2	<i>Actinomadura</i>
SM343	<i>Nonomuraea</i>
Y2UE1	<i>Kineococcus</i>
YM76	<i>Verrucosispora/ Micromonospora</i>

Table 3.4 Physiological characteristics of isolates used in this study

Genera: 1, *Nocardia*; 2, *Kineococcus*; 3, *Micromonospora*; 4, *Verrucosipora*; 5, *Kribbella*; 6, *Streptomyces*; 7, *Microbispora*; 8, *Nonomuraea*; 9, *Actinomadura*. +++, very strong positive; ++, strong positive; +, positive; +(w), weak positive; - negative; NG, no growth; ND, not determined.

	SM331	Y2UE1	SM3BL1	SM338	YM48	YPC1	YM76	YM53	YM55	YPL1	SC1	SE22	YMH1	SM359	TJ5	SM343	YPC2
Genus:	1	2	3			4	5			6			7		8	9	
Pectin hydrolysis	-	-	-	-	-	-	+(w)	-	-	-	-	+	+	-	-	-	-
H ₂ S production	+	-	+	+	+	+	+	-	+	+	+	-	+	+	+	-	-
Nitrate reduction	+	-	+	+	-	-	-	+(w)	+	+	+	-	-	+(w)	+	-	+(w)
ISP#5 (pigments)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ISP#6 (melanin)	-	-	-	-	-	-	-	-	-	-	-	NG	-	-	NG	-	NG
ISP#7 (melanin)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Degradation/Hydrolysis of:																	
Adenine	NG	-	-	-	-	-	-	NG	+	+	-	+	-	-	-	+	-
Aesculin	+	+	+	+	+	+	+(w)	+	+	+	+	+(w)	+	+	+	+	+
Allantoin	-	-	-	-	-	-	-	-	+	+(w)	-	NG	-	NG	-	-	-
Arbutin	+	+	+	+	+	+	+	-	+(w)	+(w)	+	+	+	+	+	+	+(w)
Casein	+	+	+(w)	+	+	+	+	+	+	+	+	+	+	+(w)	+	+	+
Gelatin	+(w)	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Guanine	-	-	-	-	-	-	-	-	-	+(w)	-	-	+	-	-	-	-
Hypoxanthine	-	-	-	-	-	-	-	+	+	+	-	+	+	-	+(w)	-	-
L-Tyrosine	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+(w)	+	+
Starch	-	-	+	+	+	+	+	+(w)	+(w)	+(w)	-	+	+	-	-	-	+
Tween 80	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Xanthine	-	-	-	-	-	-	-	+(w)	+	+	-	-	+	-	-	-	-
Xylan	-	-	-	-	-	NG	-	-	-	-	+(w)	-	-	-	-	-	-
Urea	+	-	-	-	-	-	-	NG	+	+(w)	+(w)	NG	-	-	-	-	-
Growth at:																	
20°C	++	+++	+	++	+	++	-	+	++	+	+++	+	+	ND	ND	+	+(w)
30°C	+++	+++	++	+++	++	+++	+++	+++	+++	+++	++	+++	+++	++	+++	+++	+++
37°C	+	++	+++	+++	+++	++	-	-	++	++	+	++	+++	++	+++	++	++
45°C	-	-	-	++	-	-	-	-	-	-	+(w)	-	++	ND	ND	-	-
2% NaCl	+++	+++	++	+++	++	+	++	++	+++	++	++	++	+	+	+++	+	++
3% NaCl	++	++	+	++	+	-	+	-	++	+++	+++	++	++	+	+	+(w)	+
4% NaCl	+	++	+(w)	+	-	-	-	-	++	++	++	+	++	+(w)	+(w)	-	-
5% NaCl	+(w)	+	+(w)	+(w)	-	-	-	-	-	+	+	+(w)	+	-	-	-	-
7% NaCl	-	+(w)	-	-	-	-	-	ND	ND	ND	+	-	+(w)	ND	ND	-	-
10% NaCl	-	-	-	-	-	-	-	ND	ND	ND	-	-	+(w)	ND	ND	-	-
pH 4.3	+(w)	++	-	+	-	+(w)	-	-	+++	+++	+(w)	+(w)	+	+(w)	+(w)	-	+(w)
pH 5	+	+++	+	+++	+	++	-	++	+++	+++	+	++	+++	ND	ND	+(w)	+
pH 7	++	+++	++	+++	++	+++	+++	++	+++	+++	++	+++	+++	+	+++	+++	+++
pH 9	+++	++	++	+++	+++	+	+	+++	+++	+++	+++	++	+++	++	+++	++	++

Table 3.5 Sole carbon and nitrogen utilisation results

Genera: 1, *Nocardia*; 2, *Micromonospora*; 3, *Kribbella*; 4, *Streptomyces*; 5, *Microbispora*; 6, *Nonomuraea*; 7, *Actinomadura*. ++, strong positive; +, positive; +/-, utilisation doubtful; -, negative

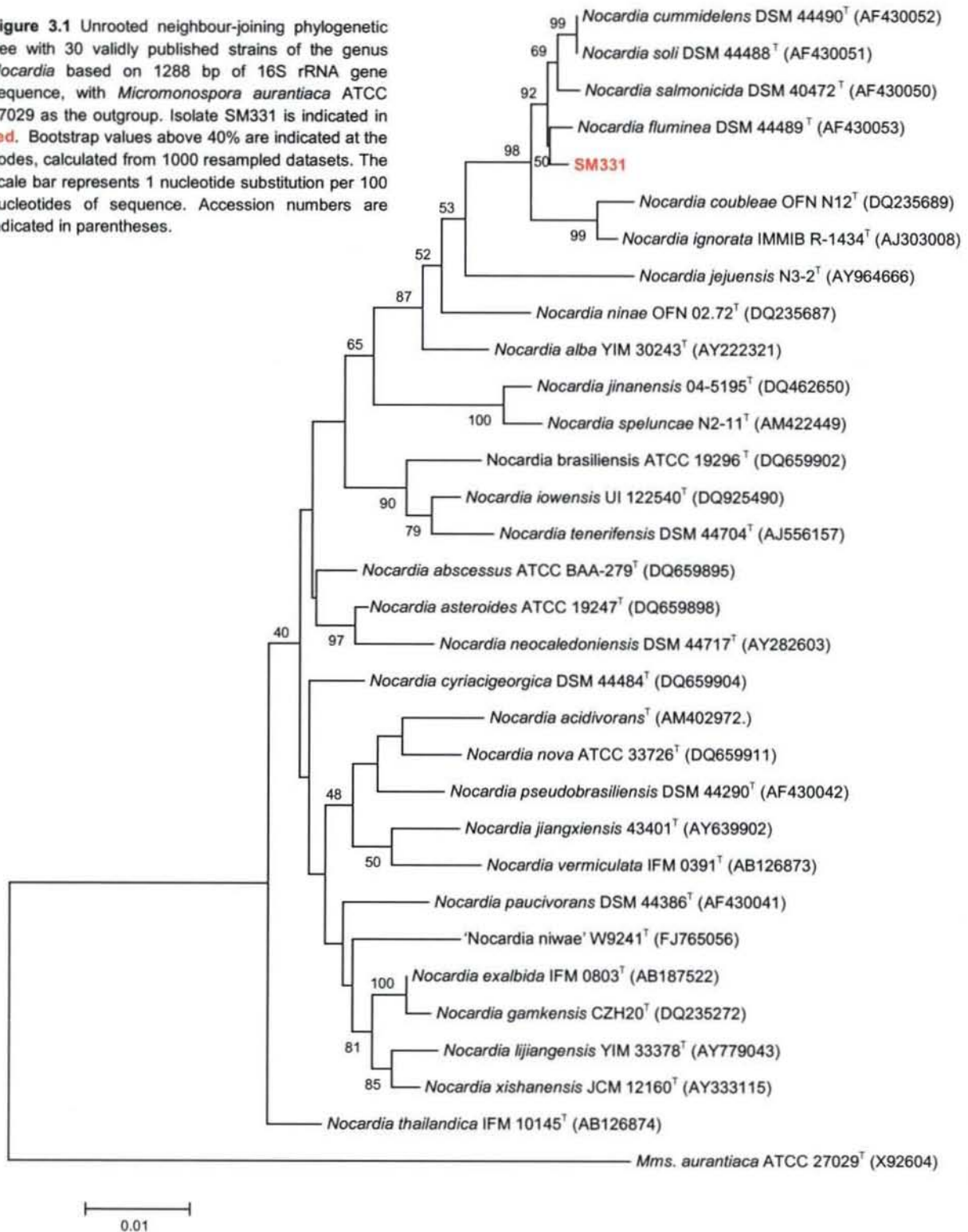
	SM331	SM3BL1	SM338	YM48	YPC1	YM53	YM55	YPL1	SC1	SE22	YMH1	SM359	TJ5	SM343	YPC2
Carbon Sources:	1	2			3			4		5		6	7		
Adonitol	-	+/-	+	-	-	++	++	++	-	-	-	-	+	+/-	++
L(+)-Arabinose	-	+	++	+	-	++	++	++	++	++	++	+	++	+	++
D(+)-Cellobiose	-	++	++	++	++	++	++	++	++	++	++	+	+	+	++
Dulcitol	-	-	+	-	-	+	-	-	-	+/-	-	+	-	++	-
D(-)-Fructose	+	+	++	++	+	+	++	++	++	-	++	++	++	+	++
D(+)-Galactose	-	+	++	++	+/-	++	++	++	++	++	++	++	++	++	-
D(+)-Glucose	++	++	++	++	++	++	++	++	+	+	++	++	++	++	++
Glycerol	++	-	+	-	-	+	+	++	++	++	++	-	+	++	+
myo-Inositol	-	-	+/-	-	-	++	++	++	++	++	++	-	-	++	+
Inulin	-	-	-	-	-	-	++	-	-	-	-	-	-	+	-
α-Lactose	-	+	++	++	-	+	++	++	++	++	+	+	++	-	+/-
Maltose	-	+	++	+	++	++	++	++	++	++	++	-	-	+	+
D(-)-Mannitol	-	+/-	+	++	-	++	++	+	++	++	++	+	++	++	++
D(+)-Mannose	-	++	++	+	++	++	++	++	+	++	++	-	-	++	++
D(+)-Melezitose	-	-	-	+/-	-	++	++	++	-	-	-	+	-	+	+
Melibiose	-	-	++	++	-	++	++	++	-	++	+/-	+	-	+	-
Propionic acid	+/-	-	+	+	+	+/-	+/-	+/-	+	+/-	+	-	-	-	-
Raffinose	-	-	++	+	-	++	++	++	+/-	++	-	+	++	++	-
L(+)-Rhamnose	-	+/-	+	-	++	++	++	++	++	++	++	-	-	+	++
Ribose	-	-	+	-	-	+	++	++	++	-	++	+	+	-	+
D(-)-Salicin	+	-	++	+/-	+	+	+	+	+	+	++	+	+	+	-
Sodium acetate	+/-	-	+/-	+	+	+	-	-	+	+/-	+	+	++	++	-
Sodium butyrate	+	-	+	+/-	+/-	+	+	+	+/-	+	+/-	+	+	-	-
Sodium citrate	+	-	+	-	-	+	-	-	+	+/-	+	+	+/-	+	-
Sodium succinate	+	+/-	+	+	-	+	-	-	+	+	+	++	++	+	-
D(-)-Sorbitol	-	+/-	+	-	-	-	-	-	-	+	-	-	++	+	-
Sucrose	-	++	++	+	++	++	++	++	-	-	+	-	+	++	++
Trehalose	-	+	++	+	++	++	++	+	++	+	++	-	+	++	+
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D(+)-Xylose	-	++	+	++	++	+	++	++	++	+	++	++	+	+	++
Nitrogen Sources:															
DL-α-amino-n-butyric acid	-	-	+/-	-	-	-	+/-	+	+/-	-	-	+/-	+	+	-
L-Arginine	+/-	++	++	++	+	+	++	+/-	++	+	+	++	++	+/-	++
L-Asparagine	++	++	++	++	+	++	++	++	++	++	++	++	++	++	++
L-Cysteine	-	-	+	-	-	-	+/-	+	+	-	-	-	+	-	+
L-Histidine	++	++	+	+/-	++	++	+	++	++	++	++	+	++	+	++
L-Hydroxyproline	-	-	+	-	-	+/-	++	+	+/-	+	-	+	-	-	-
Methionine	-	-	++	+	+/-	++	+	+	+/-	+/-	+/-	+	+	+	-
L-Phenylalanine	+/-	-	+	+	+/-	+/-	++	+	+	++	+/-	+	-	+/-	-
Potassium nitrate	+/-	-	-	++	-	++	+	++	+	++	++	++	++	+	-
L-Threonine	-	-	++	-	+/-	+/-	++	+	++	+/-	++	+	++	+	-
L-Serine	-	+	++	+/-	+/-	+	+	+	++	-	++	+	+	-	+/-
L-Valine	+	-	+	++	+/-	-	+	+	++	+	+/-	+	+	-	+/-

3.3.1 Suborder *Corynebacterineae*

3.3.1.1 *Nocardia* strain SM331

Nocardia strain SM331 was isolated from the soil sample obtained from the University of Cape Town. Colonies of strain SM331 grown on ISP 2 appeared raised and furry upon sporulation. The substrate mycelium was cream to light orange in colour, with white aerial mycelium on ISP 2. Strain SM331 produced light brown and pink pigments on ISP 6 and ISP 7, respectively, with no pigments produced on ISP 5. *Nocardia* strain SM331 was shown to contain meso-DAP with arabinose and galactose detected in whole cell-hydrolysates. A BLAST search of the GenBank database revealed the 1374 bp 16S rRNA gene sequence to have 99% sequence similarity to *Nocardia cummidelens* R89^T, *Nocardia fluminea* DSM 44489^T, *Nocardia soli* DSM 44488^T and *Nocardia salmonicida* DSM 40472^T. Pairwise sequence comparisons of strain SM331 in DNAMAN showed sequence similarities of 99.2% with *N. cummidelens*, *N. fluminea* and *N. soli*, and 99.05% with *N. salmonicida*. The closest phylogenetic neighbour, however, was determined to be *N. fluminea* (50% bootstrap support, Figure 3.1). *Nocardia* strain SM331 differs from *N. fluminea* in the following tests: positive for urea hydrolysis and degradation of casein, with negative results for hydrolysis of allantoin, degradation of tyrosine and Tween 80, and utilisation of L(+)-rhamnose (Table 3.4 and 3.5, Maldonado *et al.* 2000). Despite the physiological differences highlighted, the high sequence similarity between strain SM331 and *N. fluminea* will require DNA-DNA hybridisation to be performed to determine whether strain SM331 belongs to a distinct genomic species.

Figure 3.1 Unrooted neighbour-joining phylogenetic tree with 30 validly published strains of the genus *Nocardia* based on 1288 bp of 16S rRNA gene sequence, with *Micromonospora aurantiaca* ATCC 27029 as the outgroup. Isolate SM331 is indicated in red. Bootstrap values above 40% are indicated at the nodes, calculated from 1000 resampled datasets. The scale bar represents 1 nucleotide substitution per 100 nucleotides of sequence. Accession numbers are indicated in parentheses.



3.3.2 Suborder *Kineosporiineae*

3.3.2.1 *Kineococcus* strain Y2UE1

Kineococcus strain Y2UE1 was isolated from the surface of the seaweed *Bifurcariopsis capensis* obtained in Simon's Town, Cape Town, South Africa. Immature colonies were orange and appeared grainy on ISP 4, with mature colonies becoming smooth to mucoid. *Kineococcus* strain Y2UE1 did not sporulate on any of the media tested. No pigments were produced on ISP 5. The whole-cell hydrolysates were shown to contain *meso*-DAP and the sugars galactose, glucose, arabinose and ribose were present. A 1489 bp 16S rRNA gene sequence was used to perform a BLAST search, which showed 98% sequence similarity of strain Y2UE1 to *Kineococcus rhizosphaerae* RP-B16^T, *Kineococcus aurantiacus* IFO 15268^T and *Kineococcus radiotolerans* SRS 30216^T, with sequence similarities determined in DNAMAN to be 99.15%, 98.24% and 98.33%, respectively. The closest phylogenetic neighbour was confirmed in the neighbour-joining tree (Figure 3.2), which showed strain Y2UE1 grouping with *K. rhizosphaerae* with very high bootstrap support (96%).

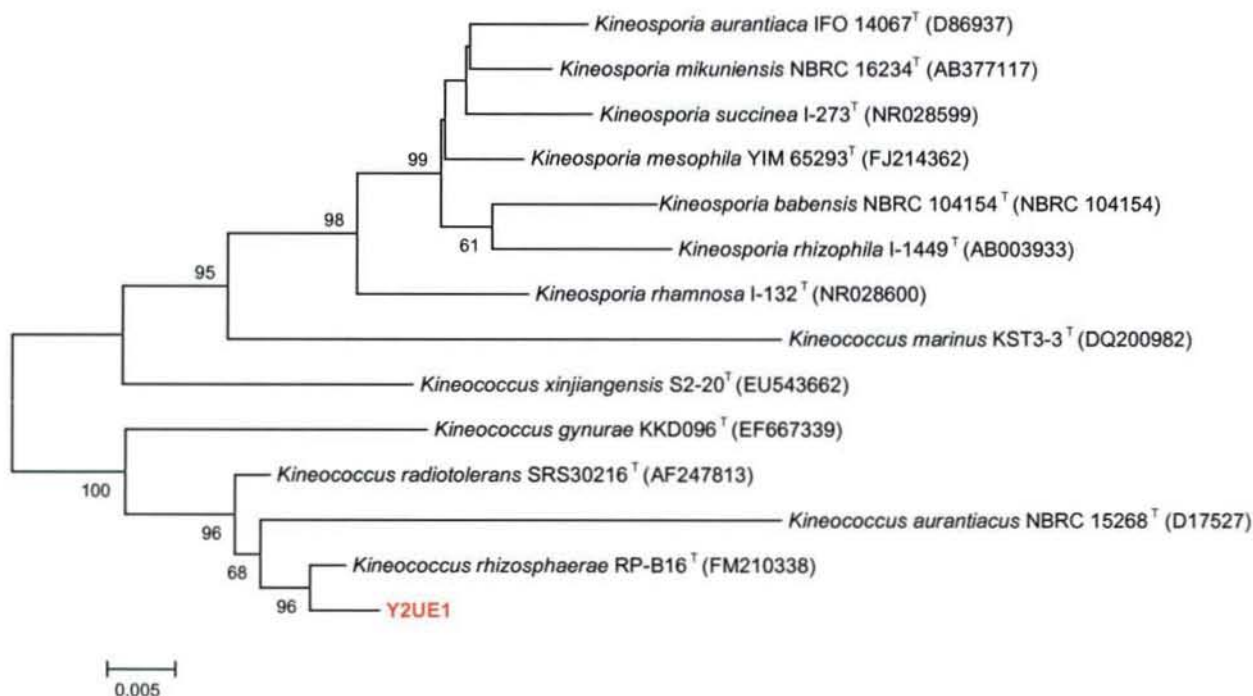


Figure 3.2 Unrooted neighbour-joining phylogenetic tree based on 1305 bp of 16S rRNA gene sequence showing all species of the genera *Kineococcus* and *Kineosporia* with validly published names. Isolate Y2UE1 is indicated in red. Bootstrap values above 40% are indicated at the nodes, calculated from 1000 resampled datasets. The scale bar represents 5 nucleotide substitutions per 1000 nucleotides of sequence. Accession numbers are indicated in parentheses.

Kineococcus strain Y2UE1 can be distinguished from *K. rhizosphaerae* by the hydrolysis of casein, and its inability to hydrolyse gelatin and urea (Table 3.4, Lee 2009). *Kineococcus* strain Y2UE1 did not grow well enough on sole carbon or sole nitrogen source media to allow for definitive results to be obtained. Due to the high sequence similarity between strain Y2UE1 and *K. rhizosphaerae*, DNA-DNA hybridisation would be required in order to ascertain whether strain Y2UE1 is a new species. Furthermore, the sole carbon and sole nitrogen source utilisation tests will have to be repeated, with modifications to the media to include a small percentage (< 0.1% (w/v)) of a nutrient rich ingredient (such as yeast extract) to encourage the growth of the isolate and allow for differentiation of the strength of growth on the media.

3.3.3 Suborder Micromonosporineae

3.3.3.1 *Micromonospora* strain SM3BL1

Micromonospora strain SM3BL1 was isolated as an endophyte from the leaves of the South African tree *Bolusanthus speciosus* obtained from a private garden in Plumstead, Cape Town. *Micromonospora* strain SM3BL1 was shown have weak antimicrobial activity against *M. aurum* A+ (Chapter 2, Table 2.7). The colonies of strain SM3BL1 were typically raised and wrinkled, and orange in colour on ISP 4. Mature colonies became black and mucoid upon sporulation. *Micromonospora* strain SM3BL1 did not produce any pigments on ISP 5. Figure 3.3(a) shows characteristic single spores on short sporophores, with a warty to blunt spiny spore surface ornamentation, when grown on Bennett medium (pH 9). Strain SM3BL1 was shown to contain meso-DAP with only the sugars xylose and glucose detected in the whole-cell hydrolysates. A BLAST search of the 1496 bp 16S rRNA gene sequence of *Micromonospora* strain SM3BL1 showed 99% sequence similarity to *Micromonospora echinospora* ATCC 15837^T in the GenBank database. The pairwise sequence alignment in DNAMAN showed 99.64% sequence similarity between *Mms. echinospora* ATCC 15837^T and strain SM3BL1. The closest phylogenetic neighbour was confirmed by the grouping of strain SM3BL1 with the type strain of *Mms. echinospora* with moderate bootstrap support (66%) in the neighbour-joining tree (Figure 3.4). *Micromonospora* strain SM3BL1 differs from *Mms. echinospora* in a number of tests, including: positive for nitrate reduction, H₂S production, degradation of L-tyrosine, and the utilisation of D(-)-fructose and α-lactose as sole carbon sources (Tables 3.4 and 3.5, Kirby & Meyers 2010). Given the high 16S rRNA gene sequence similarity, DNA-DNA hybridisation will

be required in order to determine whether strain SM3BL1 belongs to a novel *Micromonospora* species.

3.3.3.2 *Micromonospora* strain SM338

Micromonospora strain SM338 was isolated from a soil sample from the University of Cape Town, South Africa. *Micromonospora* strain SM338 showed weak antimicrobial activity against *M. aurum* A+ (Chapter 2, Table 2.7). The colonies of strain SM338 were orange in colour and appeared raised and wrinkled in appearance on ISP 4. No sporulation occurred on ISP 4, however when sporulation occurred on other test media, the surfaces of the colonies became mucoid and black in colour. No pigments were produced on ISP 5, however a brown pigment was produced on L-tyrosine plates. Figure 3.3(b) shows the moderate sporulation with smooth, rounded spores of strain SM338 when grown on Bennett medium (pH 7). The cell wall of strain SM338 was shown to contain *meso*-DAP, with only the sugar xylose being detected. A BLAST search of the 1390 bp 16S rRNA gene sequence of strain SM338 showed 99% sequence similarity to *Micromonospora aurantiaca* DSM 43813^T and *Micromonospora marina* JSM1-1^T. The pairwise sequence alignments performed in DNAMAN showed that strain SM338 has 99.71% and 99.64% sequence similarity with *Mms. aurantiaca* and *Mms. marina*, respectively. The closest phylogenetic neighbour was confirmed to be *Mms. aurantiaca* (shown in Figure 3.4) with very high bootstrap support (96%). *Micromonospora* strain SM338 differs from *Mms. aurantiaca* in a number of tests, including: starch hydrolysis, growth at 45°C, and the utilisation of glycerol, α -lactose, D(-)-mannitol, L(+)-rhamnose and D(+)-ribose as sole carbon sources (Tables 3.4 and 3.5, Kirby & Meyers 2010). Even though there are a number of phenetic differences between *Mms. aurantiaca* and strain SM338, the high 16S rRNA gene sequence similarity of 99.71% between them would require that DNA-DNA hybridisation be performed in order to determine whether *Micromonospora* strain SM338 belongs to a new species.

3.3.3.3 *Micromonospora* strain YM48

Micromonospora strain YM48 was isolated from the soil sample obtained from the University of Cape Town. Strain YM48 showed moderate antimicrobial activity against *M. aurum* A+ (Chapter 2, Table 2.7). *Micromonospora* strain YM48 had raised and convoluted/wrinkled colonies, which were orange in colour on ISP 2. YM48 did not sporulate on ISP4, however

when very sparse sporulation was observed on Bennett medium, the spore mass had a typically mucoid appearance, and was black in colour. No spores were detected by scanning electron microscopy (Figure 3.3(c)) when grown on Bennett's medium at 30°C. No pigments were produced on ISP 5, however a brown/red pigment was produced on Bennett's medium when grown at 30°C and 37°C, as well as on L-tyrosine degradation plates. *Micromonospora* strain YM48 was shown to contain meso-DAP with xylose and glucose detected in the whole cell hydrolysates. The BLAST search of the 1298 bp of 16S rRNA gene sequence against the GenBank database showed *Micromonospora* strain YM48 to have 99% sequence similarity to *Micromonospora saelicesensis* Lupac 09^T. A pairwise sequence alignment performed in DNAMAN showed a sequence similarity of 99.38%. The closest phylogenetic neighbour was confirmed to be *Mms. saelicesensis* with very high bootstrap support (93%; Figure 3.4). *Micromonospora* strain YM48 differs from *Mms. saelicesensis* in a number of physiological tests: YM48 is positive for starch hydrolysis, utilisation of trehalose and sucrose as sole carbon sources, utilisation of L-valine as a sole nitrogen source, and does not utilize myo-inositol as a sole carbon source (Tables 3.4 and 3.5, Trujillo *et al.* 2007). Although strain YM48 can be distinguished from *Mms. saelicesensis* in a number of phenotypic tests, DNA-DNA hybridisation would be required to determine whether strain YM48 belongs to a novel species.

3.3.3.4 *Micromonospora* strain YPC1

Micromonospora strain YPC1 was isolated as an endophyte from the leaves of the South African plant *Pelargonium cucullatum* obtained from Devil's Peak, Table Mountain National Park, Cape Town. Strain YPC1 showed moderate antimicrobial activity against *M. aurum* A+ (Chapter 2, Table 2.7). Colonies appeared raised and convoluted/wrinkled, with orange substrate mycelium on ISP 4. Mature colonies became mucoid and black in colour upon sporulation. Strain YPC1 did not produce any pigments. Figures 3.3(d) and 3.3(e) shows characteristic single spherical spores of strain YPC1 borne on short sporophores, with a warty spore surface, when grown on Bennett medium (pH 7). The cell wall of YPC1 contains meso-DAP and glucose, xylose, ribose and arabinose as the sugar constituents. A BLAST search in the GenBank database of the 1366 bp 16S rRNA gene sequence showed *Micromonospora peucetia* DSM 43363^T and *Micromonospora auratinigra* TT1-11^T to have the greatest sequence similarity of 99% to *Micromonospora* strain YPC1. Pairwise sequence alignments in DNAMAN (version 4.13, Lynnon Biosoft, 1999) showed a sequence similarity to YPC1 of 99.71% and

98.9% for *Mms. peucetia* and *Mms. auratinigra* respectively. The closest phylogenetic neighbour is confirmed in the neighbour-joining tree in Figure 3.4, where YPC1 groups with *Mms. peucetia* with 100% bootstrap support.

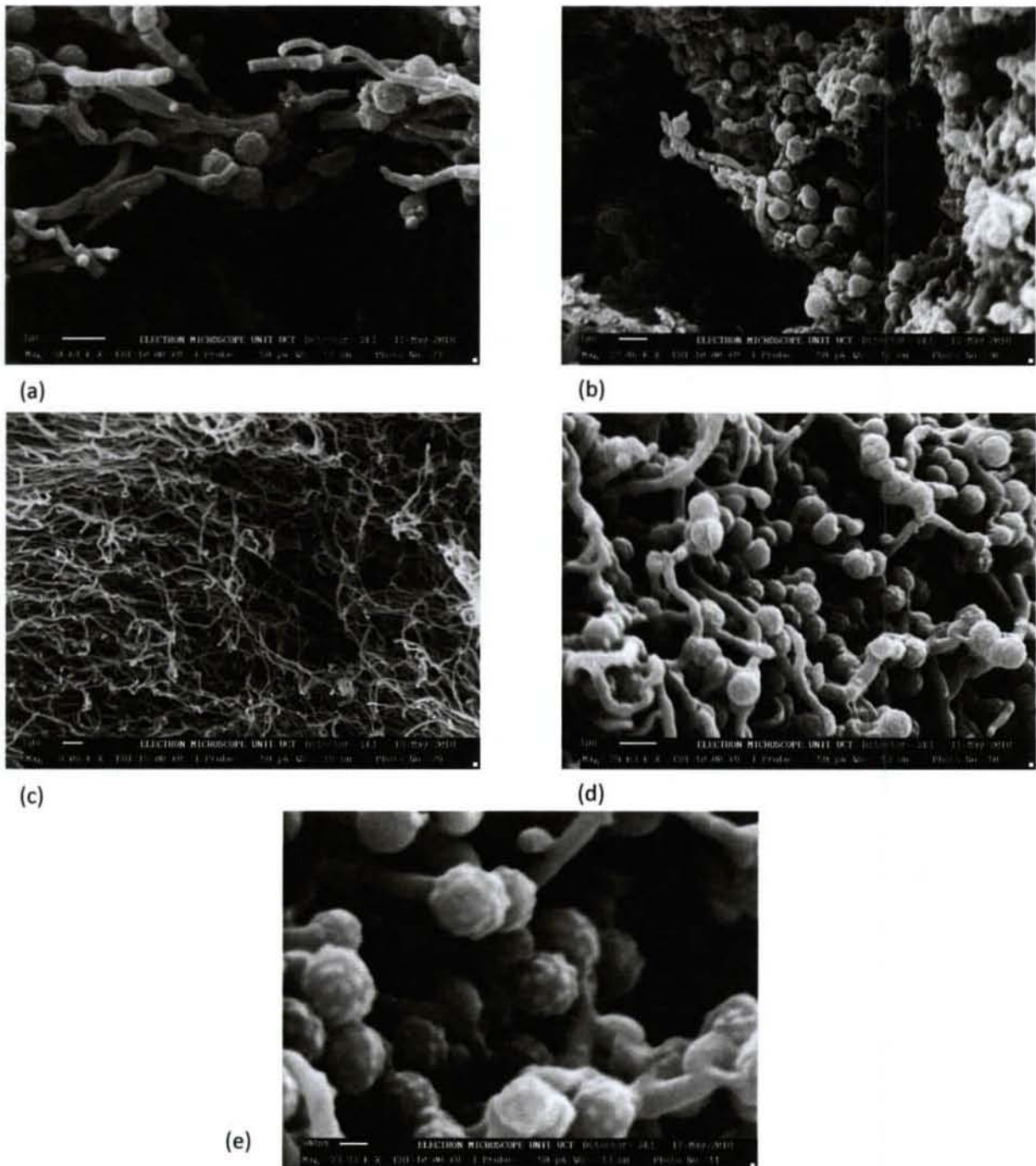
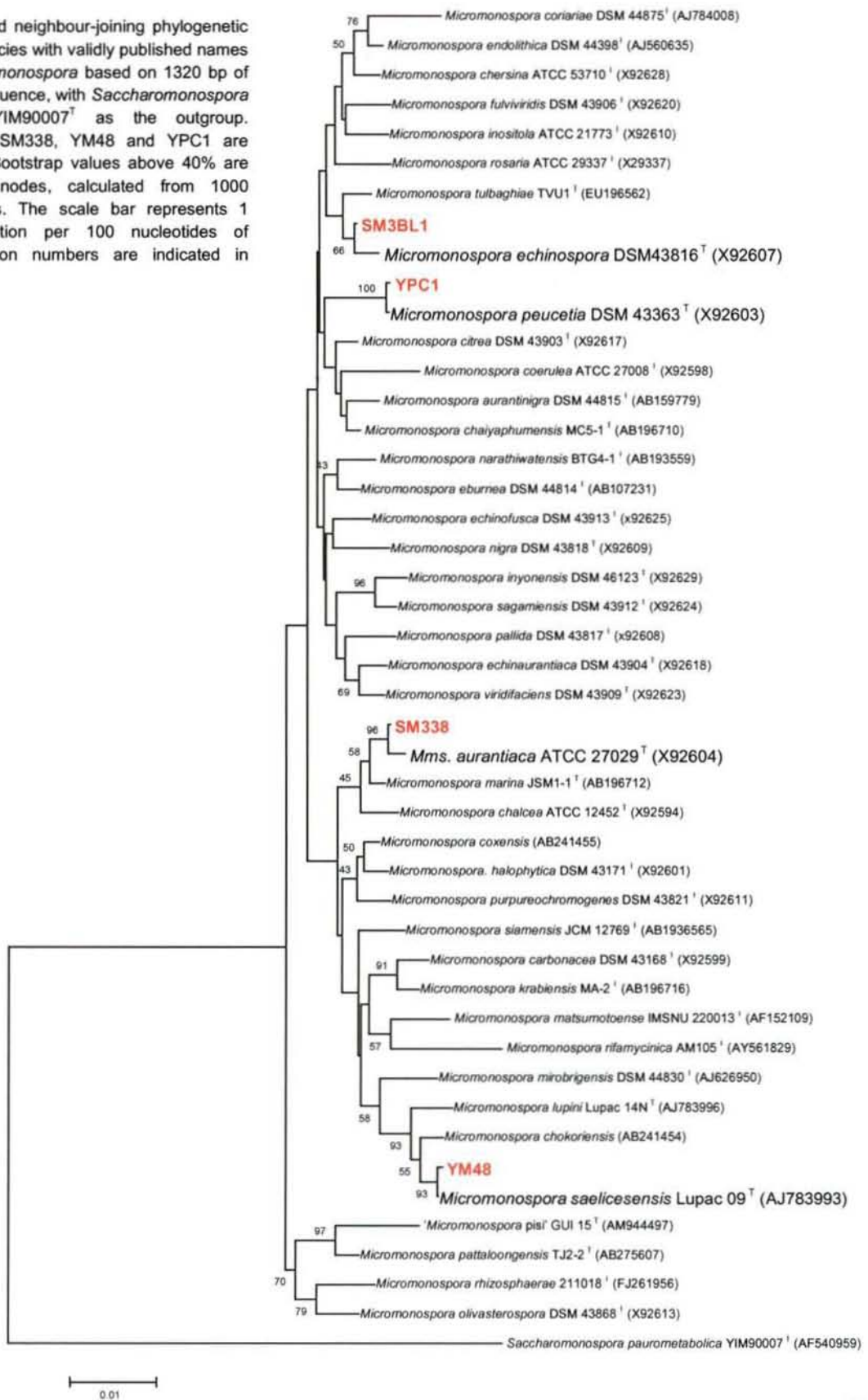


Figure 3.3 Scanning electron micrographs of *Micromonospora* strains (a) SM3BL1 (scale 1 μ m), (b) SM338 (scale 1 μ m), (c) YM48 (scale 2 μ m), (d) YPC1 (scale 1 μ m) and (e) YPC1 (scale 300 nm).

Figure 3.4 Unrooted neighbour-joining phylogenetic tree showing all species with validly published names in the genus *Micromonospora* based on 1320 bp of 16S rRNA gene sequence, with *Saccharomonospora paurometabolica* YIM90007^T as the outgroup. Isolates SM3BL1, SM338, YM48 and YPC1 are indicated in **red**. Bootstrap values above 40% are indicated at the nodes, calculated from 1000 resampled datasets. The scale bar represents 1 nucleotide substitution per 100 nucleotides of sequence. Accession numbers are indicated in parentheses.

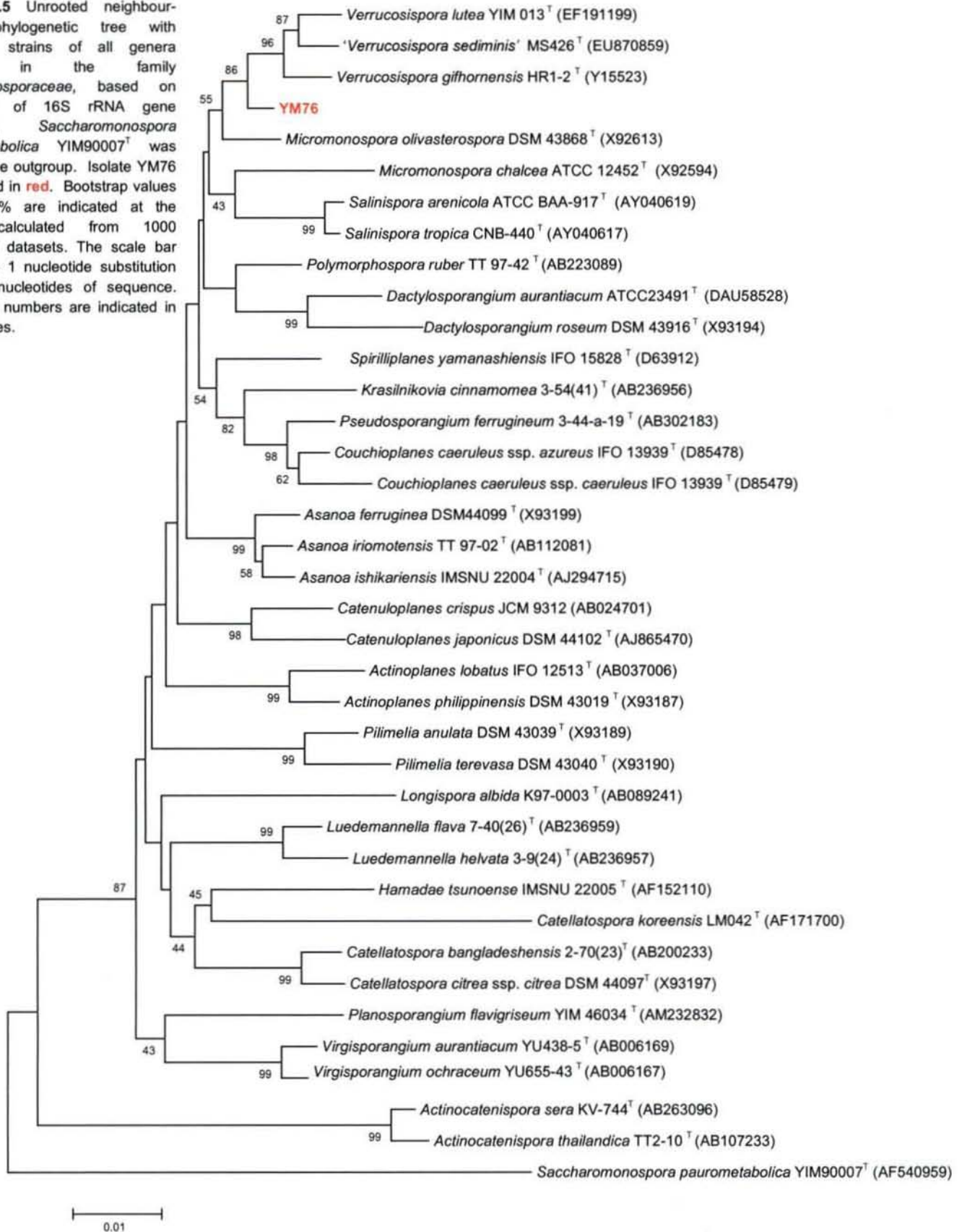


Micromonospora strain YPC1 can be distinguished from *Mms. peucetia* with respect to the colour of substrate mycelium on ISP 4, which is orange for strain YPC1 and bottle green for *Mms. peucetia*, and strain YPC1 is able to utilise D(+)-xylose and L(+)-rhamnose as sole carbon sources (Tables 3.4 and 3.5, Kroppenstedt *et al.* 2005). The high sequence similarity between strain YPC1 and *Mms. peucetia* would require DNA-DNA hybridisation to be performed to determine whether YPC1 is a novel species of the genus *Micromonospora*.

3.3.3.5 *Verrucosispora* strain YM76

Strain YM76 was isolated from the soil sample obtained from the University of Cape Town. Colonies of strain YM76 were raised and wrinkled on all media tested. The substrate mycelium was light orange in colour on ISP 4. The colony surface became brown and slightly mucoid upon sporulation. *Verrucosispora* strain YM76 did not produce any diffusible pigments on ISP 5. The whole cell hydrolysates of strain YM76 contained *meso*-DAP and glycine, with xylose, mannose, glucose and ribose detected in the whole-cell sugar analysis. The BLAST analysis of a 1334 bp 16S rRNA gene sequence indicated that strain YM76 had the greatest sequence similarity (98%) to *Micromonospora olivasterospora* DSM 43868^T, and 98% sequence similarity to *Verrucosispora gifhornensis* DSM 44337^T. The sequence similarities determined in DNAMAN between strain YM76 and the type strains of *M. olivasterospora* and *V. gifhornensis* were 98.8% and 98.72%, respectively. Strain YM76 was shown to form an outgroup of the *Verrucosispora* clade with good bootstrap support (86%) in the neighbour-joining phylogenetic tree (Figure 3.5). Strain YM76 can be assigned to the genus *Verrucosispora* based on the phylogenetic and chemotaxonomic analyses (the sugar analysis revealed a lack of arabinose, which is a characteristic sugar of the genus *Micromonospora*). The 16S rRNA gene sequence similarities between strain YM76 and *Verrucosispora sediminis* MS 426^T and *Verrucosispora lutea* YIM 013^T were 98.65% and 98.2%, respectively, which allows YM76 to be distinguished as a separate species from *V. sediminis* and *V. lutea* according to Stackebrandt & Ebers (2006). Isolate YM76 did not grow on any of the sole carbon and sole nitrogen sources plates, and there were no differences found between strain YM76 and the few physiological characteristics published for *V. gifhornensis*. In order to definitively determine the novelty of strain YM76, DNA-DNA hybridisation experiments should be performed between strain YM76 and *V. gifhornensis*, as well as obtaining definitive results for the sole carbon and sole nitrogen tests, with modifications to media to include trace amounts (< 0.1% (w/v)) of a nutrient rich ingredient (such as yeast extract).

Figure 3.5 Unrooted neighbour-joining phylogenetic tree with published strains of all genera present in the family *Micromonosporaceae*, based on 1350 bp of 16S rRNA gene sequence. *Saccharomonospora paurometabolica* YIM90007^T was used as the outgroup. Isolate YM76 is indicated in red. Bootstrap values above 40% are indicated at the nodes, calculated from 1000 resampled datasets. The scale bar represents 1 nucleotide substitution per 100 nucleotides of sequence. Accession numbers are indicated in parentheses.



3.3.4 Suborder *Propionibacterineae*

3.3.4.1 *Kribbella* strain YM53

Kribbella strain YM53 was isolated from the soil sample obtained at the University of Cape Town. Colonies were raised on agar plates and appeared convoluted and wrinkled. Fragmentation occurred in liquid media. The substrate mycelium appeared cream in colour, with white aerial mycelium on ISP 4. *Kribbella* isolate YM53 had strong antibiotic activity against *M. aurum* A+ (Chapter 2, Table 2.7). YM53 produced no diffusible pigments. Whole cell hydrolysates were shown to contain LL-DAP. The sequence similarity between strain YM53 and all members of the genus *Kribbella* with validly published names was shown to be the highest for *Kribbella hippodromi* S1.4^T and *Kribbella sandramycini* ATCC 39419^T at 99.02%, followed by 98.86% sequence similarity to *Kribbella sancticallisti* BC 633^T. In the phylogenetic tree (Figure 3.6), however, strain YM53 grouped with *K. sancticallisti*, but with low bootstrap support (<40%). Physiological differences distinguishing strain YM53 from *K. sancticallisti* include the utilisation of *myo*-inositol as a sole carbon source, no growth at 37°C or in the presence of up to 3% (w/v) NaCl, no hydrolysis of arbutin and no melanin production on ISP 7 (Table 3.4 and 3.5, Urzi *et al.* 2008). Even though a number of differences have been identified between strain YM53 and *K. sancticallisti*, in order to determine the novelty of strain YM53, it is recommended that DNA-DNA hybridisation experiments be performed with type strains having 16S rRNA gene sequence similarities of greater than 98.7% to strain YM53 (this includes the strains identified above, plus *Kribbella karoonsensis* Q41^T, *Kribbella flavida* DSM 17836^T and *Kribbella solani* DSA1^T).

3.3.4.2 *Kribbella* strain YM55

Kribbella strain YM55 was isolated from the soil sample obtained from the University of Cape Town. Colonies were typically raised and convoluted/wrinkled on agar plates. The substrate mycelium appeared cream in colour, with abundant white aerial mycelium on ISP 4. *Kribbella* strain YM55 was shown to have very strong antibiotic activity against *M. aurum* A+ (Chapter 2, Table 2.7). No diffusible pigments were produced by strain YM55 on ISP 5. The whole cell hydrolysates showed the presence of LL-DAP in the cell walls. When compared to sequences of *Kribbella* type strains with validly published names, *K. hippodromi* S1.4^T was shown to have the greatest sequence similarity (99.2%), followed by *Kribbella aluminosa* HKI 0478^T with 99.1%

sequence similarity. The closest phylogenetic neighbour (Figure 3.6) was shown to be *Kribbella swartbergensis* HMC25^T, with *K. aluminosa* and *Kribbella jejuensis* HD9^T forming part of the same clade. *Kribbella* isolate YM55 has 99% and 98.9% 16S rRNA gene sequence similarity to *K. swartbergensis* and *K. jejuensis*, respectively.

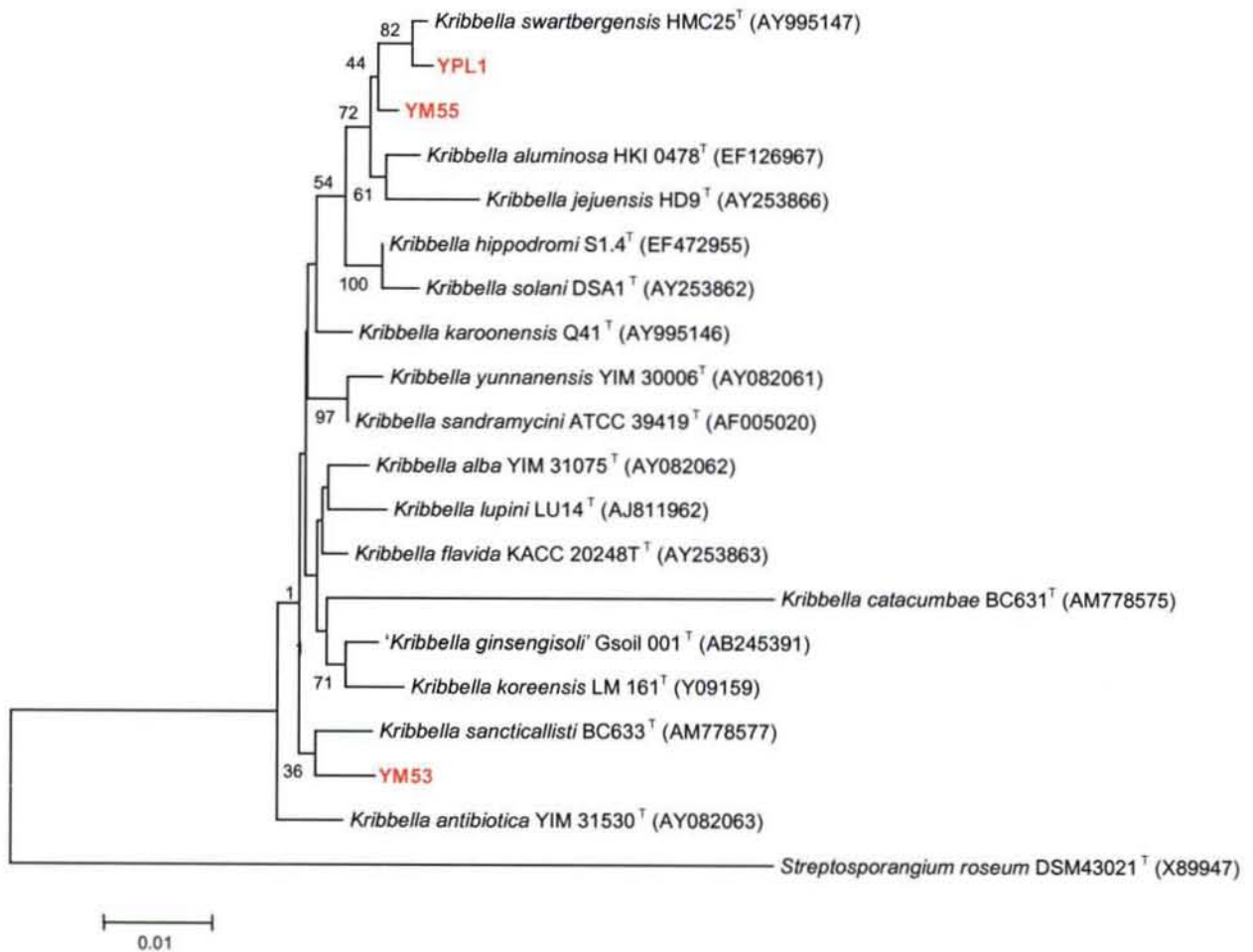


Figure 3.6 Unrooted neighbour-joining phylogenetic tree based on 1240 bp of 16S rRNA gene sequence showing all species of the genus *Kribbella* with validly published names. *Streptosporangium roseum* DSM43021^T was used as the outgroup. Isolates YM53, YM55 and YPL1 are indicated in red. Bootstrap values above 40% are indicated at the nodes, calculated from 1000 resampled datasets. The scale bar represents 1 nucleotide substitution per 100 nucleotides of sequence. Accession numbers are indicated in parentheses.

Kribbella strain YM55 differs from *K. swartbergensis* in the following tests: positive for the utilisation of inulin, negative for the utilization of sorbitol, sodium acetate and sodium succinate, as well as negative for growth at 45°C. Similarly *Kribbella* isolate YM55 can be distinguished from *K. jejuensis* in the following tests: positive for nitrate reduction, hypoxanthine, Tween 80 and L-tyrosine degradation, starch hydrolysis, growth at pH 9 and in the presence of 2-4% (w/v) NaCl (Urzi *et al.* 2008). Only one difference could be determined between *K. aluminosa* and strain YM55, where strain YM55 is positive for nitrate reduction (Table 3.4 and 3.5, Urzi *et al.* 2008, Kirby *et al.* 2006). The high sequence similarity requires that DNA-DNA hybridisation studies be performed between *Kribbella* strain YM55 and its closest phylogenetic neighbours in order to determine whether strain YM55 is a novel species.

3.3.4.3 *Kribbella* strain YPL1

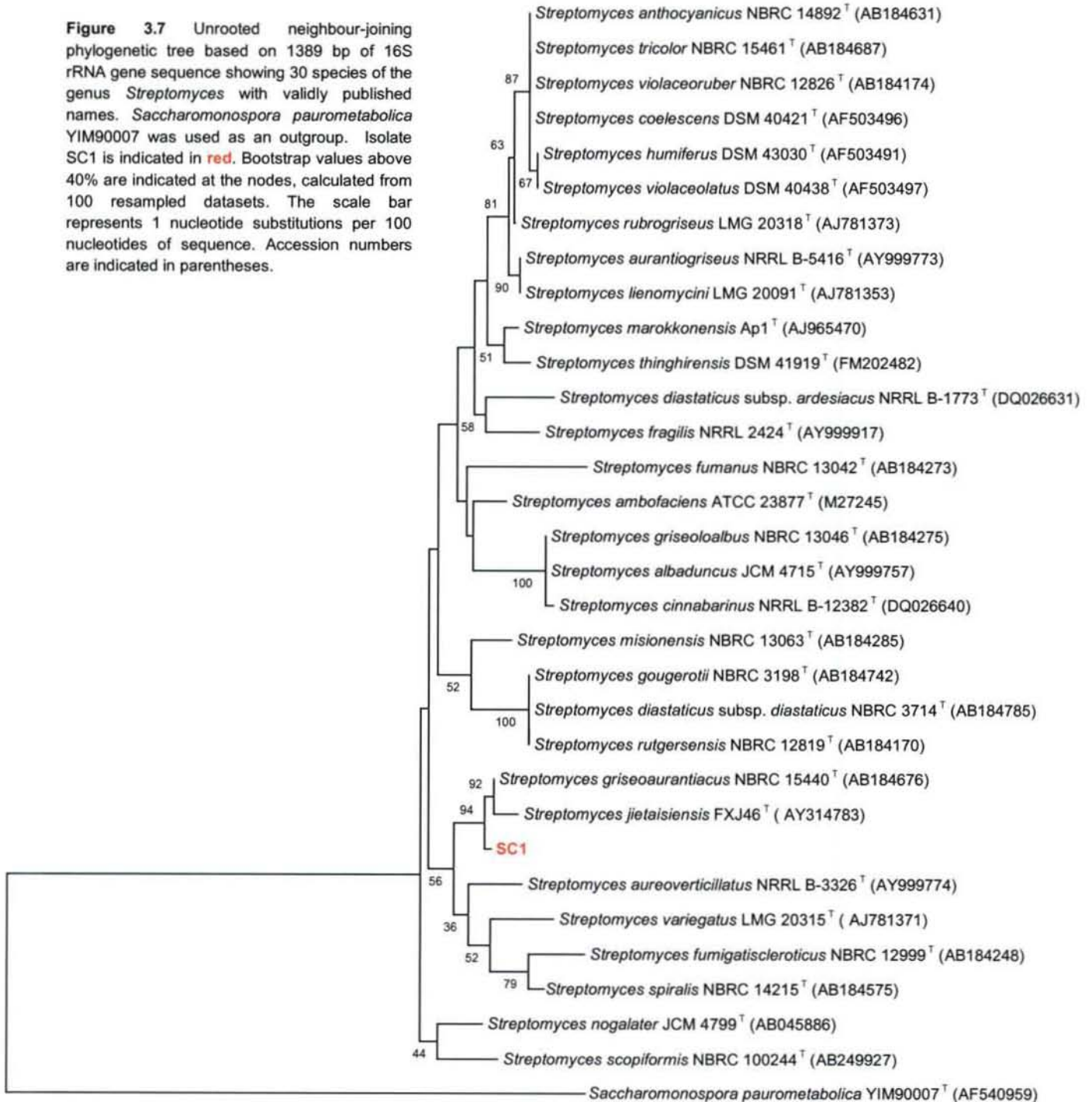
Kribbella strain YPL1 was obtained from the leaves of the South African tree *Podocarpus latifolius* obtained from Devil's Peak, Table Mountain National Park, Cape Town. Colonies were raised, irregular and wrinkled in appearance. Vegetative hyphae were cream in colour, with white aerial mycelium on ISP 4. *Kribbella* strain YPL1 was shown to have very strong antimicrobial activity against *M. aurum* A+ (Chapter 2, Table 2.7). No pigments were produced by strain YPL1 on ISP 5. The cell-wall peptidoglycan was shown to contain LL-DAP and glycine. *K. swartbergensis* HMC25^T had the highest sequence similarity of 99.6% when the 16S rRNA gene sequence of YPL1 was compared to all *Kribbella* species with validly published names, followed by 99.2% sequence similarity to *K. aluminosa*. The phylogenetic tree (Figure 3.6) showed that *Kribbella* strain YPL1 is indeed most closely related to *K. swartbergensis*, with high bootstrap support (82%). Isolate YPL1 differs from *K. swartbergensis* in the following tests: negative for the utilisation of D(-)-sorbitol, sodium acetate and sodium succinate, as well as positive for growth in the presence of 5% (w/v) NaCl. The 16S rRNA gene sequence similarity between strain YM55 and YPL1 is 99.31%. There are two phenetic differences between strains YM55 and YPL1, namely, the ability of strain YM55 to utilise inulin as a sole carbon source, and the ability of strain YPL1 to weakly degrade guanine. DNA-DNA hybridisation experiments are required in order to determine whether strain YPL1 is a novel strain of the genus *Kribbella*, as well as determining whether *Kribbella* strains YPL1 and YM55 belong to separate species.

3.3.5 Suborder Streptomycineae

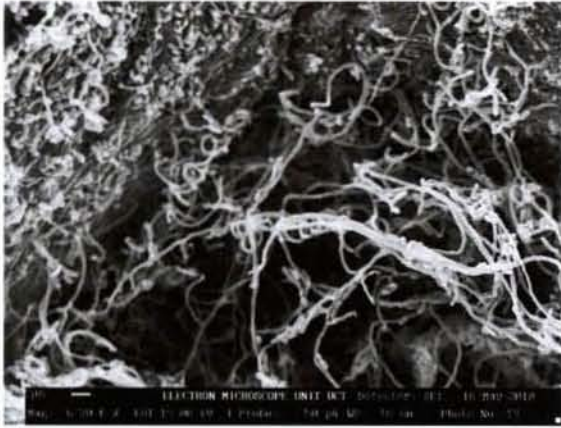
3.3.5.1 Streptomyces strain SC1

Streptomyces strain SC1 was isolated as an endophyte from the stipe portion of the seaweed, *Ecklonia maxima*, obtained in Kalk Bay, Cape Town, South Africa. Strain SC1 was shown to have moderate antimicrobial activity against *M. aurum* A+ (Chapter 2, Table 2.7). Colonies grown on ISP 4 were raised with abundant sporulation present. The substrate mycelium was dark pink in colour, with the spore mass appearing grey on ISP 4. No pigments were produced by strain SC1 on ISP 5. Figures 3.8(a) and 3.8(b) show loose spiral spore chain morphology, with smooth spores for strain SC1. The whole cell hydrolysates of SC1 contained LL-DAP, with no characteristic sugars present. The BLAST search of a 1384 bp 16S rRNA gene sequence of strain SC1 in GenBank showed 99% sequence similarity to *Streptomyces griseoaurantiacus* NBRC 15440^T and *Streptomyces jietaisiensis* FXJ46^T, while pairwise sequence similarities determined in DNAMAN were 99.86% and 99.06%, respectively. The neighbour-joining tree in Figure 3.7 shows strain SC1 grouping with both *S. griseoaurantiacus* and *S. jietaisiensis* with very high bootstrap support (94%). *Streptomyces* strain SC1 differs from *S. griseoaurantiacus* only in one phenetic test, namely, its inability to utilise sucrose as a sole carbon source (Table 3.5, He *et al.* 2005). Strain SC1 differs from *S. jietaisiensis* in its inability to grow on D(-)-sorbitol as a sole carbon source (Table 3.5, He *et al.* 2005). In order to determine the novelty of strain SC1, DNA-DNA hybridisation is required between SC1 and its closest phylogenetic relatives.

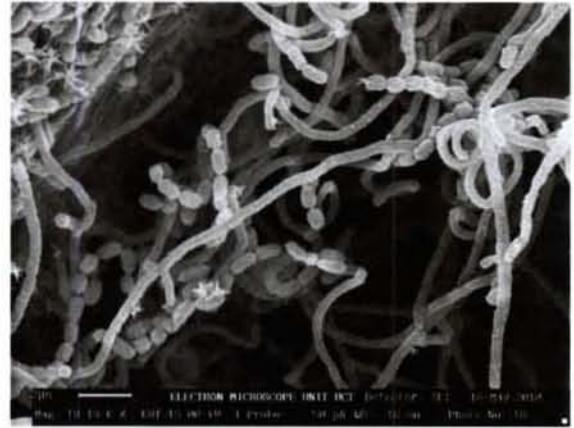
Figure 3.7 Unrooted neighbour-joining phylogenetic tree based on 1389 bp of 16S rRNA gene sequence showing 30 species of the genus *Streptomyces* with validly published names. *Saccharomonospora paurometabolica* YIM90007 was used as an outgroup. Isolate SC1 is indicated in red. Bootstrap values above 40% are indicated at the nodes, calculated from 100 resampled datasets. The scale bar represents 1 nucleotide substitutions per 100 nucleotides of sequence. Accession numbers are indicated in parentheses.



0.01



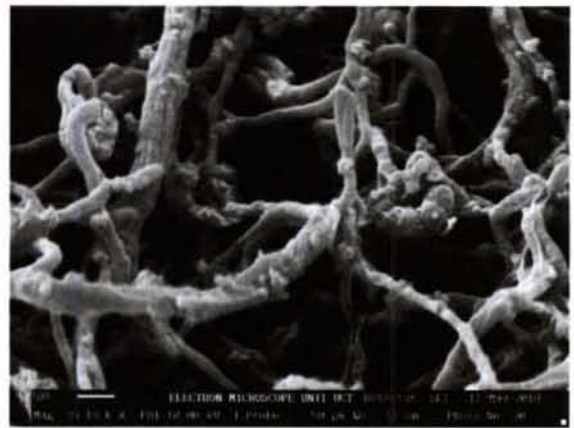
(a)



(b)



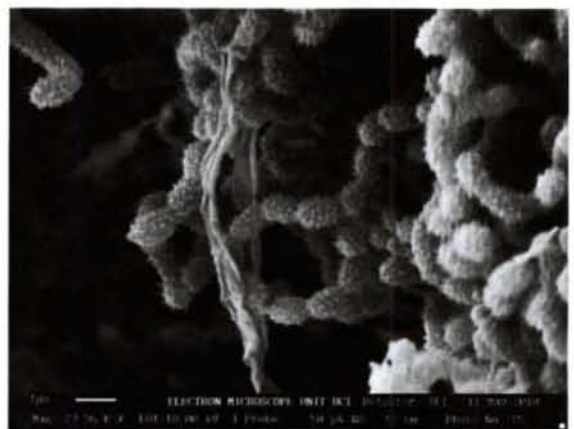
(c)



(d)



(e)



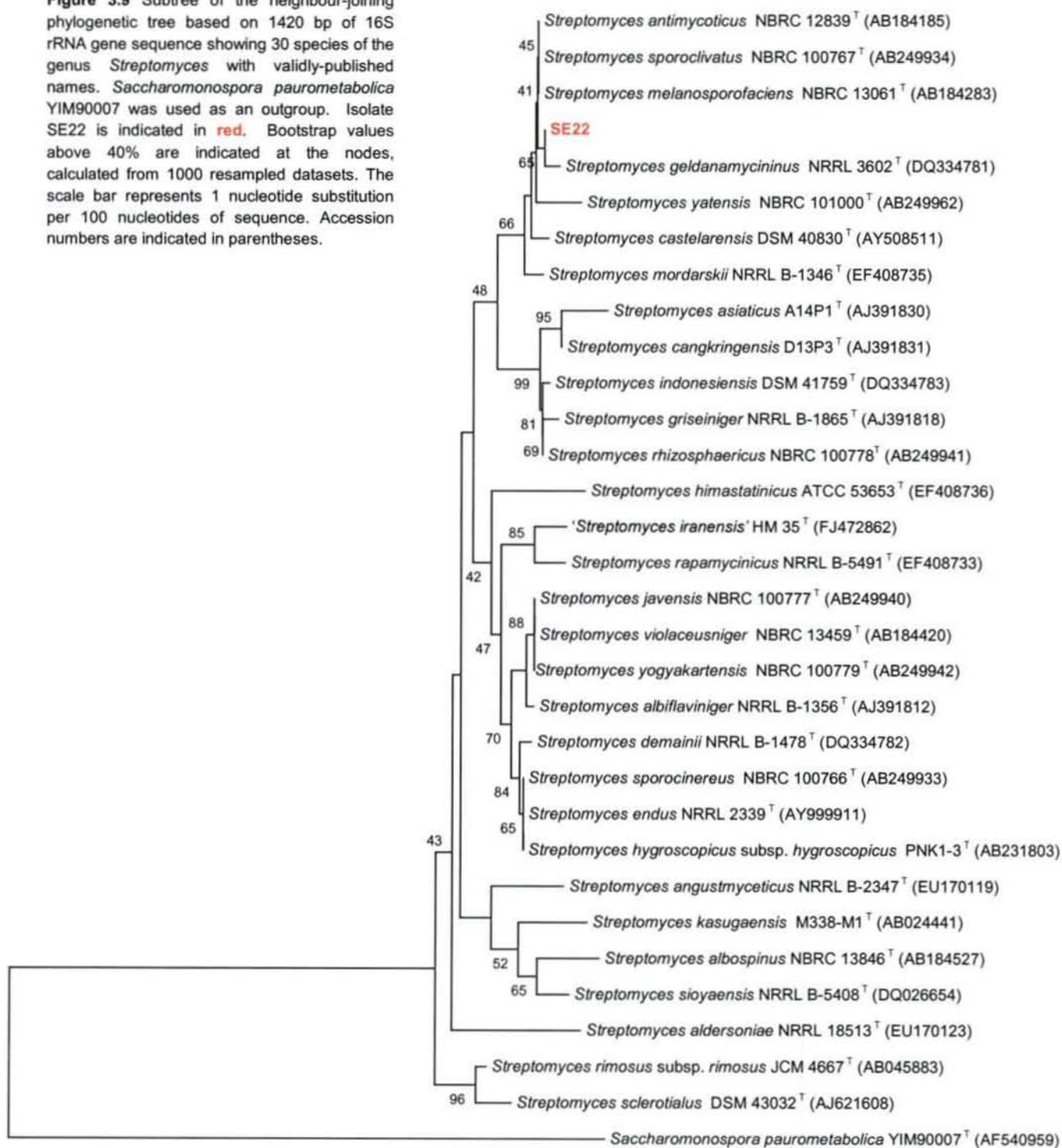
(f)

Figure 3.8 Scanning electron micrographs of *Streptomyces* isolates (a) SC1 (scale 2 μm); (b) SC1 (scale 2 μm); (c) SE22 (scale 2 μm); (d) SE22 (scale 1 μm); (e) YMH1 (scale 1 μm); (f) YMH1 (scale 1 μm).

3.3.5.2 *Streptomyces* strain SE22

Streptomyces strain SE22 was isolated from the soil sample obtained from the University of Cape Town. Strain SE22 has been shown to produce a number of antibiotics effective against *M. aurum* A+ (sections 2.3.3 and 2.3.5). Colonies appeared raised and convoluted/wrinkled on all media tested, with sparse sporulation on ISP 4. The substrate mycelium was creamy to bright yellow in colour, with a white aerial spore mass on ISP 4. *Streptomyces* strain SE22 does not produce any diffusible pigments. Figure 3.8(c) and 3.8(d) indicate the spiral nature of the spore chains, with rugose spore surface ornamentation. The whole cell hydrolysates of strain SE22 contained LL-DAP, with no characteristic sugars present. A BLAST search in the GenBank database showed the 1433 bp 16S rRNA gene sequence of SE22 to have 99% sequence similarity to *Streptomyces sporoclivatus* NBRC 100767^T, *Streptomyces melanosporofaciens* NBRC 13061^T, *Streptomyces antimycoticus* NBRC 12839^T and *Streptomyces geldanamycininus* NRRL B-3602^T. The neighbour-joining phylogenetic tree in Figure 3.9, however, grouped SE22 with *Streptomyces geldanamycininus* NRRL B-3602^T with moderate bootstrap support (65%). The 16S rRNA gene sequence similarity between strain SE22 and *S. geldanamycininus* is 99.86%. *Streptomyces* strain SE22 differs from *S. geldanamycininus* in 3 phenetic tests: its inability to utilise adonitol and melezitose, and the ability to utilise salicin, as sole-carbon sources (Tables 3.4 and 3.5, Kumar & Goodfellow 2008). Due to the high 16S rRNA gene sequence similarity between strain SE22 and *S. geldanamycininus*, and few physiological differences, DNA-DNA hybridisation experiments would be required in order to determine whether SE22 is a novel *Streptomyces* species.

Figure 3.9 Subtree of the neighbour-joining phylogenetic tree based on 1420 bp of 16S rRNA gene sequence showing 30 species of the genus *Streptomyces* with validly-published names. *Saccharomonospora paurometabolica* YIM90007 was used as an outgroup. Isolate SE22 is indicated in red. Bootstrap values above 40% are indicated at the nodes, calculated from 1000 resampled datasets. The scale bar represents 1 nucleotide substitution per 100 nucleotides of sequence. Accession numbers are indicated in parentheses.



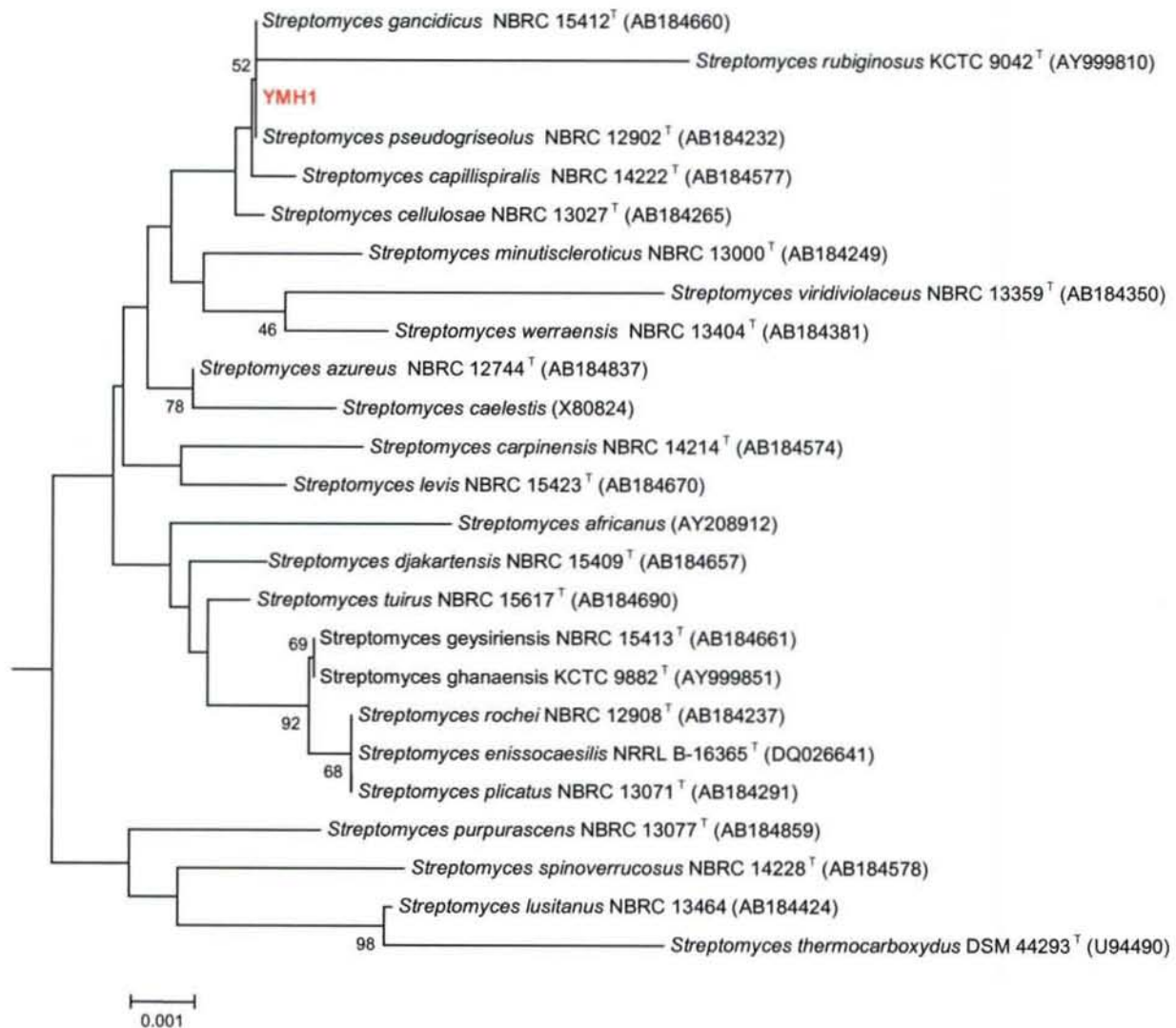


Figure 3.10 Neighbour-joining phylogenetic subtree of the genus *Streptomyces* based on 1377 bp of 16S rRNA gene sequence. Isolate YMH1 is indicated in red. Bootstrap values above 40% are indicated at the nodes, calculated from 1000 resampled datasets. The scale bar represents 1 nucleotide substitution per 1000 nucleotides of sequence. Accession numbers are indicated in parentheses.

3.3.6 Suborder Streptosporangineae

3.3.6.1 The *Microbispora* isolates

Initial phenetic tests were performed on all *Microbispora* species with validly published names, including all subspecies, but excluding *M. siamensis* (the description of which was *in press* at the time of testing).

3.3.6.1.1 *Microbispora* strain SM359

Microbispora strain SM359 was isolated by the author in 2007 as part of a B.Sc.(Honours) research project. Strain SM359 was isolated from a soil obtained in a residential garden in Rondebosch, Cape Town. Colonies of SM359 appeared convoluted and wrinkled, which turned furry upon sporulation. The substrate mycelium was dark pink to reddish-brown in colour, with peachy white aerial mycelium on ISP 4. *Microbispora* strain SM359 did not produce any diffusible pigments. The whole cell hydrolysates of strain SM359 were shown to contain *meso*-DAP and glycine. *Microbispora* strain SM359 showed the highest pairwise sequence similarity of 99.4% to *Microbispora karnatakensis* IMNSU 22065^T. As can be seen in Figure 3.11, the closest phylogenetic neighbours of strain SM359 are *Microbispora karnatakensis* IMNSU 22065^T (with low bootstrap support) and *Microbispora amethystogenes* JCM 3021^T.

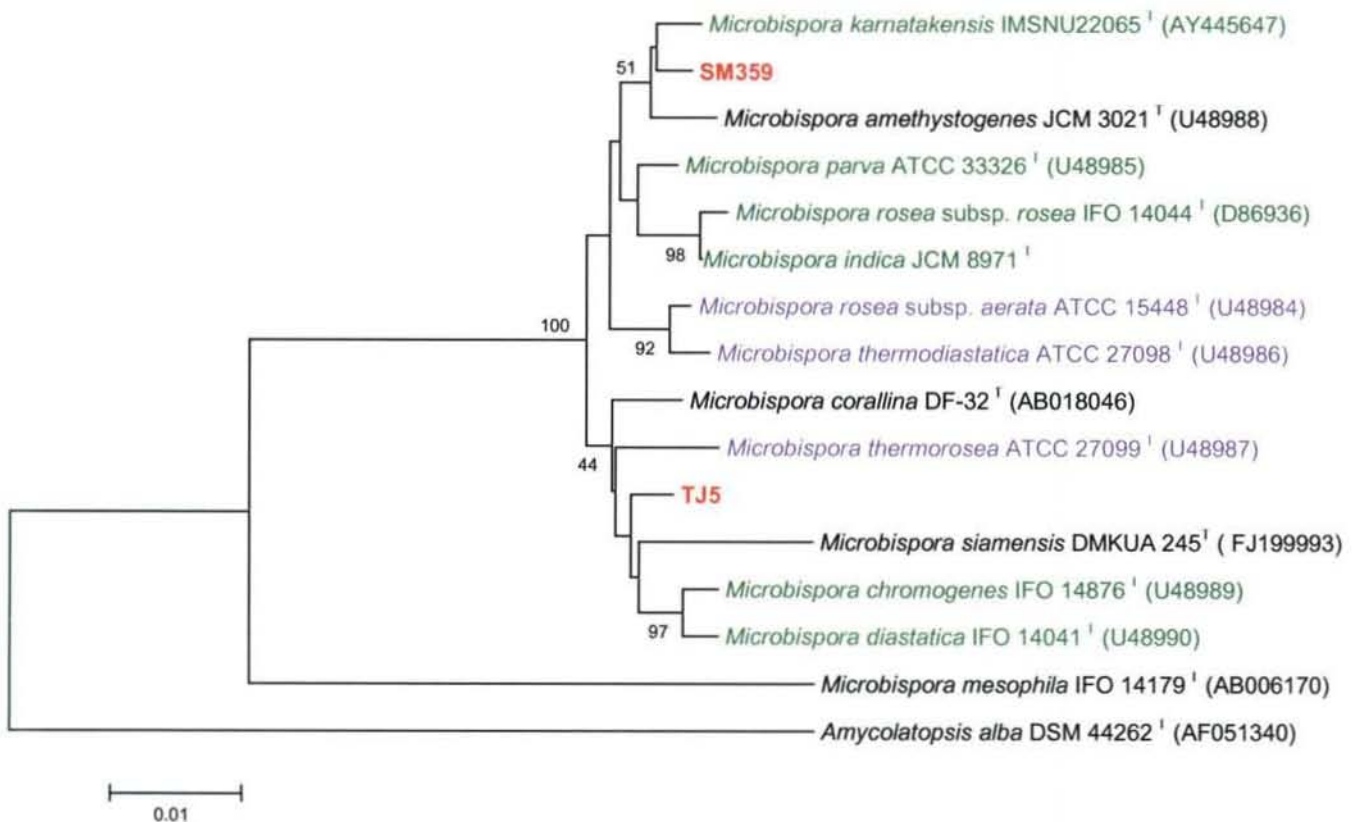


Figure 3.11 Unrooted neighbour-joining phylogenetic tree of the genus *Microbispora* based on 1359 bp of 16S rRNA gene sequence, with *Amycolatopsis alba* DSM 44262 as the outgroup. Strains are shown by their original published names. Isolates indicated in green currently recognised as *M. rosea* subsp. *rosea*; isolates in purple currently recognised as *M. rosea* subsp. *aerata*. Isolates SM359 and TJ5 are indicated in red. Bootstrap values above 40% are indicated at the nodes, calculated from 1000 resampled datasets. The scale bar represents 1 nucleotide substitution per 100 nucleotides of sequence. Accession numbers are indicated in parentheses.

The sequence similarity between strain SM359 and *M. amethystogenes* is 99.1%. *Microbispora* strain SM359 differs from *M. amethystogenes* and *M. karnatakensis* in a number of phenetic tests (Table 3.6).

Table 3.6 Physiological Characteristics of Isolates and Species within the genus *Microbispora*

1, *Microbispora rosea* subsp. *aerata* DSM 43176^T; 2, *Microbispora amethystogenes* DSMZ 43164^T; 3, *Microbispora chromogenes* NRRL B-2534^T; 4, *Microbispora corallina* DSM 44682^T; 5, *Microbispora diastatica* NRRL B-2630^T; 6, *Microbispora indica* JCM 8971^T; 7, *Microbispora karnatakensis* JCM 8972^T; 8, *Microbispora mesophila* NRRL B-16986^T; 9, *Microbispora parva* NRRL B-2629^T; 10, *Microbispora siamensis* DMKUA 245^T; 11, *Microbispora rosea* subsp. *rosea* NRRL B-2632^T; 12, *Microbispora thermodiastatica* DSM 43166^T; 13, *Microbispora thermorosea* DSM 43840^T. ND, not determined; NG, no growth; +(w), weak growth/positive reaction; +, growth/positive reaction; -, No growth/negative reaction; Y, yellow.

	SM359	TJ5	1	2	3	4	5	6	7	8	9	10*	11	12	13
Pectin hydrolysis	-	-	NG	+(w)	+	-	+	NG	+	+(w)	-	ND	NG	+	NG
H ₂ S production	+	+	-	-	+(w)	-	+	+(w)	+	+(w)	+(w)	ND	-	-	-
Nitrate reduction	+(w)	+	+	+	+	-	-	+	+	+	-	-	+	-	-
ISP#5 (pigments)	-	-	-	-	-	Y	-	-	-	-	-	ND	-	-	-
Degradation of:															
Gelatin	+	+	-	+	+	-	+	NG	+	+	+(w)	-	NG	+(w)	NG
Hypoxanthine	-	+(w)	-	+(w)	-	+(w)	+	+(w)	+(w)	+	-	ND	-	+	+
L-Tyrosine	-	+(w)	-	+	-	-	+	-	-	+	-	ND	-	+	+(w)
Starch	-	-	+	+(w)	+	+(w)	+(w)	NG	+(w)	+	-	ND	NG	+	NG
Xylan	-	-	+(w)	-	-	-	-	+(w)	-	-	-	ND	+(w)	-	+
Urea	-	-	NG	-	NG	NG	-	NG	-	+(w)	-	ND	NG	-	-
Growth at:															
50°C	-	+	++	-	-	-	-	-	++	-	+	+	-	+++	+++
55°C	-	-	+	-	-	-	-	-	-	-	-	-	-	++	++
2% NaCl	+	+++	+(w)	++	+(w)	+	++	+(w)	+(w)	++	++	ND	+	++	-
3% NaCl	+	+	-	+	-	-	+	-	+(w)	+	-	-	-	-	-
4% NaCl	+(w)	+(w)	-	-	-	-	-	-	-	+(w)	-	ND	-	-	-
pH 4.3	+(w)	+(w)	-	-	-	+(w)	++	-	+	+(w)	-	ND	-	-	+

* Data obtained from Boondaeng *et al.* 2009

3.3.6.1.2 *Microbispora* strain TJ5

Microbispora strain TJ5 was isolated as an endophyte from the leaves of the South African plant, *Geranium incanum*, by Tsungai Jongwe in 2007 as part of a B.Sc. (Honours) research project. Colonies were typically wrinkled and irregular on agar surfaces. The substrate mycelium on most media appeared brown in colour, with white aerial mycelium present on ISP 4. *Microbispora* strain TJ5 produced no diffusible pigments. Phenetic characteristics are shown in Tables 3.4 and 3.5. Phylogenetic analysis of the 16S rRNA gene sequence of strain TJ5 showed that it formed part of a clade with *M. siamensis* DMKUA 245^T, *Microbispora chromogenes* IFO 24872^T, *Microbispora diastatica* IFO 14041^T, *Microbispora thermorosea* ATCC 27099^T and *Microbispora corallina* DF-32^T (Figure 3.11), with 99% sequence similarity between strain TJ5 and *M. chromogenes*, *M. corallina* and *M. diastatica* (98.4% for *M. siamensis* and *M. thermorosea*). Differences in physiological tests determined are shown in Table 3.6.

3.3.6.1.3 *Microbispora gyrB* analysis

Due to the low bootstrap support for the grouping of isolates in the *Microbispora* 16S rRNA gene tree (Figure 3.11), a phylogenetic analysis of the *gyrB* gene was attempted in order to try and improve the robustness of the phylogenetic tree and aid in the identification of *Microbispora* strains SM359 and TJ5. The *gyrB* sequences of the *Microbispora* species with validly published names depicted in Figure 3.12 were obtained from Dr. Paul Meyers. Interestingly, the *Microbispora gyrB* tree and 16S rRNA gene tree show different topologies. The *gyrB* neighbour-joining phylogenetic tree (Figure 3.12) based on 1381 bp of *gyrB* sequence was shown to have improved bootstrap support as well as longer branch lengths, which would allow for greater resolution of closely related species. It is important to note the grouping of the three species in the *gyrB* phylogenetic tree now classified as *M. rosea* subsp. *aerata* (*Microbispora rosea* subsp. *aerata*, *Microbispora thermodiastatica* and *Microbispora thermorosea*), which was not evident in the 16S rRNA gene phylogenetic tree. In fact, all groupings shown in the *Microbispora gyrB* phylogenetic tree are stable with high bootstrap support, which is not the case for most of the 16S rRNA gene tree clusters, where only five clusters were supported by bootstrap values greater than 40% (with actual bootstrap values > 90% for three of the clusters). The three clusters were the pairings *M. rosea* subsp. *rosea*-*M. indica* and *M. rosea* subsp. *aerata*-*M. thermodiastatica*, as well as the branching of *M. chromogenes*-*M. diastatica*. These three

clusters were conserved in both the 16S-rRNA gene tree and the *gyrB* tree with high bootstrap support (>65%). The different resulting topologies and the low stability of the 16S rRNA gene tree may indicate that *gyrB* genes or/and the 16S rRNA genes may have undergone horizontal gene transfer.

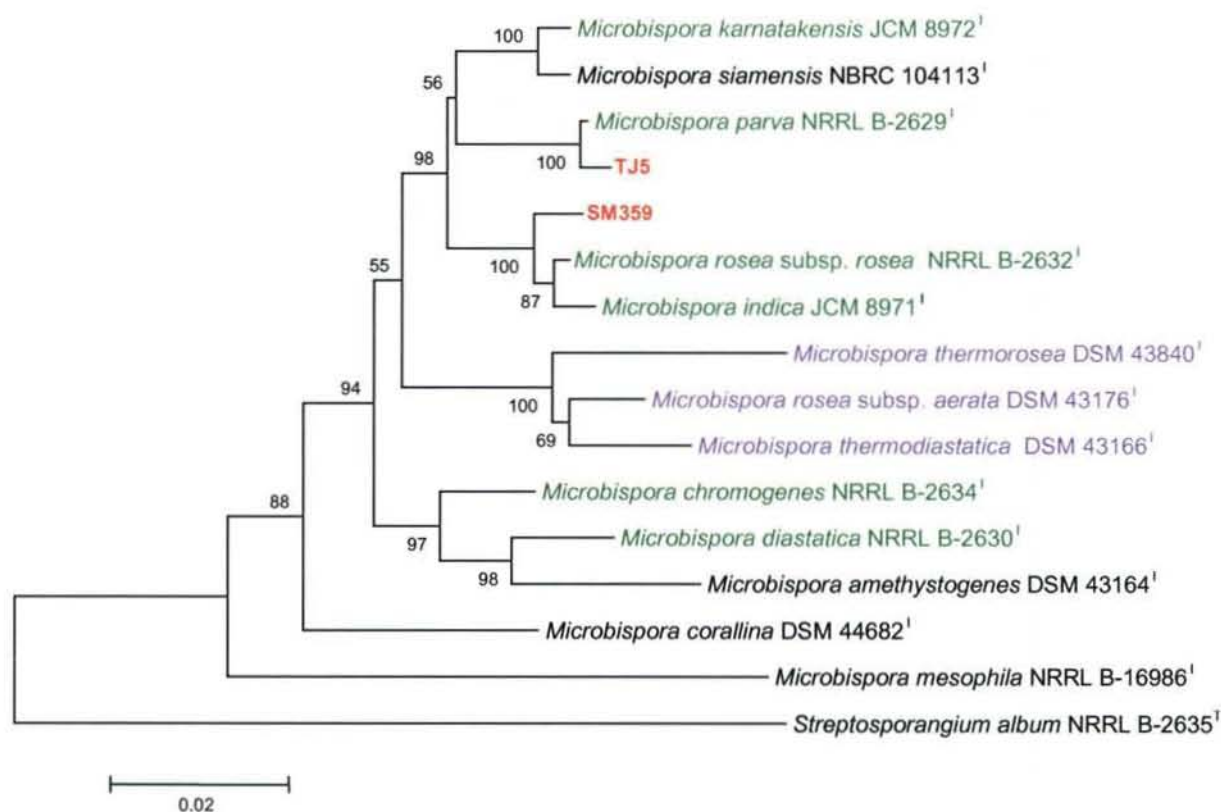


Figure 3.12 Unrooted neighbour-joining phylogenetic tree constructed from *Microbispora gyrB* gene sequences, based on 1381 bp of sequence, with *Streptosporangium album* NRRL B-2635 as the outgroup. Isolates SM359 and TJ5 are indicated in red. Isolates indicated in green currently recognised as *M. rosea* subsp. *rosea*; isolates in purple currently recognised as *M. rosea* subsp. *aerata*. Bootstrap values above 40% are indicated at the nodes, calculated from 1000 resampled datasets. The scale bar represents 2 substitutions per 100 nucleotides of sequence.

Microbispora isolate TJ5 was shown to group with *Microbispora parva* in the *gyrB* tree (Figure 3.10), with 100% bootstrap support and a *gyrB* genetic distance of 0.004 (99.6% *gyrB* sequence similarity), indicating that these two species are closely related. The 16S rRNA gene sequence similarity between strain TJ5 and *M. parva* is 99%. Similarly, *Microbispora* strain SM359 was

shown to group with *Microbispora rosea* subsp. *rosea* and *Microbispora indica* in the *gyrB* tree, with high bootstrap support and 0.01 and 0.012 *gyrB* genetic distances (99% *gyrB* sequence similarity), respectively. The 16S rRNA gene sequence similarity between strain SM359 and *M. indica* and *M. rosea* subsp. *rosea* is 99.1% and 99.3%, respectively. It is recommended that a genetic distance of >0.05 be used to indicate between two separate species within the genus *Microbispora* (Meyers & Sfarlea 2009). Thus *Microbispora* strains SM359 and TJ5 would require DNA-DNA hybridisation with the closest phylogenetic neighbours to determine whether they are novel species of the genus *Microbispora*.

On the whole, due to the apparent horizontal gene transfer within the genus *Microbispora*, it is suggested that DDH studies are performed between SM359 and TJ5 and their closest relatives in both the 16S rRNA and *gyrB* gene phylogenetic trees (where the 16S rRNA gene sequence similarity is greater than 98.7%). For this reason, comparisons should at least be made between isolate SM359 and *M. amethystogenes*, *M. indica*, *M. karnatakensis* and *M. rosea* subsp. *rosea*, and between isolate TJ5 and *M. chromogenes*, *M. corallina*, *M. diastatica* and *M. parva*, in order to determine whether they are indeed novel species.

3.3.6.2 *Nonomuraea* strain SM343

Nonomuraea species SM343 was isolated from the soil sample obtained from the University of Cape Town. Colonies were raised, tough and leathery in appearance. The colonies were typically dark cream in colour on ISP 4. No spore mass was present. No diffusible pigments were produced on ISP 5. The cell wall of isolate SM343 was shown to contain meso-DAP. The BLAST search of a 1337 bp 16S rRNA gene sequence showed 98% similarity to *Nonomuraea turkmeniaca* DSM 43926^T, *Nonomuraea salmonea* DSM 43678^T and *Nonomuraea candida* HMC10^T. The sequence similarities obtained in DNAMAN between strain SM343 and *N. turkmeniaca*, *N. salmonea* and *N. candida* were 98.8%, 98.35% and 97.45%, respectively. The neighbour-joining phylogenetic tree (Figure 3.13) however, shows *Nonomuraea* isolate SM343 grouping (with low bootstrap support) with *Nonomuraea roseoviolacea* subsp. *carminata* IFO 15903^T, *Nonomuraea roseoviolacea* subsp. *roseoviolacea* IFO 14098^T and *Nonomuraea ferruginea* IFO 14094^T, with sequence similarities of 97.3%, 97.67% and 97.97%, respectively.

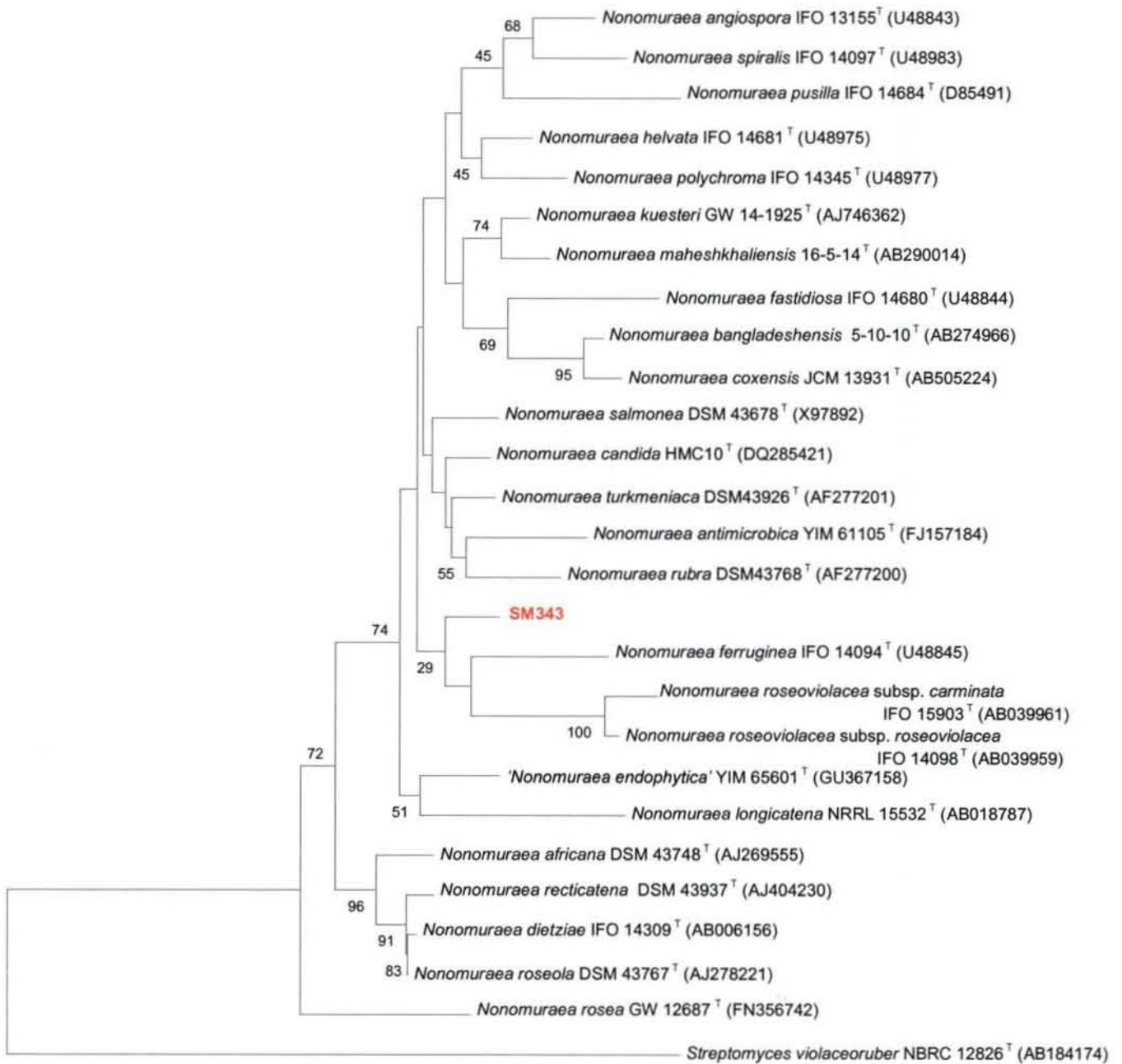


Figure 3.13 Unrooted neighbour-joining phylogenetic tree based on 1328 bp of 16S rRNA gene sequence showing all species of the genus *Nonomuraea* with validly published names, with *Streptomyces violaceoruber* NBRC 12826 as the outgroup. Isolate SM343 is indicated in red. Bootstrap values above 40% are indicated at the nodes, calculated from 1000 resampled datasets. The scale bar represents 1 nucleotide substitution per 100 nucleotides of sequence. Accession numbers are indicated in parentheses.

The sequence similarity between strain SM343 and *N. roseoviolacea* subsp. *carminata*, *N. roseoviolacea* subsp. *roseoviolacea* and *N. ferruginea* are low enough to allow for strain SM343 to be distinguished from these species without requiring DDH studies. Isolate SM343 may be distinguished from *N. turkmeniaca* by negative test results for nitrate reduction, hypoxanthine and starch degradation, and a positive result for the degradation of L-tyrosine (Kämpfer *et al.* 2010, Table 3.4). Due to the 98.8% sequence similarity, combined with the differences in the listed phenetic characteristics, isolate SM343 may be a novel species of the genus *Nonomuraea*, however DDH studies will be needed to establish this.

3.3.6.3 *Actinomadura* strain YPC2

Actinomadura strain YPC2 was isolated as an endophyte from the leaves of the South African plant *Pelargonium cucullatum* obtained from Devil's Peak, Table Mountain National Park, Cape Town. Colonies of YPC2 were raised and convoluted, with a tough leathery appearance. The substrate mycelium was creamy yellow in colour on ISP 4. No sporulation was observed on any media. Isolate YPC2 did not produce any pigments on any ISP media. The cell wall of YPC2 was shown to contain *meso*-DAP. The BLAST analysis of 1376 bp of 16S rRNA gene sequence revealed that isolate YPC2 had the highest sequence similarity of 98% with *Actinomadura bangladeshensis* 3-46-b(3)^T. A sequence similarity of 98.84% was determined in DNAMAN between YPC2 and the type strain of *A. bangladeshensis*. However, in the neighbour-joining tree shown in Figure 3.14, the closest phylogenetic neighbours were shown to be *Actinomadura cremea* subsp. *cremea* JCM 3308^T and *Actinomadura cremea* subsp. *rifamycini* IFO 14183^T, but with low bootstrap support (50%). The pairwise sequence similarity was calculated to be 97.82% and 96.92% between *Actinomadura* strain YPC2 and *A. cremea* subsp. *cremea* and *A. cremea* subsp. *rifamycini*, respectively. The sequence similarity of 96.92% is low enough to allow strain YPC2 to be deemed a separate species from *A. cremea* subsp. *rifamycini* without DNA-DNA hybridisation being required. Strain YPC2 differs from *A. cremea* subsp. *cremea* and *A. cremea* subsp. *rifamycini* with positive results for starch and L-tyrosine degradation (Meyer 1989). Isolate YPC2 may be distinguished from *A. bangladeshensis* in the following tests: YPC2 does not grow in the presence of 4% (w/v) NaCl or at 45°C, but can utilise *myo*-inositol and D(+)-mannose as sole carbon sources (Ara *et al.* 2008).

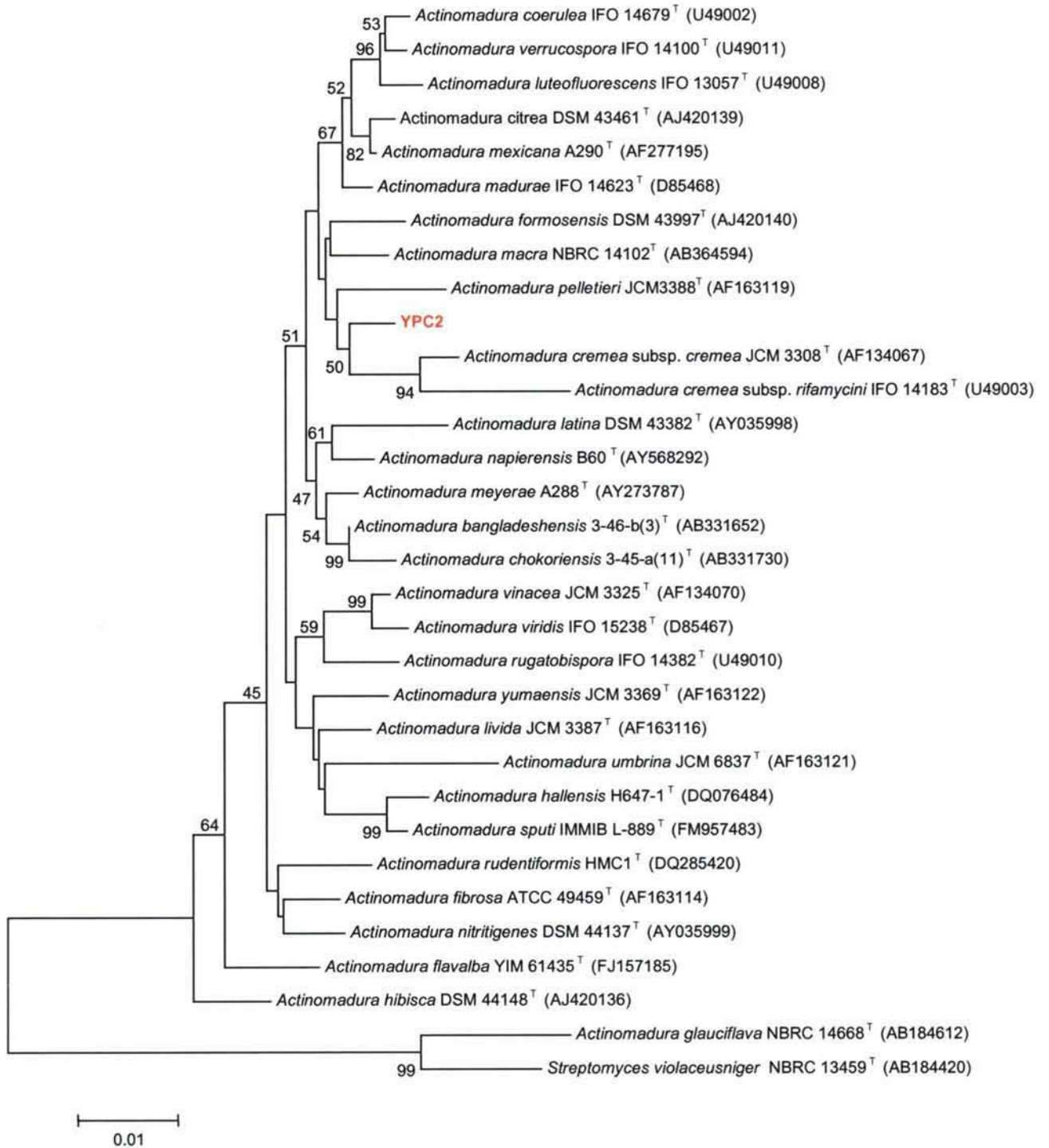


Figure 3.14 Unrooted neighbour-joining phylogenetic tree based on 1369 bp of 16S rRNA gene sequence showing 30 species of the genus *Actinomadura* with validly published names, with *Streptomyces violaceusniger* NBRC 13459 as the outgroup. Isolate YPC2 is indicated in red. Bootstrap values above 40% are indicated at the nodes, calculated from 1000 resampled datasets. The scale bar represents 1 nucleotide substitution per 100 nucleotides of sequence. Accession numbers are indicated in parentheses.

Although the strains characterised in this study have shown various phenetic differences to their closest phylogenetic neighbours, the novelty thereof can not be definitively established without further investigations being done. These include more extensive physiological and chemotaxonomic testing as well as DDH studies, which is recommended for strains that show a 16S rRNA gene sequence similarity greater than 98.7% (Stackebrandt & Ebers, 2006).

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Chapter 4

Conclusion and Future Work

The investigations into three different isolation sources, namely, various indigenous plants, indigenous seaweeds and a soil sample, proved fruitful in the isolation of actinobacteria, with the isolation of species belonging to eight different genera: *Actinomadura*, *Kineococcus*, *Kribbella*, *Micromonospora*, *Nocardia*, *Nonomuraea*, *Streptomyces* and *Verrucosipora*.

One of the main aims achieved in this project was the isolation of non-*Streptomyces* species (rarer actinobacteria) from the various sources investigated, with a total of 28 isolates obtained in the preliminary identification of actinobacteria. Of the 28 non-*Streptomyces* isolates, the majority belonged to the genus *Micromonospora* (20), followed by *Kribbella* isolates (3), both genera that include species known to produce antibiotics. The isolation of such a great percentage of *Micromonospora* is not surprising given that *Micromonospora* are considered to be one of the larger culturable groups among the actinobacteria. Further characterisation could be carried out on the remaining 16 *Micromonospora* isolates that were not a focus of this study, to potentially identify novel *Micromonospora* species. The use of *gyrB* sequence analysis in these characterisation studies is recommended, as there is a growing number of *Micromonospora gyrB* sequences available.

When considering the plant and seaweed sources, a total of seven isolates was obtained (five endophytes isolated from plants, and two isolates obtained from seaweeds, with strain SC1 as an endophyte and strain Y2UE1 as an epiphyte). Five of these isolates were identified as non-*Streptomyces* species, of which four were endophytes obtained from various plant sources (strains YPC1, YPL1, SM3BL1 and YPC2), and one was obtained from the surface of a seaweed (strain Y2UE1). Although the percentage of endophytes isolated from various plants was low (approximately 3% of the total number of isolates obtained), when compared to those obtained from the soil sample, they actually represent half of the total of eight genera isolated (strain YPC2 – *Actinomadura* sp., strain YPL1 – *Kribbella* sp., strains SM3BL1 and YPC1 – *Micromonospora* spp. and strain YMH1 – *Streptomyces* sp.). Similarly, the *Kineococcus* strain

(Y2UE1) was obtained from a seaweed sample. This is particularly interesting as these alternative sources (i.e. non-soil sources) represent 62.5% (5 of 8) of the genera isolated in this study. In this case, investigations into alternative sources such as indigenous plants and seaweeds for the isolation of non-*Streptomyces* species was successful, and it may be concluded that these alternative environments were a good source of these rarer actinobacteria. Future investigations into exploring the diversity of these alternate sources should be considered, as it seems there is good potential for isolating novel rare actinobacteria.

Antibacterial testing was performed against *M. aurum* A+ for all the non-*Streptomyces* strains (28), as well as 14 selected *Streptomyces* strains. A total of 12 isolates displayed antibacterial activity of roughly 1000 mm² or more, with seven of the 28 strains (25%) identified as belonging to non-*Streptomyces* genera, consisting of the three *Kribbella* isolates (strains YM53, YM55 and YPL1), and four *Micromonospora* isolates (including strain SE17, and three of the four strains that were characterised in Chapter 3, YPC1, SM3BL1, SM338). Interestingly, four of the six endophytes (67%) isolated in this study (strains SC1, SM3BL1, YMH1 and YPL1) had antibiotic activity against *M. aurum* A+. On the other hand, a total of only 7 of the 35 soil isolates (20%) tested against *M. aurum* A+ had antibiotic activity. In this case, the isolation of endophytes, although small in number in comparison to those isolated from the soil sample, has provided a greater percentage of antibiotic producers effective against *M. aurum* A+. This might be attributed to the production of novel secondary metabolites by the actinobacteria in these alternate plant and seaweed environments.

The most potent antibiotic producer was isolate SE22, a streptomycete, followed by the *Kribbella* strains. Focus was placed on extracting antibiotics from six of these isolates, the top four antibiotic producers, strains SE22, YM53, YM55 and YPL1 (an endophyte), and two other endophytes YMH1 (a streptomycete) and YPC1. Of these, antibiotics could only be obtained from two isolates (strains SE22 and YM55) with the solvents used. Further work could be done to encourage the production of antibiotics in liquid culture from the remaining isolates, by using alternate media.

Streptomyces strain SE22 demonstrated very strong antibiotic activity against *M. aurum* A+, as well as *S. aureus* ATCC 25923, suggesting the specificity of the antibiotics to Gram positive bacteria. The antibiotics were extracted with all the solvents used, suggesting that a number of different antibiotics with differing polarities are present. One of these may be an ansamycin type antibiotic (as suggested by the antibiotic biosynthetic gene analysis; section 2.3.4). Investigations could be done into the number of organic compounds in the antibiotic extracts using TLC followed by cerium ammonium sulphate oxidation. Although the various antibiotics could not be separated in this study to allow for column purification, further investigations into solvent systems could be done to aid in this regard. Further work should also be done in purifying the antibiotics extracted from isolate SE22 as there is an increased chance, due to the number of antibiotics produced by this isolate that are effective against *M. aurum* A+, that one could potentially be effective against *Mycobacterium tuberculosis*.

Isolate YM55 was shown to have very strong and specific antibiotic activity against *M. aurum* A+. A compound was extracted and an optimal solvent system of 70% (v/v) chloroform : 30% (v/v) acetone was determined which showed an R_f value of 0.7 for the active spot. Further studies could not be attempted due to the labile nature of the antibiotic. In future, work could be done to investigate solvent systems that may lower the R_f to the optimal 0.5. The antibiotic could then be subjected to column purification and, once isolated, tested for its activity against *M. tuberculosis*.

Assuming the purification of the antibiotics produced by isolates SE22 and YM55 is achieved, work could then be done in characterising them by methods including determining their structures by nuclear magnetic resonance (NMR), X-ray crystallography, and elemental analysis.

A total of 15 species isolated in this study plus two *Microbispora* species isolated in 2007 were subjected to further morphological, physiological and chemotaxonomic characterisation. These included the five plant endophytes and the two isolates obtained from seaweed samples, and eight other strains representing the *Kribbella*, *Micromonospora*, *Nocardia*, *Nonomuraea*, *Streptomyces* and *Verrucosipora* genera (all eight obtained from the soil sample). Although

the majority of the species characterised would require DNA-DNA hybridisation studies to determine whether they are novel species within their respective genera, at least one isolate has already been identified as likely to be novel (*Actinomadura* strain YPC2). For a number of the closest relatives of the isolates, only limited published characteristics were available for comparisons, resulting in a limited number of differences that could be highlighted between them. Further work would require repeating all the physiological tests for the isolates and their closest relative(s) in parallel to allow for more thorough comparisons to be made. These results would then aid in determining whether the DDH studies are worth pursuing.

For *Kineococcus* isolate Y2UE1 and *Verrucosipora* isolate YM76, new carbon and nitrogen utilisation media need to be prepared that are modified to include a small amount of a nutrient rich ingredient, such as yeast extract. This should hopefully encourage enough growth of the isolates to allow for the differentiation between growth and no growth on the different carbon/nitrogen sources. These results could then be used to further differentiate these isolates from their closest relatives, and thus potentially identify them as novel species, in conjunction with DDH studies.

The phylogeny of the *gyrB* gene was investigated to help resolve isolates SM359 and TJ5 within the genus *Microbispora* as a result of the unstable topology of the 16S rRNA gene phylogenetic tree. Although it was determined that *gyrB* phylogeny may be useful in predicting the novelty of an isolated strain, the suggestion of horizontal gene transfer does present a hurdle in determining the potential novelty of *Microbispora* isolates through *gyrB* gene phylogenetic studies. As such, further work could be done to include phylogenies of additional housekeeping-genes, such as the *rpoB* and *recN* genes, for the genus *Microbispora*. Subsequently, multilocus sequence analysis (MLSA) could be performed to better reflect the phylogenetic relationships within the genus *Microbispora*, which should assist in the identification of new isolates.

It has also been shown that *gyrB* phylogeny has been successfully used in phylogenetic studies involving the *Kribbella*, *Micromonospora* and, recently, the *Nocardia* genera (Kirby *et al.* 2010, Kasai *et al.* 2000, Takeda *et al.* 2010). Thus, the phylogenetic analyses of the isolates in this

study identified as *Kribbella* (strains YM53, YM55 and YPL1), *Micromonospora* (strains YPC1, SM3BL1, YM48 and SM338) and *Nocardia* (strain SM331) species could be expanded to include *gyrB* based phylogeny. It is hoped that the *gyrB* phylogeny would better resolve the phylogenetic relationships of the isolates with their closest relatives, as well as help determine whether they are novel species.

In conclusion, the strains isolated and characterised in this study are worthy of further investigations, particularly those showing antimicrobial activity against *M. aurum* A+. The potential novelty of these strains is encouraging, as the antibiotics they produce may also be novel. This is particularly important in addressing the need for novel antibiotics needed to combat antibiotic resistance.

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