The characterisation of actinomycetes isolated from diverse South African sources, with emphasis on the genus *Kribbella*

by

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Abstract

Actinomycetes were isolated from the leaves of indigenous plants, aquatic sediment and soil samples, using alternative isolation methods to select for actinomycetes belonging to the rarer genera. Thirty actinomycete strains belonging to the genera *Gordonia, Kineococcus, Kribbella, Micromonospora*, *Nocardia* and *Streptomyces* were selected for full characterisation. A polyphasic approach combining physiology, chemotaxonomy and phylogenetic analysis was used to characterise these isolates. A number of potentially novel strains belonging to the rarer genera were identified, including two *Kineococcus* and three *Micromonospora* strains. Two novel *Kribbella* species were isolated from soil samples and the species descriptions of *Kribbella karoonensis* Q41^T and *Kribbella swartbergensis* HMC25^T were published in 2006.

More than 80% of all antibiotics are produced by actinomycetes and they are potentially the source of novel bioactive compounds. In this study, all actinomycete isolates were screened for antimicrobial activity against *Mycobacterium aurum* A+. Antibiotic production by the actinomycete strains was assessed by agar overlays, solvent extraction and bioautography, as well as by PCR screening with primers that target antibiotic biosynthetic genes. Twenty four actinomycete strains produced antimicrobial compounds that inhibited *M. aurum* A+. Antibiotic production was found to be the most prevalent in strains isolated from soil, with *Streptomyces* being the most prolific antibiotic-producing genus. The antimycobacterial compound produced by *Streptomyces speibonae* strain PK-Blue^T was characterised. Based on preliminary structural and physical characterisation, it appears that this compound is a tetramic acid type antibiotic.

The 16S rRNA gene sequence similarity between members of the genus *Kribbella* ranges from 97.5% to 99.1%, and DNA-DNA hybridization is usually performed when classifying new species within this genus. The potential of the *recN* and *gyrB* genes to discriminate between *Kribbella* species was investigated. A plot of DNA relatedness versus pairwise *gyrB* sequence similarity was not linear. Kimura's 2-parameter model has been used in other actinomycete genera to determine the *gyrB*-based genetic distances and it is reported that a genetic distance of 0.014 can be used as the threshold for species delineation. In this study, the *gyrB*-based

genetic distance was determined between all *Kribbella* type strains and values were found to range from 0.0525 to 0.1495, confirming that the *gyrB* gene can distinguish between species of this genus. Based on the analysis of *gyrB*-based genetic distances versus DNA relatedness between *Kribbella* species, it is proposed that a *gyrB*-based genetic distance of greater than 0.04 be set as the threshold for species delineation within the genus *Kribbella*. Phylogenetic analysis based on the *gyrB* gene was found to provide better resolution and more robust trees than those based on the 16S rRNA gene sequence. Based on this study, the *gyrB* gene can be used to resolve species relationships within the genus *Kribbella*.

Abbreviations

A	Adenine
AFLP	Amplified fragment length polymorphism
AHBA	3-amino-5-hydroxy-benzoic acid
AIDS	Acquired immune deficiency syndrome
ANI	Average nucleotide identity
ARDRA	Amplified rDNA restriction analysis
BCG vaccine	Bacille Calmette-Guérin vaccine
Big Pharma	Large pharmaceutical companies
bp	Base pair(s)
C	Cytosine
CAS	Cerium (IV) ammonium sulphate
CDC	Centers for Disease Control, USA
CF	Culture filtrate
cm	Centimetre(s)
CZ agar	Czapek solution agar
Da	Dalton(s)
DAP	2,6-Diaminopimelic acid
DDH	DNA-DNA hybridization
DGGE	Denaturating gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate (dATP, dCTP, dGTP and dTTP)
DOI	2-deoxy-*scyllo*-inosose
DOTS	Directly observed therapy, short-course
DP	Diffusible pigments
DSMZ	*Deutsche Sammlung von Mikroorganismen und Zellkulturen* GmbH
D_20	Deuterated water
EDTA	Ethylenediaminetetra-acetic acid (disodium salt)
ESMS	Electrospray mass spectrometry
FAME	Fatty-acid methyl ester
FDA	Food and Drug Administration, USA
FT-IR	Fourier transform infrared spectroscopy
g	Gram(s)
g	Gravitational constant
G	Guanine
G + C mol%	Mole percent guanine plus cytosine
HBC	High burden countries
HIV	Human immunodeficiency virus
HM	Hacène's Medium
h	Hour(s)
HTS	High throughput screening
I	Inosine
ISP	International *Streptomyces* Project
ITS	Internal transcribed spacer
kb	Kilobase(s)
kDa	Kilodaltons(s)
km	Kilometre(s)

l	Litre(s)
LFRFA	Low-frequency restriction fragment analysis
M	Molar
MA	Modified Aretz medium (broth)
MALDI-TOF	Matrix adsorbed laser desorption/ionization time-of-flight mass spectrometry
Mb	Megabase(s)
MC agar	Modified Czapek solution agar
MDR	Multi-drug resistant
mg	Milligram(s)
MIC	Minimum inhibitory concentration
min	Minute(s)
ml	Millilitre(s)
MLEE	Multilocus enzyme electrophoresis
MLSA	Multilocus sequence analysis
MLST	Multilocus sequence typing
mM	Millimolar
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTB	*Mycobacterium tuberculosis*
MTT	thiazolyl blue tetrazolium bromide
MW	Molecular weight
m/z	Mass-to-charge ratio
ng	Nanogram(s)
NMR	Nuclear magnetic resonance
NRPS	Non-ribosomal peptide synthetase
nt	Nucleotide(s)
OD	Optical density
OD_{600}	Optical density at 600 nm
OTU	Operational taxonomic unit
PAGE	Polyacrylamide gel electrophoresis
PBP	Penicillin-binding proteins
PCR	Polymerase chain reaction
PE agar	Plant extract agar
PFGE	Pulse-field gel electrophoresis
PKS	Polyketide synthase
PKS-I	Type-I polyketide synthase
PKS-II	Type-II polyketide synthase
PyMS	Curie-point mass spectrometry
R & D	Research and development
RAPD-PCR	Randomly amplified polymorphic DNA-PCR
RBR	Relative binding ratio
rDNA	Ribosomal deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
RIS-PCR	Ribosomal intergenic spacer-PCR
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT	Room temperature (20-22°C)
s	Second(s)
SC agar	Starch-casein agar
SEM	Scanning electron microscopy
SSC	Standard short course chemotherapy
Sub	Substrate mycelium

T	Thymine
TAE	Tris-acetate-EDTA buffer
TB	Tuberculosis
TE buffer	10 mM Tris-HCl, 1 mM EDTA, pH 7.8
Temp	Temperature
TGGE	Temperature gradient gel electrophoresis
TLC	Thin layer chromatography
T_m	Melting temperature
UK	United Kingdom
UPGMA	Unweighted pair group method with arithmetic mean
USA	United States of America
UV	Ultraviolet
V	Volts
Vol	Volume
v/v	Volume per volume
VRE	Vancomycin-resistant enterococci
w/v	Weight per volume
WHO	World Health Organization
XDR	Extensively drug-resistant
YEME	Yeast extract-malt extract (ISP 2) medium
Z	Area of the zone of inhibition in mm^2
λ	Lambda DNA
µg	Microgram(s)
µg/ml	Microgram(s) per millilitre
µl	Microlitre(s)
%	Percentage
°C	Degrees Celsius
1-D	One-dimensional
2-D	Two-dimensional
7H9	Difco Middlebrook 7H9 broth/agar

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CHAPTER 1

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1.1 Biodiversity and bacterial taxonomy

1.1.1 Microbial diversity

'Biodiversity is consequently essential, maintaining the stability of living systems around us, and ensuring that we survive the death, dung and detritus of contemporaneous members of our own species as well as of other organisms.'

D. L. Hawksworth & L. A. Mound, 1991

Although the term biodiversity is synonymous with the concept of species richness, it also encompasses all aspects relating to the distribution of these organisms and their interactions with the surrounding ecosystem (Bull *et al.,* 2000). In the past, biodiversity studies have tended to be biased towards the macroscopic world with little attention given to the humble microorganisms. A clear example of this disparity can be seen from the 1997 report on global biodiversity. At that time it was estimated that there were 1.87 million described species, of which 78% were terrestrial. However, only 8% of the described terrestrial species were microorganisms. It is only in the past decade that we have realised the true extent of their vast populations. It is conservatively estimated that there are 1.9 million microbial species (Bull, 1991). It is clear that true diversity belongs to the microorganisms (Nee, 2004). Only a miniscule fraction of the world's microorganisms (including bacteria, viruses, fungi and algae) have been characterised. In fact, it is estimated that less than 1% of the bacterial species have been described (Ward *et al.,* 1990).

Microorganisms play an invaluable role in maintaining the natural balance of any ecosystem. They degrade pollutants, waste and plant matter, releasing essential nutrients back into the environment. Some fungi are a food source for insects, forming the basis of the food chain in many habitats. Fungi and bacteria colonise the roots of plants, where they act as a form of biological pest control protecting the plant from pathogenic fungi (Colwell, 1997). They are used in the production of many of our foods, pharmaceuticals and chemicals. They are used for the treatment of our waste and in bioremediation (Arenskötter *et al.,* 2004). From a human standpoint, microorganisms are of great economic importance.

However, the negative aspects of microorganisms on human health and agriculture must not be overlooked. The most common cause of blight is *Fusarium graninearum* and crop loss due to blight infections in wheat and barley was estimated to be over $6 billion for the years 1991 to 1996 (World Health Organization, 1999), while the 1996 outbreak of bovine spongiform encephalopathy (BSE) in the United Kingdom infected over 150 000 cattle and brought the UK trade in livestock to a standstill. The 2004 World Health report on human health estimated that over 25% of all deaths in 2004 were due to infectious diseases. Of these 14.7 million deaths, over 2.3 million were due to infections of the respiratory tract (World Health Organization, 2004).

In the past, conservation efforts have focused mainly on preserving plants and animals. Biologists have disregarded microorganisms believing them to be cosmopolitan, besides which "microorganisms don't go extinct" (Bull, 1991). However, studies have shown bacteria are also endemic to ecological niches. The disappearance of a *Diospyros* species resulted in the extinction of the fungus *Penicilliopsis clavariaeformis* which had formed a symbiotic relationship with the tree (Bull, 1991). Fortunately, the need to preserve microorganisms has been recognised and the 1992 Rio Convention on Biodiversity included the conservation of microbial diversity (Colwell, 1997). Ultimately, the only way to conserve microorganisms is to protect the unique habitats in which they are found.

Sadly, many of the untapped sources of novel microorganisms have already been destroyed. Only 3.2% of the Earth's landmass is protected, and these areas are slowly being encroached on by human activities (Erwin, 1991). It is estimated that the deforestation of the tropical rainforests is between 16.4 and 20.4 X 10^6 hectares per annum (Colwell, 1997). Areas earmarked for 'microbial conservation' include those with large numbers of endemic fauna and flora, the so called "biodiversity hot spots", including the rainforests and coral reefs (Knight *et al.*, 2003; Strobel & Daisy, 2003).

On the other hand, it is not only the lush forests of the world that are the home to unique microorganisms. Diverse populations of microbes have been isolated from areas which were previously thought to be barren. The thermal springs of Yellowstone National Park are extremely inhospitable environments with temperatures of 95°C, slight alkalinity and high concentrations of iron and hydrogen sulphide. Yet they have been the source of novel bacteria and Archaea (Pace, 1997). Global warming also threatens entire populations of obligate psychrophiles which inhabit the Antarctic glaciers. Some of these microorganisms have been found to produce proteases which remain active at extremely low temperatures (Colwell, 1997; Staley, 1997).

The greatest diversity of microorganisms is probably found in the ocean. It is estimated that there may be more than ten million macrofaunal species inhabiting the deep sea floors (Bull *et al.*, 2000). Microorganisms living in this environment would have to adapt to high pressures (~1100 atmospheres), anaerobic conditions and temperatures of less than 0°C (Lam, 2006). The environmental constraints placed on the microorganisms inhabiting these unique niches are likely to be reflected in the secondary metabolites they produce.

It has been recognised by both the pharmaceutical industry and biotech companies that microorganisms are an untapped source of natural products. Bull *et al.* defined biotechnology as "the search for and the discovery of exploitable biology". The ability of biotechnology to fully exploit microorganisms hinges on a full understanding of microbial ecology and taxonomy (Bull *et al.*, 2000).

As the basis of any natural product screen is to maximize the chances of finding new compounds, biotech must take full advantage of the abundant microbial biodiversity. This understanding is based on the axiom that biodiversity is "an imperfect surrogate of chemical diversity, which is actually what should be maximized" (Peláez, 2006). However, natural product screens do not have to be limited to unique ecosystems. Almost all vascular plants have been found to be colonised by endophytic bacteria and fungi, while many metabolites thought to be produced by animals, including marine invertebrates, are actually of microbial origin (Jensen &

Fenical, 1994; Davidson *et al.*, 2001; Strobel & Daisy, 2003). These must not be overlooked as sources of novel natural products.

1.1.2 Bacterial taxonomy – the species concept

"The different kinds of bacteria are not separated by sharp divisions but by slight and subtle differences in characters so that they seem to blend into each other and resemble a spectrum"

S. T. Cowan, 1974

Gordon highlighted that this spectral nature of microorganisms resulted in difficulties in identifying characteristics that could be used to define the different strains of a species under investigation (Gordon, 1978). For eukaryotic taxonomists defining a species is relatively simple, as most organisms follow the dogma that species are "groups of interbreeding or potentially interbreeding natural populations that are reproductively isolated from other such groups" (Ravin, 1963). Prokaryotes, on the other hand, are not that obliging (Stackebrandt & Goebel, 1994).

Historically, microorganisms have been named either after the disease they cause or where they are found. This lead to some confusion as strains of a single species could be referred to by two or three different names (Gevers *et al.*, 2005). Early classification schemes were based on a few morphological features resulting in the boundaries between different species groups being blurred. With the advent of molecular biology, new methods were developed which can be used to classify microorganisms more fully. These techniques are used in population genetics and biodiversity studies, and have become the driving force behind bacterial taxonomy (Gevers *et al.*, 2005).

The ad hoc committee on reconciliation of approaches to bacterial systematics defined a bacterial species as a group of strains with "approximately 70% or greater DNA relatedness and with 5°C or less ΔT_m" and recommended that "phenotypic characteristics should agree with this definition" (Wayne *et al.*, 1987). This 70% DNA relatedness was considered to correlate to 97%16S rDNA sequence similarity. This definition has resulted in two methods, 16S rDNA sequence analysis and DNA-DNA hybridization (DDH) becoming the 'gold standards' in species delineation.

1.1.2.1 16S rDNA sequencing

The ability of rRNA gene sequences to infer phylogenetic relationships has been apparent since the 1980s. The use of rDNA sequence analysis for evolutionary studies is based on the fact that rRNA genes are highly conserved due to functional constraints, are ubiquitous, rDNA is not affected by environmental factors, it is not affected by horizontal gene transfer, and it has a shared common secondary structure (Rosselló-Mora & Amann, 2001). Traditional methods of sequencing (used in the 1980s) either required the direct sequencing of cloned segments or the indirect sequencing of the rRNA by reverse transcription. These methods were not only time consuming but also limited the length of sequence obtained. For these reasons the 5S rRNA gene was initially used as it is only 120 nucleotides (nt) in length. However, as comparisons based on short sequence lengths have limited resolving power, comparisons that could be made based on 5S rDNA sequence analysis were restricted to the genus level. With the development of automated DNA sequencing technology, sequencing of 5S

rRNA genes was superseded by the sequencing of 16S rRNA genes (Olsen, 1988). The full length of the 16S rRNA gene can now be sequenced quickly and inexpensively (Rosselló-Mora & Amann, 2001).
As defined by Wayne *et al.*, two species should share a 16S rDNA sequence similarity of less than 97%. As the length of the 16S rRNA gene is approximately 1540 base pairs (bp), this 3% variation equates to ~45 nucleotide differences between the two species. These nucleotide differences are not spread evenly throughout the gene, but are mostly confined to the three hypervariable regions. As multiple base substitutions more readily occur in these hypervariable regions, phylogenetic analysis based on short segments of the 16S rDNA can over- or under estimate the levels of relatedness, resulting in a false representation of the actual evolutionary relationships. For this reason it was proposed that all species descriptions include a 16S rDNA sequence of >1300 nt (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002).

Although 16S rDNA analysis is superior for comparison at the genus level and for well-resolved species, it lacks the sensitivity to recognise recently diverged species. This was exemplified in the study published by Fox *et al.*, where, although phenetically similar strains of psychrophilic bacteria, *Bacillus globisporus* W25^T and *Bacillus psychrophilus* W16A^T were found to share 99.8% 16S rDNA sequence similarity, they only shared 23.5% DNA relatedness (Fox *et al.*, 1992).

Some authors have commented that the 97% cut off is too high and might result in the underspeciation of genera containing species that are closely related. Keswani & Whitman found that the higher cut off of 98.6% 16S rDNA similarity could still differentiate between genospecies at the 99% confidence level (Keswani & Whitman, 2001), while in a 2006 paper Stackebrandt & Ebers recommended "a 16S rRNA gene sequence threshold range of 98.7% - 99% as the point at which DNA-DNA reassociation experiments should be mandatory" (Stackebrandt & Ebers, 2006). Ultimately, one should not overlook the fact that the values as defined by Wayne *et al.* for species delineation are 'man-made' boundaries and as such are not set in stone. Researchers should not define a species based solely on genomic information - phylogenetic clustering must be substantiated by shared phenetic features (Rosselló-Mora & Amann, 2001). There are numerous studies that demonstrate the relevance of classical taxonomic methods. The 1992 study published by Labeda illustrates this point. DNA-relatedness studies were performed on a number of numerically defined *Streptomyces* species clusters. The *Streptomyces cyaneus* cluster as defined by Williams *et al.* (1983a) was found to contain two major DNA-relatedness groups with a DNA relatedness of >70%. Morphological features clearly defined the two clusters as distinct species groups, the *Streptomyces coeruleorubidus* and the *Streptomyces purpurascens* groups (Labeda, 1992). As cautioned by Stackebrandt & Goebel, "in the end it is the presence or absence of phenotypic coherency among strains that should be the deciding factor about whether to describe species at all and whether to delineate species at the 60% or at the 80% DNA-DNA similarity level" (Stackebrandt & Goebel, 1994).

1.1.2.2 DNA-DNA hybridization

An intrinsic characteristic of nucleic acids is their ability to reassociate. The basic methodology behind DDH is relatively simple. The DNA from the two test strains is mixed and denatured. The mixture of single stranded DNA is allowed to reassociate under controlled conditions and forms a heteroduplex. As the extent of pairing is

dependent on the linear order of the nucleotides on the chromosome, more similar genomes will form more stable hybrids (Rosselló-Mora & Amann, 2001). DDH detects both similarities in the gene content and nucleotide similarity between the genomes (Vandamme *et al.*, 1996; Gevers *et al.*, 2005).

Two parameters are used to measure the level of DNA-relatedness: the difference in thermal denaturation midpoint (ΔT_m) and the relative binding ratio (RBR). The T_m is defined as the temperature at which 50% of the DNA is denatured. As a heteroduplex will have fewer paired bases compared to a native homoduplex, it is less stable and will denature at a lower temperature. The ΔT_m is the difference between the T_m of the homoduplex and that of the heteroduplex. The RBR measures the amount of double-stranded DNA present after reassociation, and compares this to the amount that is present in the homoduplex, defined as 100% reassociation (Rosselló-Mora & Amann, 2001). It has been found that thermal stabilities decrease by 1% to 2.2% for every 1% nucleotide mispairing (Vandamme *et al.*, 1996).

The threshold proposed by Wayne *et al.* was based on a large number of studies which showed a strong correlation between the DNA relatedness values and phenotypic similarity based on physiology, morphology and chemotaxonomy. As stated by Stackebrandt & Goebel, the correlation between DNA similarity and 16S rDNA homology is not linear. For meaningful comparisons at the lower relationship levels, family, genus and moderately related species, 16S rDNA sequence analysis is superior. The resolving power of DDH is greater for closely related strains and above the threshold of approximately 97.5% 16S rDNA similarity, DNA-DNA reassociation values may fluctuate from as low as 10% to 100% (Stackebrandt & Goebel, 1994). It is estimated that heteroduplexes will only form when the DNA is 80% complementary, therefore DDH measures the variability in the remaining 20% of the genome and correlates this to 0% to 100% DNA-DNA relatedness (Rosselló-Mora & Amann, 2001).

There are a number of different DDH methods, including the hydroxyapatite, optical renaturation rate and S1 nuclease methods (Vandamme *et al.,* 1996). It has been reported that the values obtained for the RBR may vary depending on the hybridization method used (Grimont *et al.*, 1980). The main disadvantages of DDH are that it is time consuming, relatively expensive and, as it requires a high level of technological expertise to generate meaningful results, it is frequently performed by specialized laboratories. These drawbacks result in researchers only performing a limited number of comparisons. As it also requires a large amount of relatively high quality DNA, it can only be used to characterise culturable microorganisms. As the data generated is comparative, it prohibits the generation of a centralised database which can be accessed by other laboratories (Vandamme *et al.*, 1996; Gevers *et al.*, 2005; Stackebrandt & Ebers, 2006). Another drawback of this method is that it assumes all prokaryotes evolve at the same rate (Stackebrandt & Goebel, 1994; Keswani & Whitman, 2001).

With recent advances in molecular biology, alternative methods to determine the DNA relatedness between bacterial genomes have been developed. Gonzalez & Saiz-Jimenez (2004) proposed a novel method of using SYBR Green I and real-time polymerase chain reaction (PCR) technology to compare bacterial genomes. Using this method they were able to differentiate between species of the hyperthermophilic genera *Pyrococcus* and *Thermococcus*. The advantages of this method are that it is rapid and relatively inexpensive, which are two factors that often impede the investigation of a large number of species. The validity of this method still needs to be confirmed in other genera (Gonzalez & Saiz-Jimenez, 2004).

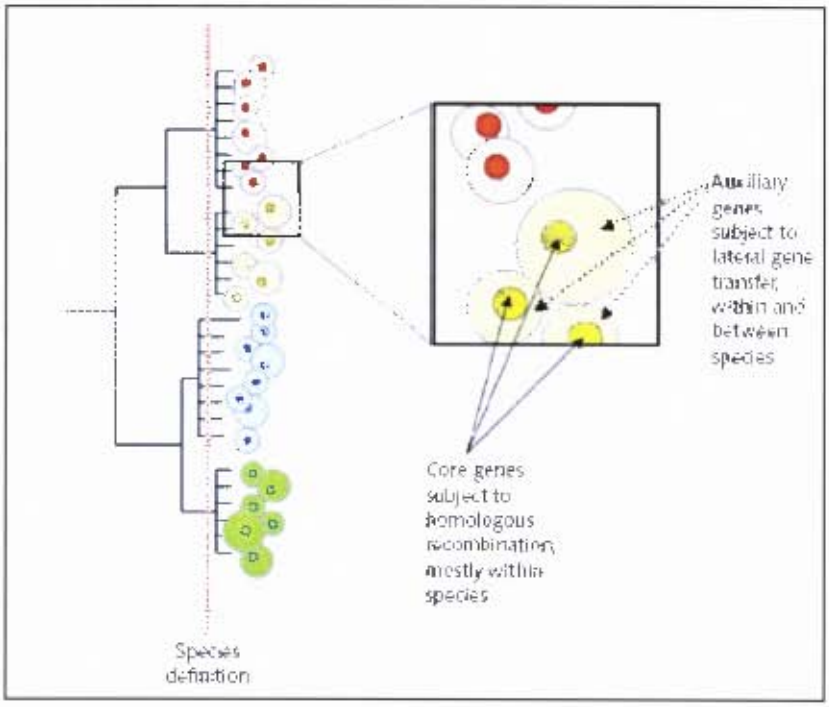
1.1.2.3 Future methods – Average nucleotide identity

There are nearly 300 fully annotated bacterial genomes available in public databases. Due to the cost involved, the vast majority of the genomes sequenced are from bacteria of medical relevance or are industrially important species. With the invention of genome sequencers, it may soon be relatively inexpensive to sequence an entire bacterial genome (http://www.454.com/enabling-technology/index.asp). Some researchers have questioned how this wealth of information can be pragmatically integrated into our current classification schemes (Rosselló-Mora, 2006).

A method to compare the gene content of bacterial genomes was proposed by Konstantinidis & Tiedje (2005). They determined the average nucleotide identity (ANI) for 70 fully sequenced genomes from closely related species. The ANI compares the gene content between genomes and it was found that an ANI of approximately 94% correlated to 70% DNA-DNA reassociation. One surprising finding was that the gene content between strains of the same species can vary by up to 30%. This variation in gene content could be attributed to the different ecological niches inhabited by the strains. As the ANI is obtained from the comparison of a large number (greater than 1000) lineage specific genes, the phylogenetic relationships identified are robust and well resolved (Konstantinidis & Tiedje, 2005). A subsequent study by Goris *et al.* recommended an ANI of 95 ±0.5% would correspond to 70% DNA-DNA relatedness and 69% conserved DNA. At this level two strains were found to share 85% conserved genes when the analysis was limited to the protein-coding portions of the genomes (Goris *et al.*, 2007). In general, ANI was found to be a robust and pragmatic approach to determining phylogenetic relationships. As more complete bacterial genome sequences become available, the application of this method may be used to resolve interspecies relationships in environmental taxonomy and epidemiological studies (Konstantinidis *et al.*, 2006). The one disadvantage of ANI is that it is restricted to species whose genomes have been fully sequenced. Within the class *Actinobacteria* this would be limited to a few species including a number of *Corynebacterium* (6), *Frankia* (2), *Mycobacterium* (16), *Nocardia* (1), *Nocardioides* (1) *Rhodococcus* (4), *Saccharopolyspora* (1) and *Streptomyces* (2) (the number of fully annotated genomes available within each genus is given in parentheses) (http://www.ncbi.nlm.nih.gov/genomes/static/eub.html). The complete genome of *Salinospora tropica* has recently been published (Udwary *et al.*, 2007).

As a 30% difference in gene content over an entire genome equates to over 1500 different genes, Lan & Reeves proposed the concept of a 'species genome'. A species genome is defined as all the genes found in strains of that species and is comprised of the core and auxiliary genes (Figure 1.1). The core genes are present in 95% or more of the strains and are responsible for the characteristic features of that species, while the auxiliary genes are found in 1%-95% of the strains and can be dependent on specific ecological niches. Theoretically, the core genes should be less 'prone' to horizontal gene transfer, while the divergence observed in the auxiliary genes could be due to the horizontal gene transfer of large DNA fragments including pathogenicity islands or the acquisition of phages and transposons (Lan & Reeves, 2000; Coenye *et al.*, 2005; Doolittle, 2006).

Figure 1.1 Graphic representation of the 'species genome' concept. This concept takes into account the microdiversity (intercluster nodes which are more closely related) seen in clusters below the species level (red hatched line). (Figure taken from Doolittle, 2006).

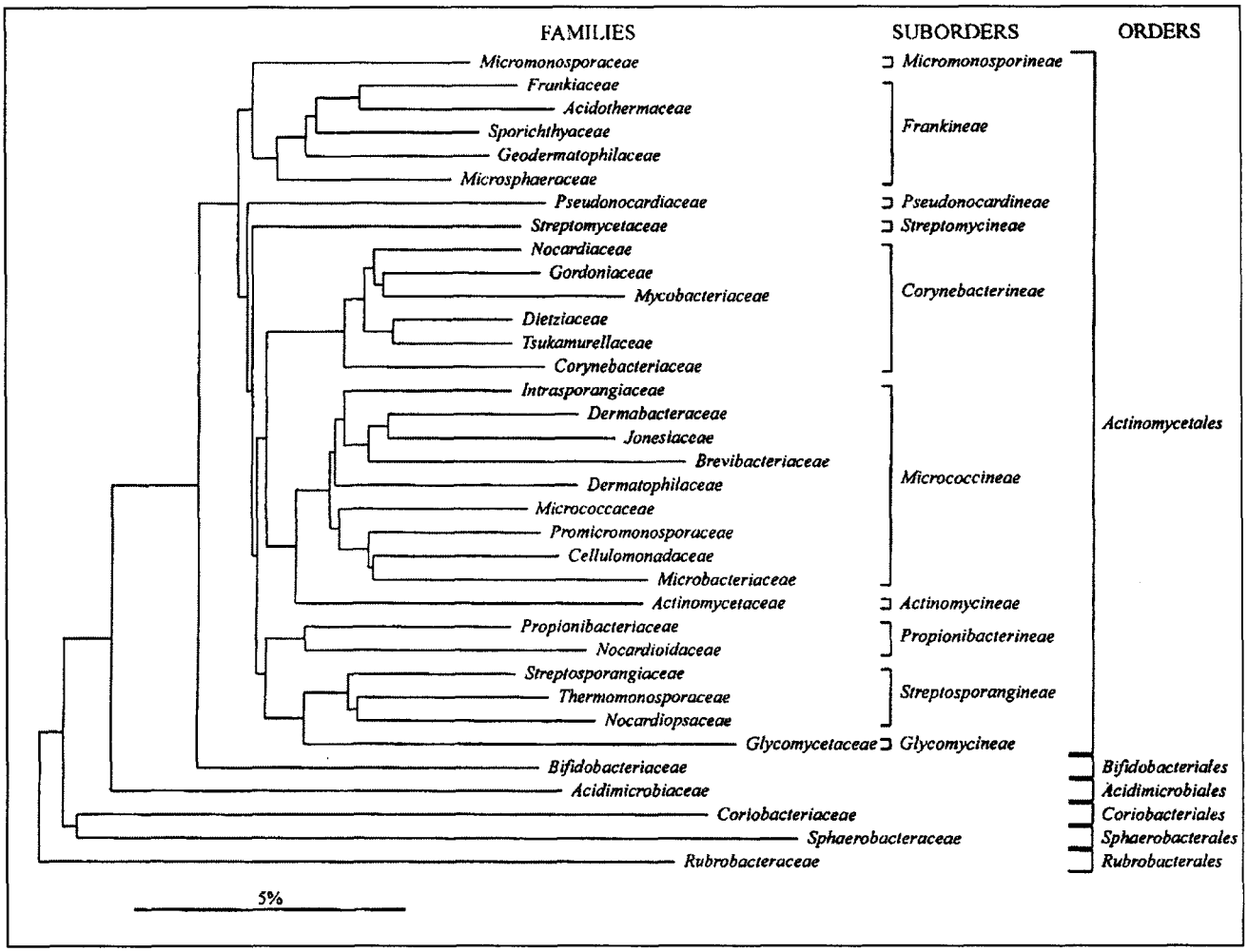


As some taxonomists still debate whether the current species definition accurately reflects the natural relationships between bacteria, a number of additional terms to define a species have been suggested including: *ecotype* – populations of genetically coherent but ecological distinct strains; *genomic species* – strains that share a high level of DNA relatedness but differ phenetically; *nomenspecies* – strains that have been grouped by numerical taxonomy; and *taxospecies* – groups of strains which form a coherent cluster based on phenotypic similarity (Stackebrandt & Goebel, 1994; Vandamme *et al.*, 1996; Rosselló-Mora & Amann, 2001; Gevers *et al.*, 2005). Others have proposed that bacteria should not be defined as a species at all but rather as operational taxonomic units (OTU), where an OTU is defined as any unit that is convenient for the required purpose, be it an entire population, a species or a cohort of subspecies (Rosselló-Mora & Amann, 2001).

1.2 Actinomycetes

According to the definitive study by Woese *et al.*, the class *Actinobacteria* clusters with the bacteria with a guanine plus cytosine content of greater than 50 mol% (Stackebrandt *et al.*, 1997) (Figure 1.2). Historically, actinomycetes have been confused with the Fungi Imperfecti or deuteromycetes. Even the name actinomycete is derived from the Greek *actinos*-ray and *mycete*-fungus.

Figure 1.2 Intraclass relatedness of *Actinobacteria* showing the presence of six orders as well as the 10 suborders of the order *Actinomycetales* based upon 16S rDNA sequence comparisons. The scale bar represents 5 nucleotide substitutions per 100 nucleotides. (Figure taken from Stackebrandt *et al.*, 1997).



Actinomycetes belong to class *Actinobacteria*, order *Actinomycetales*. As defined by *Bergey's Manual of Systematic Bacteriology* (2nd edition, 1989), actinomycetes are classified as filamentous bacteria. The classical defining features of the group are that they are Gram positive and form branching hyphae, which either remain as a stable mycelium or fragment into rod- or coccoid-shaped elements; most are aerobic. If cell motility is observed, it is due to flagella (Lechevalier, 1989). Actinomycetes are considered to be terrestrial and marine organisms (Goodfellow & Williams, 1983; Mincer *et al.*, 2002). Economically, their importance is not limited to the production of antibiotics. They produce an array of other secondary metabolites that can be used by the

pharmaceutical industry including anti-tumour drugs and immunosuppressives. Actinomycetes also produce vitamins and pigments that are used in the food industry. Members of the genus *Gordonia* are used for the treatment of raw sewage and petrochemicals (Arenskötter *et al.*, 2004).

1.2.1 Methods in actinomycete taxonomy – a polyphasic approach

In the past, actinomycete taxonomy was heavily weighted towards the phenotypic characterisation of species, first by morphological taxonomy then by chemotaxonomy. The introduction of numerical taxonomy in the 1960s was seen as the solution to many of the problems that had been plaguing actinomycete taxonomy. As this classification scheme is based on a large number of phenetic characteristics it was seen to be less subjective than previous methods (Goodfellow *et al.*, 1992). The main disadvantage of numerical taxonomy is that the traits selected to classify stains into phena may bias the frequency matrix that is generated and many readily observable features should be avoided. With the advent of molecular biology, numerical classification is rarely used in modern taxonomic studies.

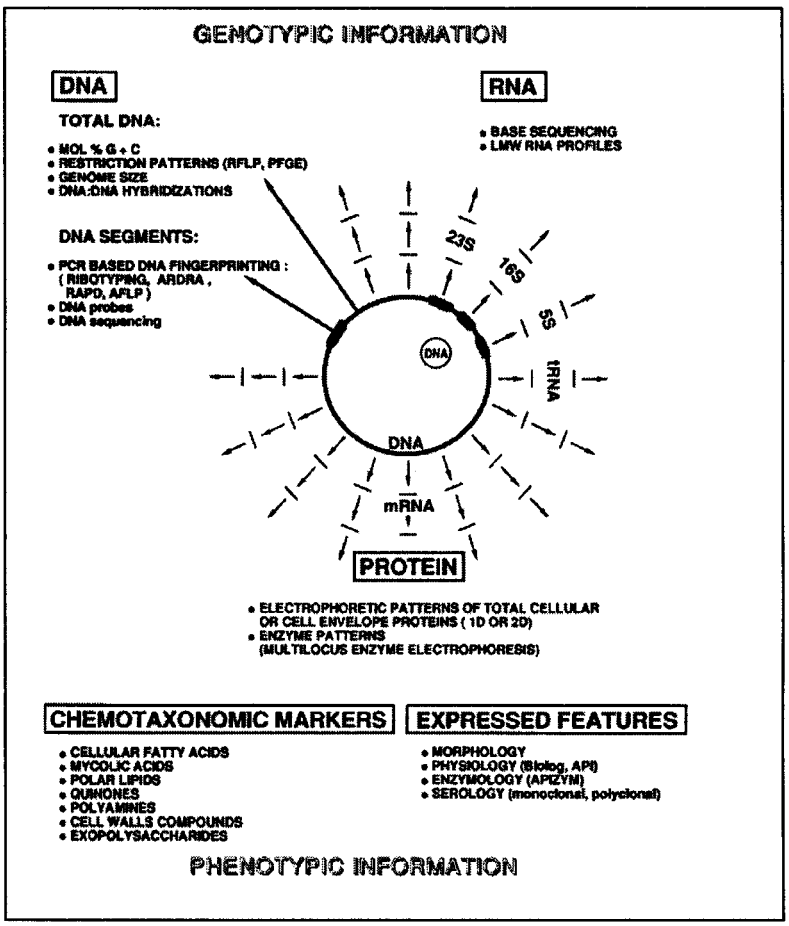
With the 'birth' of the genomics era traditional methods have been complemented by an assortment of genotypic methods (Rosselló-Mora, 2006). The integration of genotypic, phenotypic and phylogenetic studies is termed polyphasic taxonomy (Figure 1.3). Genotypic data includes all information that is derived from the nucleic acids, either directly or indirectly. Phenotypic traits include all the features that can be observed about a microorganism. Phenotypic taxonomy is concerned with the expression of genes and includes all information derived from proteins; their cellular functions, chemotaxonomic markers and other expressed features (Vandamme *et al.*, 1996; Rosselló-Mora & Amann, 2001). It is felt that "bacterial classification should reflect as closely as possible the natural relationships between bacteria, which are the phylogenetic relationships as encoded in 16S and 23S rRNA sequence data" and that this is best achieved when using a polyphasic approach to taxonomy (Vandamme *et al.*, 1996).

1.2.1.1 Genomic methods

The fact that so many current taxonomic studies are biased towards genomic methods is a reflection of our increased understanding of molecular biology and improved techniques. The one advantage of genomic taxonomy is that some methods can be used for the identification of both culturable and unculturable strains (Rosselló-Mora & Amann, 2001). Genomic taxonomy can be subdivided into whole genome methods and gene specific methods. Three different types of genes are investigated: *Informational genes* are ubiquitous within a population and the gene products encoded are essential for cellular functioning; they include genes involved in transcription, translation and regulatory pathways. Informational genes have a slow evolution rate and can be used for the phylogenetic analysis at the family and genus level. The 16S rRNA gene is an example of an informational gene. *Housekeeping genes* are ubiquitous within a population. They code for proteins which are essential for central metabolism. They have a moderate evolution rate and can be used to distinguish phylogenetic relationships at the species level. The *gyrB* gene is an example of a housekeeping gene. *Hypervariable genes* are not ubiquitous within populations. Many of the gene products they code for are niche

specific and include virulence factors and surface proteins. Their fast evolution rate allows for differentiation at the subspecies level (Cooper & Feil, 2004).

Figure 1.3 Diagrammatic representation of the polyphasic approach to taxonomy.



Abbreviations: ARDRA, amplified rDNA restriction analysis; LMW, low molecular weight; PFGE, pulse-field gel electrophoresis. Other abbreviations are defined in the text. (Figure taken from Vandamme *et al.*, 1996).

1.2.1.1.1 Whole genome methods

Mole percent guanosine plus cytosine

Determining the G + C content of a bacterium's genome was probably the first genomic method used in taxonomy. The G + C content in actinomycetes generally ranges from 68-72 mol%. The accepted variation in the G + C content within a species is 3% and 10% at the genus level. According to the ad hoc committee for the re-evaluation of the species definition in bacteriology, it is not essential to determine the G + C mol% for the valid description of a new species, but it must be determined for the type species of a new genus (Stackebrandt *et al.*, 2002). It is important to be aware of the fact that the phylogenetic relatedness between species can not be inferred by a having a similar G + C content, as this method simply measures the composition of the DNA, not the actual sequence of the nucleotides (Rosselló-Mora & Amann, 2001).

Whole-genome restriction fragment analysis

As originally described, this method involved the digestion of whole genome DNA with restriction enzymes followed by fragment separation by agarose gel electrophoresis. The resulting pattern is due to polymorphisms in the DNA generating restriction fragments of varying lengths. The disadvantage of this method is that complex patterns are obtained, which may be difficult to interpret. This method was replaced by low-frequency restriction fragment analysis (LFRFA), which uses a selection of 6- or 8-base cutter restriction enzymes that infrequently cut the genome. As actinomycetes are GC-rich, AT cutters that recognise rare recognition sites are commonly used. However, due to the large size of the generated fragments, standard agarose gels can not be used. LFRFA is combined with pulse-field gel electrophoresis (PFGE). The resulting banding pattern has improved resolution and allows for the differentiation of bacterial species. This method is frequently used in population genetics studies (Vandamme *et al.,* 1996; Anderson & Wellington, 2001). Beyazova and Lechevalier used the combined method of LFRFA-PFGE to investigate the application of this method in streptomycete taxonomy. The genomic DNA of 59 strains (eight species and the numerically classified *S. cyaneus* group) was digested with *Ase*I. Eight clusters of two to ten strains were identified with a similarity of greater than 80%. Seventeen strains were unclustered, which unexpectedly included all six *S. cyaneus* strains. The largest cluster contained ten strains which included all six *Streptomyces albus* strains. They concluded that although the congruence between physiological classification (numerical) and LFRFA similarity was variable, there was a good correlation between LFRFA similarity and levels of DNA-DNA relatedness. This probably reflects the fact that both DNA-DNA reassociation and LFRFA compare similarities between the entire genome while physiological testing only reflects the difference in a smaller, defined section of the genome (Beyazova & Lechevalier, 1993). LFRFA-DGGE can be combined with Southern blot hybridization to determine the organisation of the genome including the order of genes or the number of operons (Rosselló-Mora & Amann, 2001)

Microarray technology

Due to their sensitivity, microarrays can be used to detect a large number of polymorphisms within a bacterium's genome. However, the large amounts of data generated can be problematic and very time consuming to analyse. These factors, together with the cost, limit their use in actinomycete taxonomy (Gürtler & Mayall, 2001). Microarrays have been used to discover new biosynthetic gene clusters within different actinomycete strains and to identify mutations that result in increased antibiotic production (Lum *et al.,* 2004). Behr *et al.* published a method of using a microarray to identify gene insertions and deletions. They constructed an array of the fully sequenced genome of *M. tuberculosis* H37Rv^T and probed it with the genomes of wild type *Mycobacterium bovis* and attenuated *M. bovis* BCG vaccine strains. This method allowed them to identify genes that were common to all strains and also identify deletions in the genomes of the various *M. bovis* strains (Behr *et al.*, 1999). As there are a number of fully annotated actinomycete genomes available, this technique could be useful in identifying the presence or absence of gene clusters between sequenced strains that produce antibiotics such as *S. coelicolor* and novel isolates.

The main advantage of whole genome methods, compared to gene specific methods, is that the results obtained from whole genome comparisons are less affected by horizontal gene transfer. Homologous recombination was

once thought to be a rare event in prokaryotes, only occurring between very closely related species and had little impact in defining their genomes. Recent studies have revealed the actual extent of homologous recombination in bacteria and it has been found to occur between genomes of related species with up to 25% sequence divergence between homologous genes (Rosselló & Amann, 2001).

1.2.1.1.2 Gene specific methods

Multilocus sequence typing and multilocus sequence analysis

Housekeeping genes evolve slowly by the random accumulation of neutral mutations. As these genes evolve quicker than rRNA genes, they can be used to delineate strains to the species level. Based on an analysis of 44 sequenced genomes, Zeigler identified 32 protein-encoding genes which could potentially be used for species delineation. The *recN*, *rpoA* and *thdF* genes were found to be good predictors of whole genome relatedness. Ultimately, the *recN* gene was identified as being the most discriminatory (Zeigler, 2003). Santos & Ochman identified 39 genes that were universally conserved in bacterial genomes and designed PCR primers that could be used to amplify ten of these genes including *gyrB*, *fusA* and *lepA* (Santos & Ochman, 2004). Only *recA*, a recombination-repair gene, was common to both studies. Due to the variability of these genes it may not be possible to design universal primers and genus-specific primers may have to be designed. The gene of interest is sequenced and phylogenetic analysis performed. The one disadvantage to using protein coding genes is that prior knowledge of the gene sequence is required to design primers.

Most forms of phylogenetic analysis are based on a multiple sequence alignment, which organises sequences based on homologous residues. No distinction is made between homology, which infers a common ancestry, and sequence similarity, which may imply a common ancestry or may be due to chance (Olsen & Woese, 1993). Three algorithms are routinely used; distance matrix, maximum parsimony and maximum likelihood, which are based on different evolutionary models. The topography of the tree may vary depending on the model used (Rosselló-Mora & Amann, 2001). There are a number of programs that use distance matrices: unweighted pair group method with arithmetic mean (UPGMA) and neighbour-joining are two commonly used methods. In both these models, the distance between two sequences is the fraction of sites that differ. The multiple sequence alignment used to construct a distance matrix tree is based either on the clustering of taxa into nodes (UPGMA) (Sneath & Sokal, 1973) or directly calculates the distances between nodes (neighbour-joining) (Saitou & Nei, 1987). The maximum parsimony model assumes that the most likely tree requires the least number of changes to generate the observed alignment, while maximum likelihood models infer a tree based on an alignment that maximizes the probability or likelihood of observing the data (Hall, 2001).

Although there is sufficient allelic variation in the sequence of a single protein coding gene to differentiate between species, it lacks the sensitivity to resolve strain relationships. Another disadvantage of single locus sequencing methods is that recombination of the allele may distort the results (Spratt, 1999). Intraspecies relationships can be resolved by comparing a number of housekeeping genes, with the added benefit that the effects of homologous recombination at single loci will be "buffered" (Gevers *et al.*, 2005).

Multilocus sequence typing (MLST) was developed for epidemiological studies of the human pathogen, *Neisseria meningitidis* (Maiden *et al.*, 1998). MLST compares the allelic variation in a small number (usually

seven) housekeeping genes by directly sequencing short internal fragments (Maiden *et al.*, 1998; Gevers *et al.*, 2005). The three criteria to consider when selecting the genes to use for MLST analysis are: the genes should be distributed throughout the genome, each gene must be present in a single copy, and the selected genes should be ubiquitous. The amplified fragment is usually 450-500 bp as this allows both strands to be accurately sequenced with a single pair of primers. The corresponding sequences for each gene are directly compared and any variation is considered to be a unique allele. A matrix of pairwise differences between the allelic profiles is then used to construct a dendogram (Maiden *et al.*, 1998; Gürtler & Mayall, 2001).

Gevers *et al.* proposed the introduction of the term 'multilocus sequence analysis' (MLSA) which is described as "a method for the genotypic characterisation of a more diverse group of prokaryotes (including entire genera) using the sequences of multiple protein-coding genes". As in MLST, short internal fragments from several housekeeping genes are amplified and sequenced. These sequences are concatenated and this chimeric-gene sequence is used to construct a phylogenetic tree (Gevers *et al.*, 2005). A two-pronged approach was proposed which could ultimately be used to identify an isolate. Firstly, the 16S rDNA would be sequenced to assign the isolate to a genus. In the second step, MLSA would be performed using primers specific for that genus to assign the isolate to a particular species. Like MLST, this method also allows for the construction of databases (Gevers *et al.*, 2005). Both methods are commonly referred to as MLST. The difference between the two methods is that MLST compares the allelic variation between strains by performing cluster analysis, while MLSA analyses the sequences which are used to construct a phylogenetic tree. The idea that MLST (and MLSA) could be used to "revolutionise systematics" is shared by Cooper & Feil (2004).

A study by Devulder *et al.* has shown that the MLSA concept is technically sound (although in the paper the method is described as MLST). They applied this method to the genus *Mycobacterium*. The 16S rDNA similarity values between species in this genus range from 94.3% to 100%. They amplified and sequenced a short fragment for four genes from 81 type strains. The genes investigated were: 16S rRNA (564 bp), *hsp65* (420 bp), *rpoB* (396 bp) and *sod* (408 bp). These sequences were concatenated and the resulting 1788 bp fragment was used to determine the global phylogeny within this genus. This global tree was compared to those obtained for the four genes singly. It was found that the concatenation of sequences increased the resolution and robustness of the global tree, with each species, apart from the *Mycobacterium tuberculosis* complex, being differentiated as a distinct species. The deep node bootstrap values also increased with over 60% of the nodes having a bootstrap of above 50%, compared to the 16S rDNA tree where only 35% of the nodes had a bootstrap above 50% (Devulder *et al.*, 2005).

The lack of housekeeping gene sequences for 'non-medical' bacteria may be the one hindrance in the widespread application of this method (Stackebrandt, 2003). Until the genomes of more diverse organisms become available, this method will be limited to the few sequenced genes that offer conserved sites for primer design (Konstantinidis *et al.*, 2006). Nevertheless, there have been a number of recent studies using housekeeping genes to infer the phylogenetic relationships of different genera in the Class *Actinobacteria*. Kim *et al.* used the *rpoB* gene for the phylogenetic analysis of the genera *Streptomyces* and *Kitasatospora* (Kim *et al.*, 2004), *gyrB* has been used in the genera *Gordonia* (Shen *et al.*, 2006a) and *Micromonospora* (Kasai *et al.*, 2000), while Yoon & Park studied the *RNase P* gene in actinomycetes with LL-DAP (Yoon & Park, 2000). An

extension of the work by Kim *et al.* has recently been published by Mun *et al.* (2007). They found that the *rpoB* gene can be used to differentiate between *Streptomyces* strains that cause potato scab disease. Generally, relationships inferred from *rpoB* gene analysis correlated with those based on the 16S rRNA gene (Mun *et al.*, 2007).

Oligonucleotide probes

The order of the three bacterial rRNA genes, 16S-23S-5S, is highly conserved. Stackebrandt *et al.* identified sites in the *Streptomyces* 16S and 23S rDNA that were used to design genus- and species-specific probes. The three variable regions identified in the 16S rRNA genes are between nucleotides 158 and 203 (γ-region) (*S. coelicolor* strain A3(2) numbering), nucleotides 982 and 998 (α-region) and nucleotides 1102 and 1122 (β-region). The γ-region is highly variable and was used to design *Streptomyces* species-specific probes, while the α- and β-regions are less variable and were used to design *Streptomyces* genus-specific probes. A highly variable region between nucleotides 1518 and 1645 of the 23S rDNA was also used to design *Streptomyces* species-specific probes. Stackebrandt *et al.* proposed that these variable regions could be used to design primers that could rapidly identify an isolate to the genus, species or strain level and to investigate the structure of a bacterial community (Stackebrandt *et al.*, 1991). Genus- and species-specific primers for the rapid identification of actinomycete isolates have been designed for a number of genera including *Gordonia (*de los Reyes *et al.*, 1997), *Saccharomonospora* (Salazar *et al.*, 2000) and *Streptomyces* (Mehling *et al.*, 1995).

PCR-based fingerprinting

There are a number of PCR-based methods which generate a DNA fingerprint and do not require the sequencing of the amplified product. The advantages of these methods are that they are rapid and allow for the comparison of a large number of isolates simultaneously. The main drawback is that some methods require standardised conditions which must be strictly adhered to for meaningful comparisons to be made. Fingerprinting methods include AFLP, BOX-PCR, RIS-PCR and RFLP. They can generally be used to identify an isolate to the species level, however, with the automation of some of these techniques, intraspecies comparisons can be made. Relationships inferred from the fingerprint usually correlate to those obtained from traditional phenetic and genomic methods. Although these methods are routinely used for the identification of medical isolates, they are not widely used for the taxonomic studies of actinomycetes. The one disadvantage of some of these PCR-based fingerprinting methods is that complex banding patterns are obtained which require specialised software for gel analysis, such as Bionumerics® and True Allele II™ (http://www.biosystematica.com/bionumer.htm; http://www.cybergenetics-inc.com).

Amplified fragment length polymorphism

Amplified fragment length polymorphism (AFLP) can be used in taxonomy, clinical diagnostics and epidemiological studies to distinguish between strains at the genus, species and strain level. AFLP begins with the digestion of total genomic DNA with specific restriction enzymes. These fragments are then ligated to specific adaptors. This is followed by the selective PCR amplification of the restriction fragments with attached adaptors. The PCR is performed at a low annealing temperature to allow primer annealing to multiple sites. This is followed by gel electrophoresis of amplified products on polyacrylamide gels (Vos *et al.*, 1995; Vandamme *et al.*, 1996). The development of fluorescent AFLP lead to the automation of this method and the

digitized fingerprints can be used to construct a database. The ability to exchange data between laboratories is invaluable for epidemiological studies. Like many fingerprinting methods, no prior knowledge of the sequence is required. The disadvantages of AFLP analysis are that it is dependent on reaction conditions, DNA quality and annealing temperature (Vos *et al.*, 1995). Although generally used in the clinical setting, AFLP analysis can be used in actinomycete taxonomy. Zhao *et al.* used fluorescent AFLP to differentiate between the twenty *Micromonospora* strains they isolated from a Welsh soil sample. The AFLP fingerprint for each isolate typically contained 100 fragments ranging in size from 50 to 500 bp. All the *Micromonospora* isolates had a unique fingerprint and only a few common bands were identified. Generally, there was a good correlation between the phylogenetic analysis of the AFLP fingerprints and that of the 16S rDNA. However, cluster analysis of the AFLP fingerprints found that very few nodes had significant bootstrap values compared to the 16S rDNA sequence tree. Although this study showed that the 20 isolates could be distinguished as unique, the AFLP fingerprints obtained for these strains were not compared to the fingerprints of the type strains of the genus *Micromonospora*. Therefore, the isolates could not be identified to the species level (Zhao *et al.*, 2004).

BOX-PCR

For BOX-PCR analysis, outwardly facing primers are designed which are complementary to repeat elements that are interspersed throughout the genome. A number of different sized DNA fragments will be amplified consisting of the sequences that lie between these repeat elements. These products are separated on a gel generating a genomic fingerprint. The 2004 study by Lanoot *et al.* showed that BOX-PCR is sensitive enough to discriminate between highly related genomes. They used this method to screen 473 *Streptomyces* type strains. Three hundred and fifty strains yielded a unique banding pattern, while 75 strains were grouped into one of the 30 clusters of strains with highly similar patterns. DNA-DNA reassociation experiments were performed on seven of these clusters. The DNA relatedness for all the strains within each cluster was higher than 80% which suggests that these strains are subjective synonyms (Lanoot *et al.*, 2004).

PCR-DGGE

Heuer *et al.* combined PCR using specific primers and denaturating gradient gel electrophoresis (DGGE) to analyse the actinomycete communities from a number of environmental samples. In this study, they used both DGGE, which denatures the DNA by chemical means (urea and formamide) and temperature gradient gel electrophoresis (TGGE), where increased temperature results in the denaturation of the DNA. The fingerprints generated by these methods are reported to be similar. The advantage of this fingerprinting method is that the band of interest can be studied further by probing or the band can be excised from the gel and sequenced (Heuer *et al.*, 1997). PCR-DGGE has also been used by others to study other ecosystems, such as tidal flats (Stevens *et al.*, 2007).

Randomly amplified polymorphic DNA

The principle behind randomly amplified polymorphic DNA (RAPD)-PCR is that polymorphisms in DNA can be identified by using a single primer of arbitrary sequence that will, under non-stringent conditions, anneal randomly to the DNA and amplify fragments of varying length. The resulting profile can be used to differentiate between isolates. The advantage of this method is that no prior knowledge of the DNA sequence is required, so it can be used to identify unknown strains (Anderson & Wellington, 2001).

Mehling *et al.* used RAPD-PCR to amplify an approximately 1100 bp actinomycete-specific fragment from 27 actinomycete species. This fragment was found to contain the conserved 3' end of the 23S rRNA gene, the variable intergenic region and the putative start of the 5S rRNA gene. It was found that the conserved region could be used to design actinomycete and streptomycete-specific probes for subsequent hybridization experiments, which would allow for the identification of unknown actinomycete strains to the genus level. The main disadvantage is that RAPD-PCR requires specific conditions. Mehling reported that they initially used *Taq* and Vent (New England Biolabs) polymerase but could only amplify the 1100 bp band when using Vent polymerase. They concluded that this may be due to the fact that Vent is a high-fidelity thermophilic DNA polymerase with a 3'→5' proofreading exonuclease activity (Mehling *et al.*, 1995; http://www.neb.com/nebecomm/products/productM0254.asp). Other authors have also noted that specific conditions are required for RADP-PCR and that slight alterations to the reaction conditions may result in no amplification (Anderson & Wellington, 2001).

Restriction fragment length polymorphisms

Restriction fragment length polymorphism (RFLP) analysis is a fingerprinting method that involves the specific PCR amplification of the gene of interest, followed by digestion of the amplicon with restriction endonucleases. The digested fragments are separated on a polyacrylamide or agarose gel and visualised. In taxonomic studies, either the 16S or 23S rRNA gene is generally used for RFLP analysis as it can differentiate between strains at the genus level (Vandamme *et al.*, 1996; http://en.wikipedia.org/wiki/RFLP). Lanoot *et al.* used RFLP fingerprinting to investigate the phylogenetic relatedness between 463 *Streptomyces* and *Kitasatospora* type strains. They designed primers that amplified the 16S rDNA and adjacent 16S-23S internal transcribed spacer (ITS). The two restriction endonucleases used for the RFLP analysis, *Bst*UI and *Hae*III, have a GC-rich recognition sequence and generated a high number of unique bands (10-15 bands) per strain. A total of 59 clusters containing multiple strains were delineated. It was found that there was a good correlation between the RFLP results and those obtained from 16S rDNA sequence analysis. Due to the variability of the ITS, this method had a higher resolution than the 16S rDNA analysis (Lanoot *et al.*, 2005). Cook & Meyers developed a method whereby filamentous actinomycetes could be rapidly identified to the genus level by the sequential single-enzyme digestion of the amplified 16S rRNA gene with selected restriction endonucleases (Cook & Meyers, 2003). Amplified rDNA restriction analysis (ARDRA) has also been used to identify thermophilic actinomycetes that cause hypersensitivity pneumonitis. Digestion of partial 16S rDNA fragments with *Taq*I and *Hha*I combined with selected physiological tests allowed for the identification of *Saccharopolyspora*, *Saccharomonospora* and *Thermoactinomyces* from environmental samples (Harvey *et al.*, 2001)

RIS-PCR

As the ribosomal intergenic spacer (RIS) region evolves at a faster rate than the rRNA genes, analysis of the RIS can be used to discriminate between species. The advantage of RIS-PCR is that the flanking rRNA genes are highly conserved so allow for the design of specific primers. Park and Kilbane (2006) combined RIS-PCR with DGGE to generate *Streptomyces*-specific fingerprints, which could identify isolates to the species level. They designed PCR primers to the 16S-23S rRNA-RIS. As the forward primer bound to the 16S rRNA gene between nucleotides 1197-1213, the amplified RIS also included 350 bp of the 3' end of the 16S rRNA gene. This could be sequenced and used for 'traditional' 16S rDNA sequence analysis. This approach successfully

differentiated between all the *Streptomyces* species they tested and was able to amplify the desired fragment from DNA isolated from a soil sample (Park & Kilbane, 2006).

Ribotyping

There are typically six copies of each rRNA gene present in *Streptomyces* (seven have been identified in *Streptomyces venezuelae*) (Wenner *et al.*, 2002). Ribotyping is similar to other fingerprinting techniques in that total genomic DNA is digested with restriction enzymes and the resulting fragments are separated on a gel. This is followed by Southern blotting with 16S or 23S rRNA-gene probes to generate a DNA banding pattern. This technique can be performed manually or by robot and is commonly used for the identification of clinical isolates (McNeil & Brown, 1994). Automated riboprints are highly reproducible and sufficiently sensitive to identify an isolate to the species and subspecies level (Stackebrandt, 2003). Due to the cost of the equipment, this method is not routinely used for identification of isolates in non-medical settings. However, it is offered as a service by a number of institutions including the *Deutsche Sammlung von Mikroorganismen und Zellkulturen* GmbH (DSMZ), Braunschweig, Germany (http://www.dsmz.de/identification/).

1.2.1.2 Phenotypic characterisation – physiology, morphology and chemotaxonomy

**"One requires patience when working with actinomycetes,
and one must have the ability to plan and run several experiments
concurrently to avoid wasting time."**

T. Cross, 1989

Phenetic characterisation has been described as "classical" taxonomy and encompasses chemotaxonomy, morphology and physiology. Although it can not be used to determine the phylogenetic relationships between groups of organisms, it can be used to assign bacteria to the lower taxa based on certain key features. The main drawbacks of these methods are that they are time consuming, require a standardised methodology to obtain comparative results and some features such as determination of spore colour are subjective (Williams *et al.*, 1989; Rosselló-Mora & Amann, 2001). Another drawback of phenetic characterisation is that it requires the isolation of the strain in pure culture, therefore can only be used to classify culturable strains. Phenetic classification may also over-estimate relatedness as numerous genetic variations can account for a single observable phenotype (Olsen & Woese, 1993). To make a meaningful comparison, as many tests as possible should be performed (Vandamme *et al.*, 1996; Van Waasbergen, 2004). Some genera such as *Streptomyces* and *Nocardia* have well defined methods for the phenetic classification of an isolate. For other genera, especially the ones with only a few species, the classification strategies could be described as sporadic with little consistency in the phenetic characterisation (Cross, 1989).

1.2.1.2.1 Physiology

In layman's terms, physiological characterisation determines what a bacterium 'can do', while morphology is 'what it looks like'. For the physiological characterisation of an actinomycete, a range of features are considered. The most routinely tested features are the different growth conditions, including growth over a range of different temperatures and pH values (Williams *et al.*, 1989). If a strain was isolated from a specific environmental niche, "atmospheric conditions" including the ability to growth at high pressures or anaerobically

can also be included (Vandamme *et al.*, 1996). The metabolic ability and enzyme activity of the isolate is also investigated. Metabolic features include the ability to utilise a range of carbon and nitrogen sources, as well as acid production from carbohydrates (Gordon *et al.*, 1974). Enzyme activity includes chitinolytic activity and β-lactamase production, while a range of degradative tests including casein, starch, L-tyrosine and xylan can also be included. The ability to grow at different NaCl concentrations and antibiotic resistance is usually determined, while the testing of growth in the presence of other inhibitory compounds is often limited to the classification of *Streptomyces* species. The production of diffusible pigments and melanin can also be determined as these features can be diagnostic in some genera. In the known antibiotic-producing genera, the antimicrobial activity against a selection of test organisms may also be investigated (Vandamme *et al.*, 1996; Williams *et al.*, 1989).

As physiological testing can be very time consuming, it is essential that one first considers which features would be the most useful to differentiate between the species of a particular genus. Once it is determined that an isolate is phenetically unique, more extensive physiological testing can be performed. Although there are commercially available kits (API ZYM and RapID ANA II) which can rapidly test a large number of metabolic and enzymatic features, they are only routinely used for diagnostic purposes. The use of the classical plating techniques are still widely used for the taxonomic studies of environmental actinomycete isolates (Rosselló-Mora & Amann, 2001).

1.2.1.2.2 Morphology

Actinomycete taxonomy is unique in that it still requires extensive morphological description of a species. When describing an actinomycete, it is necessary to look at the morphology of the cells and that of the colony. The basic morphological features of an actinomycete colony are either observed with the naked eye or with a light microscope. When describing these features, the isolate is usually grown on a minimal medium such as inorganic salts starch agar (International *Streptomyces* Project medium No. 4), as aerial mycelium may not form on rich media and the colonies will appear hard and leathery (Cross, 1989; Lechevalier, 1989). Features that are used to describe the substrate mycelia include whether they are well developed and branched or rudimentary. The substrate mycelium of some genera fragments. If fragmentation has occurred, the shape of the fragments and whether they are motile can be important distinguishing features. For motile spores, the number and distribution of the flagella can be observed under a transmission electron microscope (Itoh *et al.*, 1989). The presence or absence of aerial mycelium is a key morphological feature. A number of genera do not produce aerial mycelium including *Actinoplanes, Gordonia, Kineosporia* and *Micromonospora*. The genus *Sporichthya* is unique as its lacks substrate mycelium. When observing an actinomycete under a light microscope, it is best to streak the strain in a cross-hatched pattern as one will be able to observe mature hyphae at points where streak lines intersect (Cross, 1989).

In many actinomycete genera, the morphology of the spores is critical for the morphological classification of genera. Sporulation can alter the appearance of the colony. *Micromonospora* colonies appear mucoid when they sporulate, while the spore mass of *Kineosporia* species may appear in tufts which are accompanied by the production of a polysaccharide-like matrix. The production of spores in other genera may give the colony a dusty appearance (Goodfellow & Lechevalier, 1989; Itoh *et al.*, 1989; Kawamoto, 1989).

The distinguishing features of the spores are viewed under a scanning electron microscope (SEM). Traditionally, to prepare a sample for SEM, agar blocks containing the sporulating actinomycete are fixed with osmium tetroxide, followed by serial dehydration in an ethanol gradient (Cross, 1989). However, samples can be frozen and viewed directly by cryo-SEM. Spores may be found singly, in pairs or in short to long chains. *Thermomonospora* and *Micromonospora* are two genera that bear single spores, the spores of the former being thermostable endospores, while the formation of pairs of conidia on the aerial mycelium is a distinguishing feature of the genus *Microbispora* (Lechevalier, 1989).

Spore chains are described as either short, which generally refers to chains of 3 to 20 spores, or long chains which are generally referred to as 20 to up to 100 spores. Many genera fall into both the short and the long chain categories. Genera that form short chains include *Amycolatopsis, Nocardia, Pseudonocardia*, some *Streptomyces* (including those formerly classified as *Streptoverticillium* and *Microellobosporia*) and *Sporichthya*. Genera that bear long chains of spores include *Amycolatopsis, Nocardia, Nocardioides* and *Streptomyces* (Lechevalier, 1989).

The spore surface ornamentation is also of taxonomic use. In the genus *Streptomyces,* spores can be defined as being hairy, rugose, smooth, spiny or warty. The spore surface in many other genera is generally described as being either smooth or rough. The spores of the genus *Micromonospora* can be smooth or blunt-spiny (Kawamoto, 1989; Lechevalier, 1989).

The formation of sporangia, which are spore-filled sacs, is a characteristic feature of some genera. They can develop on the aerial mycelium or, in the absence of aerial mycelium, on the surface of the colony. The sporangia of *Actinoplanes* and *Dactylosporangium* are borne on the colony surface, while the sporangia of *Spirillospora* and *Streptosporangium* are borne on aerial hyphae. The formation of sporangia is also characteristic of the genus *Kineosporia,* however, they are generally formed within the agar (Lechevalier, 1989). Some genera form large (100 µm in diameter), globose sacs formed by a dense mesh of branching hyphae called sclerotia which may be confused with sporangia. However, sclerotia contain lipid filled cells not spores (Lechevalier, 1989). The formation of sclerotia occurs in some *Streptomyces* species including *Streptomyces ochraceiscleroticus* (homotypic synonym of *Chainia olivacea*) (Euzéby, 2007). The spore-bearing mycelium can also combine to form synnemata which release motile spores. The formation of synnemata is a characteristic of the genus *Actinosynnema*, of which there are currently two validly described species (Lechevalier, 1989; Euzéby, 2007).

1.2.1.2.3 Chemotaxonomy

Chemotaxonomy is defined as the use of analytical methods to investigate the chemical make-up of the cell (Vandamme *et al.,* 1996). In the 1970s, chemotaxonomic methods began to supersede morphological and physiological methods as, unlike these methods, chemotaxonomy generated "quantitative information". The use of biochemical features to identify an organism was favoured due to their genetic stability and uniformity in some genera. However, it must always be kept in mind that these features are not stable and a mutation in a single gene may result in a different expressed phenotype (Kutzner, 1981). Few of the 'traditional' chemotaxonomic methods can resolve relationships below the genus level and chemotaxonomy is seen as archaic by some researchers.

Cell wall-DAP and sugars

The classification of the cell wall into nine different chemotypes based on the diaminopimelic acid (DAP) isomer and whole cell sugars was proposed by Lechevalier & Lechevalier (1970) and is still widely used. Like all eubacteria, the cell wall of actinomycetes contains peptidoglycan, which consists of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid units (the glycan moiety) cross-linked by a peptide moiety. Variations in the peptide unit due to the presence of different key amino acids can affect the structure of the peptidoglycan. The key amino acid found in actinomycetes is 2, 6-diaminopimelic acid (DAP), which has three isomers LL-, DD- and *meso*-DAP (DL-DAP). The isomer of DAP can be identified by the separation of whole cell hydrolysates using paper chromatography or cellulose thin-layer chromatography (TLC). Only the LL-DAP and *meso*-DAP isomers are useful in taxonomy. The glycan moiety found in some actinomycetes contains a muramic acid with the amino base replaced by a glycolyl group and the resulting 'glycolyl type' can be used for identification (Staneck & Roberts, 1974; Komagata & Suzuki, 1987).

Phospholipids

Phospholipids are a component of the bacterial cell membrane. They affect its permeability and also play a role in the regulation of the movement of compounds across the membrane. They are characterised by the presence of both a hydrophobic and a hydrophilic moiety. In bacterial taxonomy, phospholipids are identified by the comparison of fingerprints generated by two-dimensional (2-D) TLC (Komagata & Suzuki, 1987). Lechevalier *et al.* (1977) investigated the phospholipid profiles of actinomycetes and identified five types. Most strains where found to contain diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. However, the presence of certain marker phospholipids can be used to group the actinomycetes. Type PII, which is characterised by the presence of phosphatidylethanolamine, is found in the genera *Actinoplanes, Micromonospora, Nocardia* and *Streptomyces,* while type PIII, characterised by the presence of phosphatidylcholine, is found in the genera *Kribbella* and *Nocardiopsis* (Shaw, 1968; Lechevalier *et al.*, 1977).

Isoprenoid quinones

Aerobic bacteria usually have isoprenoid menaquinones and/or isoprenoid ubiquinones as a component of their cytoplasmic membranes, which plays a vital role in the electron transport chain and oxidative phosphorylation during respiration. Gram positive bacteria only have menaquinones. Both the type of quinone and the number of isoprene units is a characteristic feature of a genus. When identifying an actinomycete either mass spectrometry or high-performance liquid chromatography can be used to determine which menaquinone is present (Collins *et al.,* 1977; Vandamme *et al.*, 1996).

Mycolic acids

Mycolic acids are 2-alkyl-3-hydroxy fatty acids with long alkyl chains (the number of carbons ranges from 24-90). Mycolic acids are found in bacteria with a cell wall type IV (*meso*-DAP, arabinose and galactose) and is a characteristic feature of a number of actinobacterial genera including *Corynebacterium, Dietzia, Gordonia, Mycobacterium, Nocardia, Rhodococcus, Skermania, Tsukamurella* and *Williamsia.* The presence of mycolic acids in the cell wall is thought to be responsible for the acid fastness of these genera. Mycolic acids are found in the cell wall as free acids, part of glycolipids or complexed with polysaccharides (Komagata & Suzuki, 1987). A method

proposed by Minnikin *et al.* uses 1-D TLC to identify the type of mycolic acid present and can be used in species identification (Minnikin *et al.,* 1980).

Fatty acids

The majority of the fatty acids are located in the cell membrane as components of glycolipids and polar lipids. As the fatty acid composition is affected by growth conditions, standardised methods including temperature, media and cultivation period are essential. Fatty acids can be divided into two main types, those that contain iso-/anteiso-branched fatty acids and those that contain mainly straight-chain fatty acids (Komagata & Suzuki, 1987). Due to the inherent lack of reproducibility, fatty acid analysis has limited use in taxonomy. However, the determination of fatty-acid methyl ester (FAMEs) profiles has been used to resolve intrageneric relationships within the genus *Streptomyces* (Saddler *et al.*, 1987). FAME patterns are generated by gas chromatography and the data is analyzed by different statistical programs. A study investigating the taxonomy of the *S. cyaneus* species group and a number of blue-spored soil isolates, divided the strains into three clusters. The first contained the majority of the *S. cyaneus* group, the second cluster contained the remaining *S. cyaneus* strains and streptomycetes belonging to other species groups, while the third cluster contained the blue-spored soil isolates (Saddler *et al.*, 1987; Anderson & Wellington, 2001).

Whole cell analysis

Curie-point pyrolysis mass spectrometry (PyMS) can be used to analyse whole actinomycete cells. Pyrolysis is the degradation of organic matter (in this case whole cells) in an inert atmosphere at high temperature, to produce a mixture of volatile, lower molecular weight fragments which are then analysed by mass spectrometry. The resulting fingerprint can be used to discriminate between strains. The main advantages of using PyMS to identify isolates are that the results are quantitative, it is rapid with a run time of less than 2 minutes per sample, and it is relatively cost effective. Sanglier and co-workers found that PyMS was sensitive enough to resolve species relationships. They could readily identify potentially novel species when they compared environmental micromonosporae to recognised species. They also showed that PyMS could be used in strain dereplication as they were able to identify replicated strains when looking at *Saccharopolyspora* and *Streptomyces* species (Sanglier *et al.*, 1992; Anderson & Wellington, 2001).

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FT-IR) can be used to identify bacterial species. It measures the absorption of infrared light by the sample (mainly detecting the vibrations of functional groups and highly polar bonds). An FT-IR spectrometer measures the frequencies and the intensity of the absorptions, generating a biochemical fingerprint of all components of the cell including DNA, RNA and proteins, as well as the components of the cell wall (Zhao *et al.*, 2004). The advantages of this method include: it is very rapid (one to ten seconds per sample), only a small amount of biological material is needed, whole cells are used so there is very little sample preparation required, it can be automated with a batch size of over 300 samples, and it has excellent resolving power. This method could readily be used in high throughput screening for species dereplication (Haag *et al.,* 1996; Zhao *et al.*, 2004). A study by Zhao *et al.* revealed its potential in taxonomy. The FT-IR fingerprint generated for each of the twenty new *Micromonospora* isolates clearly differentiated them as unique strains. There was also a good correlation between the dendrograms generated from FT-IR spectrograms and the 16S rDNA

phylogenetic analysis at the species level (Zhao *et al.*, 2004). Haag *et al.* constructed a database of FT-IR profiles for 39 actinomycete species belonging to 12 genera. This database was then used to identify four test strains, of which 89% were correctly identified and they were also able to classify strains to the subspecies level. The results correlated well with those obtained using classical taxonomic methods and fatty acid profiling. Haag *et al.* pointed out that for the results to be meaningful, standardised growth conditions must be defined and the results obtained are only as good as the database itself (Haag *et al.*, 1996).

Matrix adsorbed laser desorption/ionization time-of-flight mass spectrometry

Matrix adsorbed laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry can be used to compare the different constituents of a bacterial colony and could potentially be used in species identification. Kroppenstedt *et al.* used MALDI-TOF to compare eight novel *Micromonospora* isolates to a number of *Micromonospora* type strains. Whole cell mass was analysed over a mass (m/z) range of 2.000-20.000 Da. It was found that the number of significant peaks ranged from 14 to 43. The number of identical peaks for each pairwise strain comparison was determined and the percentage similarity between them was used to generate a dendogram. MALDI-TOF profiling was able to recognise each strain as a unique species. Species with a 16S rDNA similarity of greater than 99% were also found to have similar MALDI-TOF mass spectra profiles (Kroppenstedt *et al.*, 2005).

Although some chemotaxonomic methods such as PyMS, FT-IR and MALDI-TOF allow for the rapid identification of strains at the species level, their widespread application is limited by the need for specialised equipment and standarised growth conditions.

1.2.1.2.4 Protein profiling

Multilocus enzyme electrophoresis

Multilocus enzyme electrophoresis (MLEE) looks at the variations in approximately 20 core metabolic genes (Spratt, 1999; Gürtler & Mayall, 2001; Cooper & Feil, 2004). Native enzymes are electrophoretically separated on a starch gel and stained for enzyme activity. Multiple polymorphisms between strains are detected by the differing mobilities of the enzymes (Vandamme *et al.*, 1996; Cooper & Feil, 2004). MLEE was the predecessor of MLST and MLSA. The main benefits of these sequencing-based methods compared to MLEE are that they allow for the creation of databases for inter-laboratory comparisons, and are more sensitive, as every variation is detected whereas in MLEE two different gene products with the same mobility would be seen as the same allele (Maiden *et al.*, 1998).

Protein gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) of whole-cell proteins generates complex banding patterns which can be used to differentiate between species and subspecies groups. One-dimensional (1-D)-PAGE can be used to identify strains, while two-dimensional (2-D)-PAGE enhances the separation of the different proteins. Due to its increased sensitivity, 2D-PAGE can not be used to evaluate fast evolving proteins and its use is often limited to investigating bacterial ribosomal proteins (Anderson & Wellington, 2001).

The bacterial ribosome has a complex structure consisting of three types of ribosomal RNA (5S, 16S and 23S) and over 50 types of ribosomal (r)-proteins. Due to structural and functional constraints imposed on the ribosome, the

r-proteins have a low evolution rate, therefore are ideal to investigate the evolutionary relationship between organisms. Ochi looked at the r-proteins from eleven *Streptomyces* strains by 2-D-PAGE. Five streptomycete r-proteins with highly variable migration patterns and at least ten with moderately variable migration patterns were identified. It was found that each species generated a specific profile. The validity of this method was confirmed as an isolate was successfully identified as being a strain of *Streptomyces lavendulae* using both traditional taxonomic techniques and 2-D-PAGE. The ability of the AT-L30 r-protein to differentiate between genera was also confirmed (Ochi, 1992).

Table 1.1 summarises some of the methods that can be used in polyphasic taxonomy. However, of these techniques, 16S rDNA sequence analysis and phenetic characterisation are always performed. Many chemotaxonomic methods are unable to resolve relationships at the species level, therefore their application is limited. The use of SEM is often limited to genera such as *Streptomyces* and *Micromonospora*, where the spore surface ornamentation is of diagnostic value.

Table 1.1 A summary of methods that can be used in polyphasic taxonomy to resolve genus, species and strain level relationships. (Figure adapted from Bull *et al.*, 2000).

Source of information	Method	Use for taxonomic rank		
		Genus or above	Species	Subspecies or below
Genotypic data				
Chromosomal data	Base composition (mol% G+C)	•	•	
	DNA-DNA hybridization		•	•
	Restriction patterns (LFRFA-PFGE, AFLP)		•	•
	Microarray	•	•	•
	Whole-genome sequences	•	•	•
Gene specific	DNA probes	•	•	•
	DNA sequencing (single genes, MLSA, MLST)	•	•	•
	PCR-based DNA fingerprints (BOX, RIS, RAPD, RFLP)		•	•
Ribosomal RNA	Nucleotide sequences	•	•	
	Ribotyping		•	•
Phenotypic data				
Proteins	Electrophoretic patterns (1D/2D-PAGE)		•	•
	MLEE			•
Chemical markers	Fatty acids	•	•	
	Menaquinones	•		
	Mycolic acids	•	•	
	Peptidoglycan	•		
	Phospholipids	•	•	
	Monosaccharides	•	•	
Whole-organism chemical fingerprinting	FT-IR		•	•
	MALDI-TOF		•	•
	PyMS		•	•
Whole organism expressed features	Morphology	•	•	
	Physiology	•	•	
	Rapid enzyme tests		•	•

Abbreviations are defined in the text.

1.2.2 An overview of selected members of the Class *Actinobacteria*

1.2.2.1 The family "*Kineosporiaceae*"

The family "*Kineosporiaceae*" encompasses three genera namely, *Cryptosporangium, Kineococcus* and *Kineosporia* (Euzéby, 2007). The genera *Kineococcus* and *Kineosporia* have been found to be phylogenetically closely related but members of these genera can be distinguished from one another on the basis of morphological and chemotaxonomic features (Philips *et al.*, 2002; Lee, 2006).

The genus *Kineococcus* was proposed by Yokota *et al.* (1993), and there are currently three validly described species, which were isolated from three diverse sources. The type species, *Kineococcus aurantiacus* IFO 15268^T, was isolated from soil collected in India, as part of a study investigating motile actinomycetes from natural sources (Yokota *et al.*, 1993). *Kineococcus marinus* was isolated from beach sand, collected from the island of Jeju, Republic of Korea. Although phylogenetic analysis showed that *K. marinus* was more closely related to members of the genus *Kineosporia*, key morphological and chemotaxonomic features supported its placement within the genus *Kineococcus* (Lee, 2006). *Kineococcus radiotolerans* was isolated from a shielded cell facility, at a centre used to store high-level γ-radioactive waste, where radiation levels fluctuated between 0.18 Gy/h and 3.5 Gy/h (1 Gy = 100 rads). *K. radiotolerans* was found to be resistant to radiation levels up to 3.5 kGy, and the difference in mean survival was less than 1 log between it and *Deinococcus radiodurans* (Philips *et al.,* 2002).

Kineococci are morphologically distinct in that the cells cluster in pairs, tetrads or octads surrounded by a capsular polysaccharide-like layer. This layer appears to prohibit the motility of the cells; however, cells become motile within a few hours after incubation in a phosphate buffer containing 10% soil extract (Yokota *et al.*, 1993). The coccus-shaped cells are 1.0 to 1.5 μm in diameter and have a polar tuft of flagella. Kineococci do not form aerial mycelium. The colonies are circular and are generally rough in appearance. Colony colour ranges from cream to deep-orange. Kineococci are strictly aerobic and mesophilic, with a growth optimum at 27°C. The cell wall peptidoglycan contains *meso*-DAP, alanine and glutamic acid. The cell wall of *K. aurantiacus* contains two sugars, arabinose and galactose, which are found as an arabinogalactan polymer (Yokota *et al.*, 1993). The major menaquinone present is MK-9(H_2). Kineococci do not contain mycolic acids and the characteristic polar lipids are diphosphatidylglycerol, phosphatidylglycerol and an unknown glycolipid. The G + C content ranges from 73.9 %mol to 76.6 %mol (Yokota *et al.*, 1993; Lee, 2006).

The genus *Kineosporia* was proposed by Pagani & Parenti (1978) to describe a motile actinomycete with LL-DAP and glycine in its cell wall. In the paper by Kudo *et al.* describing four new *Kineosporia* species, they questioned the original chemotaxonomic findings of Pagani and Parenti (Kudo *et al.,* 1998). Subsequently, Itoh and co-workers performed chemotaxonomic and morphological studies on the type species, *Kineosporia aurantiaca* and published the amended description of the genus (Itoh *et al.,* 1989). The defining feature of the genus is the presence of both isomers of DAP. LL-DAP is present in the mycelium, while *meso*-DAP is present in the spores. The colonies form dome-like structures on the agar surface. Vegetative hyphae radiate from the colonies and spore clusters may form. Aerial mycelium is not formed. Immature colonies appear rough and crumbly but upon maturation a polysaccharide-like matrix develops giving them a glossy appearance. The spores are spherical to ovoid and are either found at the tips of the hyphae or clustered around the central dome. The chemotaxonomic

features of the genus include: a phospholipid type PIII, the presence of galactose, glucose and ribose in the cell hydrolysate and the main menaquinone is MK-9(H_4). The G + C content is 69-71 mol% (Itoh *et al.*, 1989; Kudo *et al.*, 1998). There are presently five validly described *Kineosporia* species. *K. aurantiaca* was isolated from a soil sample, whereas *Kineosporia mikuniensis, Kineosporia rhamnosa, Kineosporia rhizophila* and *Kineosporia succinea* were all isolated from plant matter (Pagani & Parenti, 1978; Kudo *et al.*, 1998).

1.2.2.2 The genus *Gordonia*

Taxonomically, the genus *Gordonia* has had a colourful history. The genus *Gordona* was first proposed by Tsukamura in 1971. The generic epithet was in honour of the American bacteriologist, Ruth Gordon. The genus was subsequently reclassified as belonging to the genus *Rhodococcus* (Goodfellow & Alderson, 1977). However, studies of the mycolic acids and menaquinones of the gordoniae revealed that they were deserving of genus status, resulting in reinstatement of the genus *Gordona* (Stackebrandt *et al.*, 1988). The final name change occurred in 1997 when the etymologically correct name *Gordonia* was proposed (Stackebrandt *et al.*, 1997). The type species of the genus is *Gordonia bronchialis* IFO 16047^T.

Gordonia is the type genus of the family *Gordoniaceae*, which forms part of the suborder *Corynebacterineae*. The families *Corynebacteriaceae, Dietziaceae, Mycobacteriaceae, Nocardiaceae, Tsukamurellaceae* and *Williamsiaceae* are also encompassed within this suborder (Stackebrandt *et al.*, 1997). In 2005, the family *Segniliparaceae* was added to the suborder with the description of the novel genus *Segniliparus* (Butler *et al.*, 2005). Chemotaxonomically, the genus *Gordonia* is characterised by: the presence of mycolic acids (44-66 carbons), the predominant menaquinone is MK-9(H_2) and the predominant polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides. Morphologically, *Gordonia* are nocardioform actinomycetes. The term nocardioform is used to describe the fragmentation of the vegetative hyphae into coccoid or rod-shaped elements. The nocardioform actinomycetes include the genera *Amycolatopsis, Gordonia, Nocardia, Nocardioides* and *Rhodococcus*. The term is intended to be descriptive, it does not imply these genera are phylogenetically closely related. The colour of *Gordonia* colonies ranges from whitish-yellow through to orange or pinkish-red, with the colony morphology ranging from smooth and slimy to irregular and rough.

The pigmentation of some *Gordonia* species is affected by light: colonies initially appear pale beige when cultured in the dark, but darken when exposed to light. *Gordonia* are aerobic, slightly acid-fast and nonmotile. They are generally Gram positive, however may be Gram variable (Lechevalier & Lechevalier, 1970; Lechevalier, 1989; Linos *et al.*, 1999; Arenskötter *et al.*, 2004). The G + C content ranges from 63-69 mol%. Shen *et al.* confirmed that there is a high level of interspecies relatedness, with the 16S rDNA similarities ranging from 96.2 to 99.7% and DNA relatedness values ranging from 3.7% to 52% (Arenskötter *et al.*, 2004; Shen *et al.*, 2006a).

The increased interest in the genus *Gordonia* can be attributed to their many potential industrial applications including the desulphurisation of fuels and degradation of hydrophobic pollutants. Their ability to degrade a wide

range of environmental pollutants and xenobiotics is useful for bioremediation purposes. They also have the ability to synthesize a number of compounds which are potentially useful in the pharmaceutical and food industries. '*Gordonia jacobacea*' MV-1 has been found to synthesize the carotenoids canthaxanthin and astaxanthin, which have been approved by the Food and Drug Administration (FDA), USA, as food additives for poultry and fish food. *Gordonia rubropertincta* and *Gordonia terrae* produce imidazol-2-yl amino acids which are used by the pharmaceutical industry as antihistamine drugs (Arenskötter *et al.,* 2004). Gordoniae have been isolated from such benign sources as soil and mangrove rhizospheres, to industrial sources including biofilters and activated sludge foam as well as from clinical settings (Takeuchi & Hatano, 1998; Arenskötter *et al.*, 2004; Iida *et al.* 2005; Shen *et al.*, 2006b). Although *Gordonia* have been linked to bacteraemia due to a central venous catheter or infections of sternal wounds, such infections generally occurred in immuno-compromised individuals and gordoniae should be regarded as opportunistic pathogens (Richet *et al.*, 1991; Buchman *et al.*, 1992). However, their pathogenicity should not be underestimated. Iiada *et al.* examined 235 pathogenic actinomycete strains isolated from clinical specimens in Japan over a 3 year period and found that over 6% could be identified as belonging to the genus *Gordonia* (Iida *et al.,* 2005).

1.2.2.3 The genus *Kribbella*

The family *Nocardioidaceae,* suborder *Propionibacterineae*, was proposed by Nesterenko *et al.* (1985) emend. Rainey, Ward-Rainey and Stackebrandt, 1997, and currently encompasses nine genera namely: *Actinopolymorpha; Aeromicrobium; Friedmanniella; Kribbella; Marmoricola; Micropruina; Nocardioides; Propionicicella;* and *Propionicimonas* (Stackebrandt *et al.*, 1997; Euzéby, 2007). Many of the genera in this family are represented by a single species. The genus *Pimelbacter* was initially included in the family *Nocardioidaceae.* However, with the subsequent reclassification of *Pimelbacter jensenii* and *Pimelbacter simplex* as *Nocardioides jensenii and Nocardioides simplex,* respectively, and the transfer of a third species, *Pimelbacter tumescens,* to the genus *Terrabacter* as *Terrabacter tumescens,* this genus is no longer recognised (Collins *et al.*, 1989).

The genus *Kribbella* was first proposed to accommodate nocardioform actinomycetes that contain LL-DAP in their cell walls (Park *et al.*, 1999). Sohn *et al.* subsequently transferred the type species of the single membered genus *Hongia* Lee *et al.*, 2000, to the genus *Kribbella* as *Kribbella koreensis* (Lee *et al.*, 2000; Sohn *et al.,* 2003). The genus currently contains 11 species. The type species is *Kribbella flavida* NBRC 14399^{T}.

Morphologically, kribbellae are characterised by the formation of extensively branched vegetative hyphae which penetrate the agar. Vegetative hyphae fragment into rod-shaped or coccoid elements. They form abundant aerial hyphae, which readily fragment into rod-shaped elements. Hyphal fragments give rise to new mycelial growth. Colonies are generally white to cream or pale yellow, have a pasty appearance and are lichenous in shape with irregular edges. Hyphal swellings resulting in bud formation have been reported. Diffusible pigments are generally not produced. Kribbellae are Gram positive, however may appear as Gram variable depending on the stage of growth. Kribbellae are non-acid fast, catalase- and oxidase-positive. Cells are non-motile. Chemotaxonomic features of this genus include the presence of LL-DAP and glycine in their cell wall (cell wall

chemotype I). Although there are no diagnostic whole cell-sugars, mannose and ribose are present in many *Kribbella* species. Trujillo suggested that the cell wall sugar composition could potentially be used to differentiate between members of this genus. The predominant menaquinone is MK-9(H_4) and the dominant polar lipid is phosphatidylcholine (phospholipid type PIII). Mycolic acids are not present. The G + C content is 68-70 mol% (Park *et al.*, 1999; Li *et al.*, 2006; Trujillo *et al.*, 2006a).

Although nine of the eleven described species have been isolated from soil collected at different geographical locations, recent publications have revealed that kribbellae are not solely soil dwelling organisms. *Kribbella solani* was isolated from a potato tuber with scab lesions and *Kribbella lupini* was isolated from the root nodules of *Lupinus angustifolius* (Song *et al.*, 2004; Trujillo *et al.*, 2006a). The former was isolated as part of an investigation into the taxonomy of potato scab caused by streptomycetes and is not regarded as a pathogen.

Kribbella sandramycini was initially isolated in a screening program looking for novel antitumor agents. *K. sandramycini* produces the novel antibiotic sandramycin which is structurally related to the cyclic depsipeptides, luzopeptins A-D. Sandramycin was found to inhibit the growth of selected Gram positive organisms and had moderate activity in the P388 tumour model (Matson & Bush, 1989). *Kribbella antibiotica* is reported to have antifungal activity (Li *et al.*, 2004). Antimicrobial activity has not been reported for the other members of the genus. Results presented in Chapter 3 of this study, show that two novel *Kribbella* strains, *Kribbella karoonensis* and *Kribbella swartbergensis*, produce an antimicrobial compound that is effective against a number of bacteria. The production of this compound was found to be dependent on the culture medium.

Table 1.2 shows the distinguishing features for the nine members of the family *Nocardioidaceae*. It is clear that the family is heterogeneous and genera can readily be differentiated based on a limited number of phenetic tests.

1.2.2.4 The genus *Micromonospora*

The genus *Micromonospora* was first proposed by Ørskov (1923), and is the type genus of the family *Micromonosporaceae* Krasil'nikov 1938, emend. Stackebrandt *et al.*, 1997. The family *Micromonosporaceae* presently includes 15 genera: *Actinocatenispora, Actinoplanes, Asanoa, Catellatospora, Catenuloplanes, Couchioplanes, Dactylosporangium, Longispora, Micromonospora, Pilimelia, Polymorphospora, Salinispora, Spirilliplanes, Verrucosispora* and *Virgisporangium* (Euzéby, 2007). Table 1.3 lists key phenetic differences between the genera included in the family *Micromonosporaceae*. The genus *Micromonospora* is presently comprised of 30 validly described species and the type species is *Micromonospora chalcea* ATCC 12452^T.

Two distinguishing features of the genus *Micromonospora* are the formation of single, non-motile spores on the vegetative mycelium and the absence of aerial mycelium (Kawamoto, 1989). Micromonosporae form well-developed, septate substrate mycelia which do not fragment. Immature *Micromonospora* colonies are generally orange in colour but mature into a range of colours including orange, red, brown, blue-green or purple. The mycelial pigmentation is not regarded as a diagnostic feature of the genus. However, the mycelial pigments produced by two species, *Micromonospora echinospora* and *Micromonospora coerulea* are pH indicators. With

the production of spores, the colonies darken and may become mucoid. Spores may be black, brownish-black or greenish-black (Kawamoto, 1989; Koch *et al.,* 1996a). The spores may be borne sessile or on short sporophores, and are spherical, oval or ellipsoidal in shape (Koch *et al.,* 1996a) The surface of the spores is commonly described as being smooth, warty or blunt-spiny (Koch *et al.,* 1996a; Hirsch *et al.*, 2004; Trujillo *et al.*, 2005). The cell walls are characterised by the presence of *meso*-DAP and glycine (cell wall type II). The presence of xylose and arabinose in whole cell hydrolysates (whole cell sugar pattern D) is a diagnostic feature of the genus. The predominant phospholipids detected are phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides (phospholipid type PII). Mycolic acids are not produced (Kawamoto, 1989).

After streptomycetes, the genus *Micromonospora* is likely to be the second most prolific producer of antibiotics. The first antibacterial activity by a member of this genus was reported in 1942 by Welsch. However, it was the isolation of the aminoglycoside gentamicin from *Micromonospora purpurea* (reclassified as *M. echinospora* by Kasai *et al.,* 2000) in 1963, which sparked the widespread screening of this genus. The initial interest in gentamicin was that it had an extended antibacterial spectrum and improved safety compared to other aminoglycosides such as neomycin and kanamycin. Many of the *Micromonospora* species described in the 1960s and 1970s were isolated from antibiotic screening programs (Wagman & Weinstein, 1980).

The studies by Koch *et al.* (1996b) and Kasai *et al.* (2000) resolved the taxonomic relationships within the genus *Micromonospora.* Koch *et al.* sequenced the 16S rRNA genes of the 14 validly published *Micromonospora* species, four subspecies and 19 other strains. This study confirmed the species status of the 14 type strains but questioned the classification of some strains as subspecies. The study by Kasai and collaborators investigated the ability of the *gyrB* gene to resolve the intrageneric relationships between members of the genus *Micromonospora.* This study resulted in the reclassification of a number of species to the level of subspecies. They found that the *gyrB*-based classification agreed with the DDH studies, and *gyrB* was in fact superior to 16S rDNA for evolutionary studies in this genus. They concluded that the evolutionary rate of the 16S rRNA gene within this genus was not uniform due to different selective pressures imposed on it (Kasai *et al.,* 2000).

Micromonosporae appear to be widely distributed in nature and have been isolated from a number of diverse sources, including a Thai peat swamp forest (Thawai *et al.*, 2005), water samples (Trujillo *et al.*, 2005) and Antarctic sandstone (Hirsch *et al.*, 2004). A recently described species, *Micromonospora coriariae*, was isolated from the root nodules of a Mediterranean shrub (Trujillo *et al.*, 2006b).

Table 1.2 Phenetic characteristics of the genera included in the family *Nocardioidaceae*.

Genus	Morphology	DAP	Menaquinones	Polar lipids	G + C content (mol%)	Type species	Number of species	Reference
Actinopolymorpha	Irregular shaped, form branched hyphae	LL-DAP	MK-9(H_6)	PIM, PI, DPG, PG	69	*Actinopolymorpha singaporensis*	1	Wang *et al.*, 2001
Aeromicrobium	Rods	LL-DAP	MK-9(H_4)	PG, PE	71-73	*Aeromicrobium erythreum*	6	Miller *et al.*, 1991
Friedmanniella	Cocci, clusters	LL-DAP	MK-9(H_4)	PI, PG, DPG, PL	73	*Friedmanniella antarctica*	4	Schumann *et al.*, 1997
Kribbella	Hyphae fragment into rods/cocci	LL-DAP	MK-9(H_4)	PC	68-70	*Kribbella flavida*	11	Park *et al.*, 1999
Marmoricola	Cocci, singly, pairs or clusters	LL-DAP	MK-8(H_4)	PI, PG, DPG	72	*Marmoricola aurantiacus*	1	Urzi *et al.*, 2000
Micropruina	Cocci, singly, pairs or clusters	*meso*-DAP	MK-9(H_4)	ND	70	*Micropruna glycogenica*	1	Shintani *et al.*, 2000
Nocardioides	Hyphae fragment into rods	LL-DAP	MK-8(H_4)	DPG, PG, PG-OH,PL	66	*Nocardioides albus*	20	Lee *et al.*, 2000; Prauser 1976
*Propionicicella**	Rods	*meso*-DAP	MK-9	ND	69	*Propionicicella superfundia*	1	Bae *et al.*, 2006
*Propionicimonas**	Pleomorphic rods	*meso*-DAP	MK-9(H_4) and MK-10(H_4)	ND	67-69	*Propionicimonas paludicola*	1	Akasaka *et al.*, 2003

Abbreviations: DAP, diaminopimelic acid; DPG, diphosphatidylglycerol; ND, not determined; PC, phosphatidylcholine; PG, phosphatidylglycerol; PG-OH, phosphatidylglycerol containing 2-hydroxy fatty acids; PI, phosphatidylinositol; PIM, phosphatidylinositol mannosides; PL-unknown phospholipids; *, facultative anaerobes.

Table 1.3 Phenetic characteristics of the genera included in the family *Micromonosporaceae*.

Genus	G+C%	Cell wall type	Whole cell sugar	Phospho -lipid type	Presence of aerial mycelium	Spores	Spore motility	Type species	Number of described species	Reference
Actinocatenispora	72	II	Ara, Xyl	PII	+	Multiple, in chains	-	*Actinocatenispora thailandica*	1	Thawai *et al.*, 2006
Actinoplanes	70-73	II	Ara, Xyl	PII	-	Multiple, in coils	+	*Actinoplanes philippinensis*	25	Kawamoto, 1989
Asanoa	71-72	II	Ara, Gal, Xyl	PII	-	Sporulation rare	-	*Asanoa ferruginea*	3	Lee & Han, 2002
Catellatospora	70-72	II	Ara, Gal, Xyl	PII	-	Single or chains	-	*Catellatospora citrea*	7	Lee & Han, 2002
Catenuloplanes	70-73	VI	Xyl	PIII	-	Clustered in chains	+	*Catenuloplanes japonicus*	7	Kudo *et al.*, 1999
Couchioplanes	70-72	VI	Ara, Gal, Xyl	PII			+	*Couchioplanes caeruleus*	1	Tamura *et al.*, 1997
Dactylosporangium	72-73	II	Ara, Xyl	PII	-	Few, in rows	+	*Dactylosporangium aurantiacum*	6	Kawamoto, 1989
Longispora	70	II	Ara, Gal, Xyl	PII	-	Multiple, in chains	-	*Longispora albida*	1	Matsumoto *et al.*, 2003
Micromonospora	71-73	II	Ara, Xyl	PII	-	Single	-	*Micromonospora chalcea*	30	Kawamoto, 1989
Pilimelia	ND	II	Ara, Xyl	PII	-	Multiple, in rows	+	*Pilimelia terevasa*	3	Kawamoto, 1989
Polymorphospora	70	II	Gal, Gluc, Man,	PII	-	Few in short chains	-	*Polymorphospora rubra*	1	Tamura *et al.*, 2005
Salinispora	70-73	II	Ara, Gal, Xyl	PII	-	Singly or clusters	-	*Salinispora arenicola*	2	Maldonado *et al.*, 2005
Spirilliplanes	69	II	Gal, Xyl	PII	+	Short chains	+	*Spirilliplanes yamanashiensis*	1	Tamura *et al.*, 1997
Verrucosispora	70	II	Man, Xyl	PII	-	Singly or chains	-	*Verrucosispora gifhornensis*	1	Rheims *et al.*, 1998
Virgisporangium	71	II	Ara, Gal, Man, Rham, Xyl	PII	-	Rows of 6 or more spores	+	*Virgisporangium ochraceum*	2	Tamura *et al.*, 2001

Abbreviations: Ara, arabinose; Gal, galactose; Man, mannose; Rham, rhamnose; Xyl, xylose.

1.2.2.5 The genus *Nocardia*

A feature that is synonymous with the genus *Nocardia* is that they are nocardioform actinomycetes. This genus is a member of the family *Nocardiaceae*, which is currently comprised of three genera; *Nocardia, Rhodococcus* and the single member genus *Smaragdicoccus*. Although the genus *Micropolyspora* was initially included in the family *Nocardiaceae*, its standing in taxonomy has been questioned. Of the five validly described species four, including the type strain *Micropolyspora brevicatena*, have been transferred to other genera (Euzéby, 2007). A number of species initially classified as *Nocardia* have subsequently been transferred to other genera including *Amycolatopsis*, *Gordonia* and *Rhodococcus*. There are currently 61 recognised *Nocardia* species.

Nocardia asteroides ATCC 19247^T is the type species of the genus. Even though nocardiae are readily isolated from soil, the majority have been isolated from clinical samples. The true diversity of this genus is not fully understood, as is their role in the natural environment (Wang *et al.*, 2001; Zhang *et al.*, 2003). Comprehensive taxonomic studies are required to aid in the classification of environmental *Nocardia* strains (Chun & Goodfellow, 1995; Gürtler *et al.*, 2001).

Morphological features of the genus *Nocardia* include the formation of either simple or extensively branched vegetative hyphae. Fragmentation may either be due to mechanical disruption or occur *in situ*. Nocardiae usually form aerial hyphae, at times these may be sparse and only visible with a microscope. The aerial mycelium may mature into short or long chains of conidia which are nonmotile. Although they can produce a wide range of cell pigments, *Nocardia* colonies are commonly orange to pinkish-red to brown in colour. In the absence of visible aerial mycelium the colonies will appear smooth, but generally they have a chalky appearance. Aerial mycelium can range from white in colour to grey or orange-pink. *Nocardia* are Gram positive to Gram variable and aerobic (Gordon *et al.*, 1974; Goodfellow & Lechevalier, 1989).

Characteristic chemotaxonomic features of the genus *Nocardia* include the presence of *meso*-DAP, arabinose and galactose in the cell wall (cell wall chemotype IV), a phospholipid pattern type PII, and the presence of mycolic acids with 46-60 carbons. The predominant menaquinones are MK-8 (H_4) or MK-9(H_2) (Goodfellow & Lechevalier, 1989). Unlike some genera, the methodology for the physiological characterisation of a *Nocardia* strain is well defined, which allows for consistency in species comparisons. The techniques outlined by Goodfellow (1971) and Gordon *et al.* (1974) are routinely used.

Some *Nocardia* species are pathogenic to humans and lower animals including cats, dogs and birds. *Nocardia salmonicida* is a known fish pathogen, while *Nocardia vaccinii* is a plant pathogen and causes galls on blueberry bushes (Goodfellow & Lechevalier, 1989; Isik *et al.*, 1999). In humans, the three diseases most often associated with nocardiae are actinomycotic mycetomas, nocardiosis and localized subcutaneous infections. Actinomycotic mycetomas are usually caused by *Nocardia brasiliensis* and *Nocardia transvalensis*, pulmonary nocardiosis is most frequently caused by strains of *N. asteriodes, N. brasiliensis, Nocardia farcinica, Nocardia nova*, *Nocardia otitidiscaviarum* and *N. transvalensis*, while subcutaneous infections can be caused by *N. asteriodes* or *N. brasiliensis*. *Nocardia africana* was isolated from Sudanese patients with pulmonary infections that were not responding to antitubercular chemotherapy, *Nocardia cyriacigeorgici* was isolated from a patient with chronic bronchitis, while *Nocardia veterana* was cultured from a patient with a history of tuberculous pleurisy. Like all

actinomycete pathogens, nocardiae appear to be opportunistic pathogens (Goodfellow & Lechevalier, 1989; Chun & Goodfellow, 1995; Gürtler *et al.*, 2001; Hamid *et al.*, 2001; Yassin *et al.*, 2001). There have been a number of studies identifying isolates in a clinical setting. Steingrube *et al.* performed RFLP analysis of the 65 kDa heat shock protein gene which allowed for the separation of pathogenic species, while Laurent *et al.* designed genus-specific 16S rRNA gene primers for the rapid identification of *Nocardia* species from clinical samples (Steingrube *et al.*, 1995; Laurent *et al.*, 1999).

1.2.2.6 The genus *Streptomyces*

The genus *Streptomyces* was first proposed by Waksman and Henrici (1943) and is the type genus of the family *Streptomycetaceae*, suborder *Streptomycineae*. The type species is *Streptomyces albus* ATCC 25426^T and there are currently over 500 validly described *Streptomyces* species (Euzéby, 2007).

Traditionally, streptomycetes are defined as producing extensively branched vegetative mycelium that rarely fragments. Their multinucleated aerial mycelium (sporophores) mature into chains of 3 or more spores (conidia), by the formation of cross walls. These spores are non-motile. Streptomycetes form discrete, leathery colonies which may initially appear smooth surfaced but take on a granular, powdery or velvety appearance with the formation of aerial mycelium. Streptomycetes produce a wide variety of secondary metabolites including pigments and antibiotics. These pigments account for the vivid colours of their substrate and aerial mycelium. Some species also produce diffusible pigments. Three features of the aerial mycelium are used to identify streptomycetes, namely, gross morphology of the spore chains, the colour of the spore mass and the spore surface ornamentation. The three spore chain morphologies are: straight to flexuous (*Rectiflexibiles*); hooks, loops or coils with one or two turns (*Retinaculiaperti*); and spirals (*Spirales*). The classification of spore chain morphology can be problematic as there may be two different morphological types present in a single culture and the differentiation between *Retinaculiaperti* and *Spirales* is subjective. The seven-colour wheel is used to classify a streptomycete's spore mass. The colours recognised are blue, grey, green, red, violet, white or yellow. The colour of the aerial spore mass and the spore chain morphology can readily be identified with the naked eye and a light microscope, respectively. To determine the spore surface ornamentation, a streptomycete must be examined under a SEM. Although a transmission electron microscope can be used, it may be difficult to distinguish the features clearly. The spore ornamentation is actually borne on the spore sheath, not on the spore itself and may be described as being hairy, rugose, smooth, spiny or warty (Williams *et al.*, 1989; Anderson & Wellington, 2001).

Streptomycetes share a number of key chemotaxonomic features. Their cell wall is characterised by the presence of LL-DAP, glycine and the absence of characteristic sugars (cell wall type I). The predominant menaquinones are either hexa- or octahydrogenated with nine isoprene units [MK-9(H_6) MK-9(H_8)] and they lack mycolic acids. *Streptomyces* cell membranes contain a number of phospholipids including diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides (phospholipid pattern type PII). Streptomycetes are non-fastidious and utilise a wide range of carbon and

nitrogen sources. Cells are non-acid fast and Gram positive. Their G + C content ranges from 69-78 mol% (Williams *et al.*, 1989).

The isolation of actinomycin and streptomycin in 1944 from streptomycetes sparked frenetic screening programs by a number of pharmaceutical companies. This resulted in the number of described species increasing exponentially from the 40 species pre-1940 to over 3 000. This overspeciation of the genus can be attributed to the patenting of novel compounds (the producing strain was thus classified as a new species) (Kutzner, 1981; Anderson & Wellington, 2001).

For years the status of the genus *Streptomyces* was in flux. Many attempts were made to classify streptomycetes. Pridham and co-workers created the subdivision 'morphological section', a category between genus and species. These seven sections were further subdivided into six 'series' based on the colour of the spore mass. Any new isolate could therefore potentially be classified into 42 new divisions! It quickly became apparent that classification based solely on morphology would be impossible (Pridham *et al.*, 1958; Kutzner, 1981).

The initiation of the International *Streptomyces* Project (ISP) in 1964 brought some order to the taxonomic chaos. The aim of the ISP was to define a standard set of methods that could be used to identify isolates. With a standard set of criteria used to identify species, a sound identification scheme could be created. These methods are still the benchmark used to classify novel streptomycetes (Williams *et al.*, 1989; Anderson & Wellington, 2001). The introduction of chemotaxonomy to streptomycete taxonomy in the 1970s allowed for a refinement of the identification process. The main advantages of using chemotaxonomic markers were that they were seen as genetically stable, less subjective than morphological characterisation and uniform within taxa (Kutzner, 1981). The development of molecular techniques resulted in the classification of this genus being further revised.

A number of studies performed between the 1960s and 1980s attempted to use numerical taxonomy to classify members of the genus *Streptomyces* (Goodfellow *et al.*, 1992). Williams *et al.* conducted an extensive taxonomic study of the genus *Streptomyces.* They used 139 unit characters to compare 475 strains, which included 394 *Streptomyces* type strains from the ISP and other genera. Statistical analysis assigned the strains to 19 major clusters (containing six to 71 strains), 40 minor clusters (two to five strains) and 18 single membered clusters, with the 394 *Streptomyces* type strains being assigned to nine clusters. The largest cluster identified was the *Streptomyces albidoflavus* species group, which contained the subgroups for the species *S. albidoflavus, Streptomyces anulatus* and *Streptomyces halstedii* (Williams *et al.*, 1983a). This classification scheme was used to construct a probability matrix for the identification of 23 phena, which included 19 *Streptomyces*, two *Streptoverticillium* and one *'Nocardia mediterranea'* (subsequently reclassified as *Amycolatopsis mediterranei*) major clusters and the *Streptomyces fradiae* minor cluster using 41 unit characters (Williams *et al.*, 1983b). This classification was further refined by Goodfellow *et al.* who used 273 unit characters to compare 252 strains of numerically defined species and species-groups. Their findings confirmed the results of Williams *et al.* (1983a) (Goodfellow *et al.*, 1992).

These three phases of taxonomy (morphology, chemotaxonomy and genomics) have resulted in many modifications to the genus *Streptomyces* and the family *Streptomycetaceae* over the last few decades. These changes resulted in the inclusion of the genera *Actinopycnidium*, *Actinosporangium*, *Chainia*, *Elytrosporangium*, *Kitasatoa* and *Microellobosporia* within the genus *Streptomyces* (Anderson & Wellington, 2001). The genus *Kitasatosporia* was initially incorporated within the genus based on similarities in the 16S rDNA sequences. However, later phylogenetic studies by Zhang *et al.* resulted in the reinstatement of this genus as *Kitasatospora*. They found that when the full length of 16S rDNA was compared, members of this genus formed a monophyletic clade separate from the streptomycetes (Zhang *et al.*, 1997). The genera *Kineosporia* and *Sporichthya* were originally included in the genus, however, 16S rDNA sequencing has resulted in them both being recognised as independent genera. The genus *Sporichthya* is now classified as belonging to the family *Sporichthyaceae* and the genus *Kineosporia* is a member of the family "*Kineosporiaceae*". Both these families are members of the suborder *Frankineae* (Stackebrandt *et al.*, 1997; Anderson & Wellington, 2001; Euzéby, 2007).

There has been much debate about the status of the genus *Streptoverticillium*. After extensive research by Witt & Stackebrandt, this genus was finally recognised as a junior synonym of the genus *Streptomyces*. The amended species description thus stated that the aerial mycelium either forms long chains of spores or the "aerial mycelium consists of long, straight filaments bearing at more or less regular intervals branches (3-6) arranged in whorls (verticils)...each branch of the verticil produces at its apex an embel that consists of two to many chains of spherical to ellipsoidal spores". The aerial mycelium of these species have a barbed wire-like appearance under the light microscope and they are often referred to as the 'whorl-forming *Streptomyces*' (Witt & Stackebrandt, 1990). These reclassifications resulted in the genera *Streptomyces* and *Kitasatospora* being the only members of the family *Streptomycetaceae* for over a decade. A new genus within the family *Streptomycetaceae* was proposed by Kim *et al.* to accommodate a novel acidophilic actinomycete. The genus *Streptacidiphilus* is thus the third member of this family (Kim *et al.*, 2003) and contains five validly described species (Euzéby, 2007).

Despite the development of new biochemical and genomic techniques to identify bacteria, streptomycete taxonomy still depends on the proper phenetic characterisation of an isolate. Table 1.4 lists several physiological tests that are routinely used to classify a new *Streptomyces* species.

Streptomycetes are considered to be terrestrial organisms, where they are thought to play a role in the decomposition of plants and other organic matter. The production of two secondary metabolites, geosmin and methylisoborneol, by streptomycetes has been linked to the development of earthy tastes and odours in reservoirs and water supplies (Williams *et al.*, 1989). Streptomycetes are thought to play an important role in the biological control of fungal pathogens in plants, especially in the rhizosphere. In a study by Cao *et al.*, 131 endophytic actinomycetes were isolated from banana roots, of which 18.3% were found to inhibit the growth of *Fusarium oxysporum* f. sp. *cubense*, the causative agent of fusarium wilt disease of banana. The most abundant strain, S96, was found to be most similar to *Streptomyces griseorubiginosus*. The potential of using actinomycetes as biocontrol agents was also identified, as the weight of plantlet was found to increase when treated with the antagonist strain S96 (Cao *et al.*, 2005).

Table 1.4 Selected phenetic tests used in the identification of a *Streptomyces* species. (Adapted from Williams *et al*., 1989).

Phenetic characteristic	Character states
Morphology	
Spore chain morphology	*Rectiflexibiles*, *Retinaculiaperti*, *Spirales*
Spore surface ornamentation	Hairy, rugose, smooth, spiny, warty
Colour of spore mass	Blue – bluish to greyish blue Green – greenish to greyish green Grey – grey to brownish Red – tan, pink to rose shades Violet White Yellow – yellowish to greenish yellow
Colour of substrate mycelium	Yellow-brown, blue, green, red-orange, or violet, pH sensitivity of pigment
Production of diffusible pigments	Colour – yellow-brown, blue, green, red-orange or violet; pH sensitivity of pigment
Physiology	
Melanin production	Peptone-yeast extract-iron agar (ISP 6) Tyrosine agar (ISP 7)
Enzyme activity	Hydrolysis of hippurate and pectin. Nitrate reduction. Hydrogen sulphide production. Lecithinase, lipolysis and proteolysis on egg yolk medium.
Degradation activity	Adenine, aesculin, allantoin, arbutin, casein, gelatin, guanine, hypoxanthine, starch, Tween 80, L-tyrosine, urea, xanthine and xylan
Antibiotic resistance (µg/ml)	Cephaloridine (100), gentamicin (100), lincomycin (100), neomycin (50), oleandomycin (100), penicillin G (10 I.U.), rifampicin (50), streptomycin (100), tobramycin (50) and vancomycin (50).
Growth temp (°C)	4, 10, 37 and 45
Growth pH	4.3
Growth in presence of inhibitory compounds (% w/v)[a]	Crystal violet, phenol, 2-phenylethanol, potassium tellurite, sodium azide, sodium chloride and thallous acetate.
Sole carbon sources (1% w/v)[b]	Adonitol, L-arabinose, D-cellobiose, D-fructose, D-galactose, *meso*-inositol, inulin, D-lactose, D-mannitol, D-mannose, D-melezitose, D-melibiose, raffinose, L-rhamnose, sucrose, trehalose, xylitol and D-xylose
Sole nitrogen sources (0.1% w/w)[b]	DL-α-amino-*n*-butyric acid, L-arginine, L-cysteine, L-histidine, L-hydroxyproline, L-methionine, potassium nitrate, L-phenylalanine, L-serine, L-threonine and L-valine.

a-NaCl tested at 4, 7, 10, 13% w/v; b-Only the carbon and nitrogen sources that are routinely tested are listed.

The 'generalised' features of the genus *Streptomyces* are that they are aerobic, mesophilic, neutrophilic, non-pathogenic, terrestrial microorganisms. However, within this very large genus, there always appears to be an exception to the rule. Although they have traditionally been isolated from soil, increasing numbers of streptomycetes are being isolated from marine sediments, as plant endophytes and from clinical settings. Although numerous studies have shown that streptomycetes are frequently isolated from aquatic environments, the prevailing view has been that these strains are the result of 'wash in' from terrestrial environments, and that they have survived as dormant spores. With the description of the first obligate marine actinomycetes, there is renewed interest in these 'aquatic' streptomycetes (Ward & Bora, 2006). There are also many examples of streptomycetes that are capable of growth at high temperatures. Work by Kim *et al.* verified the classification of three thermophilic alkalitolerant streptomycete species, *Streptomyces thermodiastaticus*, *Streptomyces*

thermoviolaceus and *Streptomyces thermovulgaris*. These species are capable of growth between 25°C and 55°C and can grow at pH 10 (Kim *et al.*, 1999).

Streptomyces acidiscabies, *Streptomyces caviscabies*, *Streptomyces turgidiscabies* and others are phytopathogenic streptomycetes. They cause scab disease in potatoes (*Solanum tuberosum*) and other root vegetables. This disease is characterised by the formation of corky lesions on the surface of potatoes and is problematic in some of the world's main potato growing countries including the USA and parts of Europe (Miyajima *et al.*, 1998). Streptomycetes can be pathogenic to humans. *Streptomyces somaliensis* is the causative agent of actinomycetoma, a chronic granulomatous infection of the subcutaneous tissues. Although rare in temperate climates, actinomycetoma is a problem in tropical and subtropical countries (El Hassan *et al.*, 2001).

1.3 Antibiotics

The inherent nature of an antibiotic is encompassed in its very name, which is derived from the Greek *antibios* (*anti*-against, *bios*-life). Antibiotics can be classed by their mode of action, mechanism or chemical class. The two modes of action are defined as being either bactericidal (leading to cell death) or bacteriostatic (inhibiting the growth of the bacterium). The three mechanisms of action are inhibition of protein synthesis, inhibition of cell wall synthesis and inhibition of nucleic acid replication (Neu, 1992; Levy, 1998; Mandelstam & Dale, 2000).

1.3.1 The history of the antibiotic era

Chemotherapeutics have a long history, however, the discoveries made by the 'fathers of modern chemotherapy'; Ehrlich (salvarsan), Fleming (penicillin) and Waksman (streptomycin), heralded the beginning of the antibiotic era (Mandelstam & Dale, 2000). Streptomycin was the first antibiotic to be routinely used in the treatment of human disease. The 1940s and 1950s are remembered as the golden era of antibiotic discovery. With each new successful discovery, the public perception grew. Antibiotics were the 'magic bullets' which could eliminate any infection (Levy, 1998). The development of streptomycin was rapidly followed by the discovery of most of the classes of antibiotics: tetracyclines and chloramphenicol (1949); aminoglycosides (1950); macrolides (1952); glycopeptides (1958); streptogramins and quinolones (1962) (Norrby *et al.*, 2005; Overbye & Barret, 2005). Approximately a dozen antibiotics served as the chemical scaffolds for the second generation antibiotics of the 1970s and 80s (Peláez, 2006). This has resulted in the over two hundred antibiotics currently available being based on a handful of core molecules (Barret, 2005). Modifications to these lead compounds resulted in new antibiotics with an improved antibacterial spectrum, potency or pharmacokinetics (Travis, 1994; Shlaes *et al.*, 2004; Peláez, 2006).

Since the start of the 20th century, the number of deaths due to infectious diseases has been declining. This can be attributed to the introduction of antimicrobials, the implementation of community based immunization programs and improved housing and sanitation (World Health Organization, 2002; Overbye & Barret, 2005). By the mid-1960s it was felt that the fight against infectious diseases had been won, even prompting the US Surgeon General to announce "we had essentially defeated infectious diseases and could close the book on them".

1.3.2 Antibiotics produced by the actinomycetes – past, present and future

1.3.2.1 Past discoveries

Since the discovery of penicillin, microorganisms, especially the actinomycetes and fungi, have been an important source of antibiotics. More than 70% of the antibiotics marketed from 1982 to 2002 were based on natural products (Peláez, 2006).The first antibiotic isolated from an actinomycete was streptothricin in 1942. However, it was the isolation of streptomycin from *Streptomyces griseus* in 1944 which lead to systematic screening of actinomycetes (Bérdy, 1974; Watve *et al.*, 2001). It is estimated that 80% of the antibiotics isolated between 1955 and 1962 were isolated from actinomycetes, with the majority being produced by the streptomycetes. After the initial discoveries in the 1940s, the next two decades saw the discovery of 70-80% of

all known antimicrobial compounds, including many of the main classes of antibiotics such as the aminoglycosides, cephalosporins and macrolides (Bérdy, 1974).

1.3.2.2 Important classes of antibiotics produced by actinomycetes

Table 1.5 shows some of the main antibiotic classes isolated from actinomycetes. From past discoveries, it is clear that actinomycetes can produce most types of antibiotics. A brief summary of some of the important classes is given below.

Table 1.5 Important classes of antibiotics produced by actinomycetes.

Antibiotic class	Antibiotic	Producing strain	Reference
Aminoglycosides	gentamicin	*Micromonospora echinospora*	Wagman & Weinstein, 1980
	kanamycin	*Streptomyces kanamyceticus*	Korzybski *et al.*, 1967
	neomycin	*Streptomyces fradiae*	Korzybski *et al.*, 1967
	streptomycin	*Streptomyces griseus*	Korzybski *et al.*, 1967
Ansamycins	halomicin	*Micromonospora halophytica* *Micromonospora nigra*	Wagman & Weinstein, 1980
	rifamycin	*Amycolatopsis mediterranei*	Blanchard, 1996
β-lactams	clavulanic acid	*Streptomyces clavuligerus*	McGowan *et al.*, 1998
	thienamycin	*'Streptomyces cattleya'*	McGowan *et al.*, 1998
Chloramphenicol	chloramphenicol	*Streptomyces venezuelae*	Korzybski *et al.*, 1967
Glycopeptides	teicoplanin	*Actinoplanes teichomyceticus*	Somma *et al.*, 1984
	vancomycin	*Amycolatopsis orientalis*	Somma *et al.*, 1984
Macrolides (Type I PKS)	erythromycin A & B	*Saccharopolyspora erythraea*	Korzybski *et al.*, 1967
	rosaramicin	*Micromonospora rosaria*	Wagman & Weinstein, 1980
Lipopeptide	daptomycin	*Streptomyces roseosporus*	Peláez, 2006
Type II (aromatic) PKS	actinorhodin	*Streptomyces coelicolor*	Hutchinson, 1999
	angucyclines	*Streptomyces aureofaciens* *Streptomyces fradiae*	Metsä-Ketelä *et al.*, 2002
	anthracyclines	*Streptomyces nogalater*	Metsä-Ketelä *et al.*, 2002
	chlortetracycline	*Streptomyces aureofaciens*	Korzybski *et al.*, 1967
	oxytetracycline	*Streptomyces rimosus*	Korzybski *et al.*, 1967

Inhibition of protein synthesis

Many aminoglycoside antibiotics are produced by actinomycetes, including gentamicin by *Micromonospora* species, and neomycin and streptomycin by *Streptomyces* species. Although structurally diverse, all aminoglycosides contain amino sugars and a cyclohexane ring and inhibit protein synthesis by binding to the small ribosomal subunit (Prescott *et al.*, 1999). Due to their toxicity and increased levels of bacterial resistance, aminoglycoside antibiotics are not routinely prescribed. However, streptomycin is still prescribed for the treatment of resistant strains of *Mycobacterium tuberculosis* (Mandelstam & Dale, 2000).

Macrolide antibiotics are synthesised by a type I polyketide synthase (PKS) and are characterised by the presence of a lactone ring, that usually contains 12 to 20 carbons, which is linked to a sugar moiety. Erythromycin is a naturally produced macrolide. Many of the second generation macrolides such as clarithromycin and azithromycin are semisynthetic derivatives of erythromycin. Macrolides target the 50S ribosomal subunit by binding to the 23S ribosomal RNA. This results in the premature dissociation of the

peptidyl-tRNA from the ribosome during translocation (Chu *et al.*, 1996; Prescott *et al.*, 1999; Mandelstam & Dale, 2000).

A number of tetracyclines and other type II (aromatic) polyketides are produced by actinomycetes. All tetracycline-type antibiotics possess a four-membered ring, with a variety of side chains, which are responsible for their different properties (Mandelstam & Dale, 2000). Tetracyclines bind to the 16S rRNA, disrupting its secondary structure. This reduces the affinity of the binding of the aminoacyl-tRNA to its acceptor site on the 30S ribosome. Translation of new proteins ceases (Chu *et al.*, 1996).

Inhibition of nucleic acid synthesis

Ansamycins are macrocyclic polyketides (encoded by a type I PKS) and can be classified by the presence of either a benzenic or naphthalenic chromophore attached to this ring. Geldanamycin, rifamycin and streptovaricin are examples of ansamycins that have been isolated from actinomycetes. However, the most commonly prescribed ansamycin is rifampicin, a semi-synthetic modification of rifamycin, which was initially isolated from *Amycolatopsis mediterranei* (August *et al.*, 1998). Rifampicin is one of the frontline drugs used in tuberculosis chemotherapy and its mechanism of action is to inhibit bacterial DNA transcription by binding to the *rpoB*-encoded subunit of RNA polymerase (Blanchard, 1996).

Inhibition of cell wall synthesis

β-Lactam antibiotics are characterised by the presence of a β-lactam ring (McGowan *et al.*, 1998). Their mechanism of action is to bind to the penicillin-binding proteins (PBPs) found in the bacterial cytoplasmic membrane and inhibit a number of cellular functions, including cell elongation which involves a number of transpeptidation reactions. Inhibition of the PBPs will also cause the peptidoglycan to be insufficiently cross-linked, resulting in increased cell wall permeability. This can lead to cell death due to osmotic lysis. Bacterial resistance is due to the production of β-lactamases which hydrolyse the β-lactam ring rendering them ineffective. β-Lactamase inhibitors can be combined with β-lactam antibiotics (e.g. amoxicillin combined with clavulanate - Augmentin® GlaxoSmithKline) to successfully treat infections caused by resistant organisms. Clavulanic acid, a β-lactamase inhibitor, and thienamycin are two β-lactams produced by *Streptomyces* species (McGowan *et al.*, 1998).

Glycopeptide antibiotics are synthesised by non-ribosomal peptide synthetases (NRPS). Common glycopeptides produced by actinomycetes include vancomycin and teicoplanin. In many Gram positive bacteria, the peptidoglycan precursor consists of *N*-acetylmuramic acid linked to the pentapeptide L-Ala-D-Glu-L-Lys-D-Ala-D-Ala. This pentapeptide can then link to *N*-acetylglucosamine and undecaprenylpyrophosphate and in this form it can be transported to the extracellular side of the cell membrane. The mode of action of glycopeptides is to bind to the terminal D-Ala-D-Ala thereby blocking subsequent transpeptidation and carboxypeptidation reactions, resulting in the inhibition of cell wall biosynthesis (Chu *et al.*, 1996).

Many of the antibiotics produced by actinomycetes are encoded by a PKS or a NRPS. There are a number of studies using PCR screening to rapidly identify isolates which possess these genes. Such methods are of interest as they can be used to fast track screening and for strain de-replication. They can also be used to

identify novel gene clusters for combinatorial chemistry (August *et al.*, 1998; Metsä-Ketelä *et al.*, 2002; Ayuso *et al.*, 2005; Wood *et al.*, 2007).

1.3.2.3 Actinomycete derived antibiotics currently in development

There are a number of antibiotics currently in development that have been isolated from actinomycetes. Daptomycin is a cyclic-lipopeptide produced by *Streptomyces roseosporus*. Daptomycin represents the only truly novel structural type to be launched in the past 30 years. Cubicin® (daptomycin) was approved in 2003 for the treatment of skin infections caused by a number of Gram-positive organisms including *S. aureus* (drug susceptible and methicillin-resistant strains) and susceptible strains of *Enterococcus faecalis*. It also exhibits potent *in vitro* activity against vancomycin-resistant *S. aureus* (VRSA) and vancomycin resistant enterococci (VRE) (Bush *et al.*, 2004; Barret, 2005; Miao *et al.*, 2005). Ramoplanin is a novel glycolipodepsipeptide antibiotic produced by *Actinoplanes* species ATCC 33076. It has activity against a number of aerobic and anaerobic Gram-positive bacteria including VRE and multi-drug resistant (MDR)-*S. aureus*. The exact mode of action has not been fully elucidated but it appears to inhibit bacterial transglycosylases. In 2004, oral ramoplanin received fast track status by the FDA for treatment of *Clostridium difficile*-associated diarrhoea (Bush *et al.*, 2004).

Due to the exhaustive screening of terrestrial streptomycetes by the pharmaceutical industry in the past, it is felt that the chance of isolating novel compounds is slim. The pharmaceutical and biotechnology companies that are still screening have shifted their attention towards isolating actinomycetes from new, unexploited sources or focusing on the rare genera of actinomycetes (Lazzarini *et al.*, 2000). The isolation of actinomycetes from unexplored sources has already been fruitful. In 2005, Maldonado and co-workers described the genus *Salinispora*, the first obligate marine actinomycete genus. *Salinispora tropica* has been found to produce salinosporamide A, a novel β-lactone-γ-lactam with anticancer activity. A novel polycyclic polyketide antibiotic, abyssomicin C, has been isolated from a marine *Verrucosispora* strain. The mode of action of abyssomicin C appears to be the inhibition of PABA biosynthesis which results in the inhibition of folic acid production. Abyssomicin C is active against MDR-*S. aureus* and VRSA (Maldonado *et al.*, 2005; Lam, 2006).

1.3.3 The current status of antibiotic research

While many will reminisce about the 1940s being the heyday of antibiotic research, the last two decades of the 20th century will be remembered for the decline in antibiotic research. By the end of the 1990s, with so many antibiotics isolated, many of the large pharmaceutical companies (Big pharma) either pulled out completely or de-emphasized antibiotic research and development (R & D), including Aventis, Eli Lilly, GlaxoSmithKline and Procter & Gamble. Others recognise the need for new antibiotics and still promote antibacterial drug discovery programs (e.g. Johnson & Johnson, Merck and Pfizer). Antibiotic research has been replaced by the search for new drugs that can be used in the treatment of chronic diseases such as cardiovascular disease, diabetes, depression and other 'life-style' diseases. These 'pill a day for life' diseases are favoured over antibiotics, which are only used to treat acute infections for a short period (Overbye & Barret, 2005). The lack of R & D is apparent when one considers that the FDA only approved 47 new antibiotics between 1983 and 2001, and of the nine

antibiotics released since 1998 only two, linezolid and daptomycin, have novel mechanisms of action (Barret, 2005).

Fortuitously, while the 1990s witnessed big pharma scaling down their antibiotic research efforts, it also saw the 'birth' of the biotech industry. Many new biotech companies were formed with the goal of using the new platforms of genomics and high throughput screening (HTS) to find new antibiotics. There are early signs that biotech may be able to fill the void left by big pharma. Even though there are over 600 drugs in clinical development, only a handful are antibiotics. Of these, only three have novel structures and all are being developed by biotech companies (Barrett, 2005).

The lure of genomics and HTS

The growth in the biotech industry saw a "paradigm shift" in the pharmaceutical industry from the screening of natural products to one focused on combinatorial chemistry, HTS and genomics. The initial interest in genomics and HTS was their potential to identify either compounds with novel mechanisms or identify potential targets in a pathogen (Peláez, 2006).

The advantage of HTS is that a large collection of chemical compounds (the library) can be screened simultaneously against a specific bacterium or isolated receptor. These screening methods have identified many potential inhibitors of novel bacterial targets. However, most are not viable as they are either too weak or they are unable to permeate the cell membrane so can not reach their targets (Peláez, 2006).

In the early 1990s, genomics was heralded as 'the solution" to the antibiotic crisis. Genomics based methods including metagenomics, DNA microarrays, proteomics and genome sequencing of pathogenic bacteria have identified hundreds of new targets (Overbye & Barret, 2005; Peláez, 2006) However, of the antibiotics in development or pre-clinical trials, none has been developed on the gene-to-target-to-lead approach, the basis of genomic methods (Thomson *et al.,* 2004). Although these methods are theoretically sound, the fact that no genomic-based targets are currently under development has led people to question their feasibility (Knight *et al.*, 2003; Peláez, 2006).

It is clear that genomics will never completely replace traditional medicinal chemistry. Challis & Ravel predicted the structure of coelichelin, a novel peptide produced by *Streptomyces coelicolor* M145, from the sequence of its non-ribosomal peptide synthetase (Challis & Ravel, 2000). However, subsequent work by Lautru *et al.,* combining genome mining and traditional structural determination methods, showed that the predicted structure was only partially correct as the final steps in the synthesis of coelichelin are unique (Lautru *et al.*, 2005).

1.3.4 Antibiotic resistance

"Micro-organisms can do anything, micro-organisms are cleverer than chemists."

D. Perlman, 1980 (quoted in Hamilton-Miller, 2004)

Antibiotics have been described by Levy as "society drugs" in that they not only affect the person taking them, but also the bacteria in the environment surrounding the treated person. The acquisition of resistance by bacteria is inherent in an antibiotic's mode of action. They 'target' the susceptible strains, while selecting the mutants that are resistant (Levy, 1998). There are a number of theories as to the origin of resistance genes. These include the possibility that when bacteria were first exposed to antibiotics, certain housekeeping genes may have evolved to modify antibiotics, or the ability to inactivate antibiotics may have developed in producing strains as a method to protect themselves (Davies, 1994). Resistance to a new antibiotic is generally reported within four years of its FDA approval. Vancomycin had an extended 'honeymoon period': it was approved in 1972, but the development of resistance was only reported 15 years later (Bush, 2004). It is unfortunate that the decline in R & D by big pharma was coupled with the rise in antibiotic resistance. The lack of new compounds, especially those with novel mechanisms, combined with the emergence of antibiotic resistance in a number of important human pathogens pose a major threat to public health (Barret, 2005).

Bacteria can either acquire resistance via spontaneous genetic mutation or via the exchange of resistance genes. Resistance arising due to spontaneous mutation is a rare event, although resistance in certain antibiotic classes, including fluoroquinolones, has been observed to occur via mutation of the bacterial chromosome (Davies, 1994). The three main methods of resistance-gene exchange are conjugation, transformation and transduction. Transduction is the transfer of genes via bacteriophages. Transformation is the uptake of naked DNA fragments from the environment. Conjugation involves the transfer of plasmids between bacteria via cell-to-cell contact. The transfer of resistance between different Gram-positive genera frequently occurs. Although the transfer of resistance from Gram-positive to Gram-negative species does occur, due to differences in cell wall morphology transfer of resistance from Gram-negative species to Gram-positive species is rare (Neu, 1992).

The three mechanisms of antibiotic resistance are prevention of access to target, alteration of the target and inactivation of the antibiotic via enzymatic modification. The two mechanisms of preventing an antibiotic finding its target are to either reduce its uptake into the cell or actively expel it from the cell.

Worldwide, the mortality rates for many 'curable' diseases, which had been steadily declining over the last few decades, are once again rising. Amongst these, methicillin-resistant *Staphylococcus aureus* (MRSA), VRE and multi-drug resistant (MDR) *Mycobacterium tuberculosis* are of increasing public concern as they are common "hospital acquired infections" (World Health Organization, 2002). The emergence of resistance has also resulted in the development of 'new' deadly infectious diseases, including infections caused by *Acinetobacter* species which are prevalent in immuno-compromised individuals (Go *et al.*, 1994; Levy, 1998).

Even though the emergence of resistance is inevitable, the inappropriate use of antibiotics has accelerated its spread. The Centre for Disease Control (CDC), USA, estimated that of the 150 million annual outpatient prescriptions a third were unwarranted and were often the result of patients demanding antibiotics for viral

infections. Lack of compliance or saving antibiotics for self medication or to give to other people is another factor that has lead to an increase in resistance as this exposes bacteria to sub-lethal doses of antibiotics, allowing resistance to arise (Levy, 1998). Another major factor leading to the development of resistance is the use of antibiotics in farming. Of the estimated 50 million pounds of antibiotics produced annually in the USA, 50 to 70% are used in animal husbandry, mainly as growth promoters (Levy, 1998; Nicolaou & Boddy, 2001). Ultimately, the main way to reverse the resistant trend is by the judicious use of antibacterials.

The development of antibiotic resistance has highlighted the need for increased natural product research to discover novel antibiotics with new targets. Chemical modification of existing compounds are merely stalling tactics, as resistance to these modified compounds is likely to arise as readily as it did for their forerunners (Travis, 1994; Shlaes *et al.*, 2004; Peláez, 2006).

1.3.5 Future prospects – the boundless potential

"The bottleneck in any isolation strategy is not to obtain a high number of sufficiently diverse isolates but to apply sufficiently discriminating techniques that would rapidly separate taxonomically novel from known strains"

E. Stackebrandt, 2003

1.3.5.1 Natural product screening: Are actinomycetes still a source of novel antibiotics?

The decline in antibiotic research seems unwarranted when one considers the success of natural product derived drugs in the past. In 2002 alone, 17% of the top 100 best-selling drugs worldwide were derived from natural products (US$28.9 billion) (Knight *et al.*, 2003). It is also clear that there is still a market for antimicrobials when one considers that anti-infective drugs are surpassed only by drugs to treat cardiovascular disease and those that target the CNS on the list of global sales (Bush, 2004).

So, why continue screening natural products especially when looking for new antibiotics?

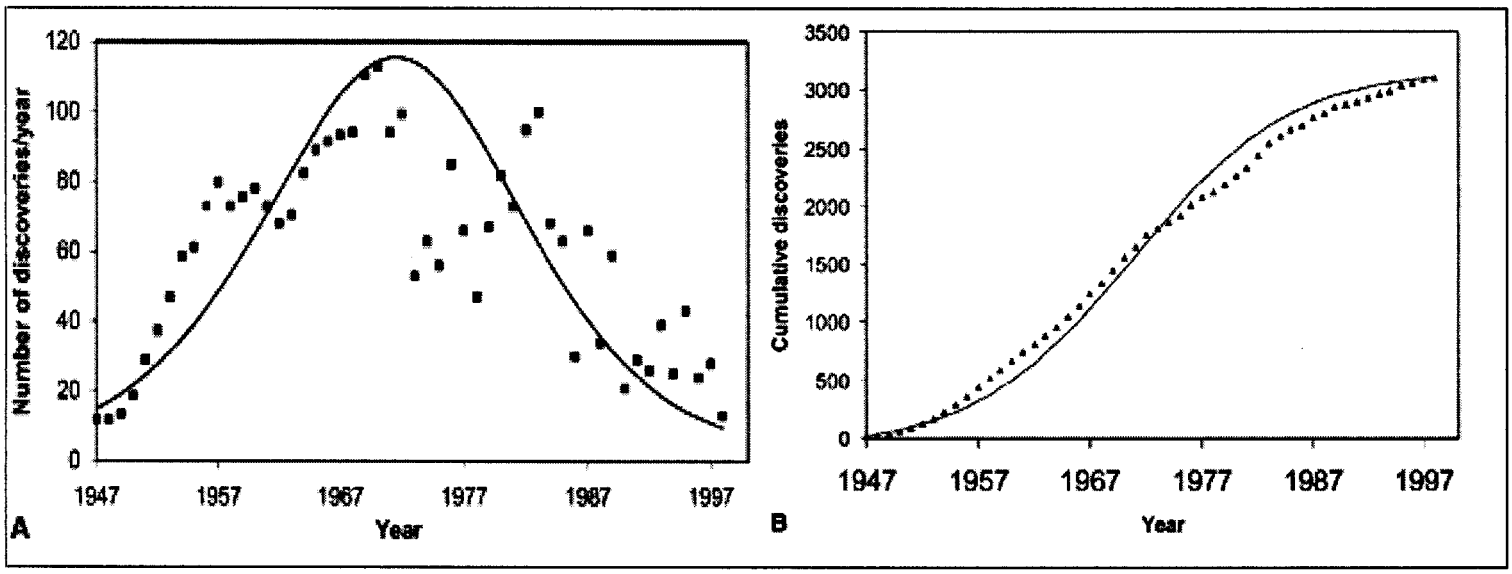
- Natural products usually have a higher molecular weight, contain more rings and are more sterically complex than synthetic compounds (Lawrence, 1999).
- Many organisms have fastidious growth conditions and are unsuitable for HTS, especially slow growing strains such as the rare actinomycetes (Lawrence, 1999).
- 40% of the natural products are not represented in synthetic libraries (Nisbet & Moore, 1997; Knight *et al.*, 2003).
- 99% of bacteria are unexplored and, with advances in microbiology, many of those deemed 'unculturable' can in fact be cultured (Watve *et al.*, 2001).

Several methods have been developed to selectively isolate the rare antibiotic-producing genera including *Actinoplanes, Amycolatopsis, Micromonospora* and *Streptosporangium* (Lazzarini *et al.*, 2000). Chemoattraction and pollen baiting have been used to isolate *Actinoplanes* species (Hayakawa *et al.*, 1991). Nocardioform actinomycetes can be selectively isolated by air drying the sample prior to plating and incorporating rifamycin into the isolation medium, while the addition of novobiocin and spectinomycin can selectively enhance the

isolation of *Micromonospora* species (Horan, 1999). A study published by Lazzarini *et al.* (2000) estimated that of the 8000 antimicrobials in the Antibiotic Literature (ABL) database (Biosearch Italia), 45% were produced by streptomycetes and 16% were produced by rare actinomycete genera. The three most prolific families of rare actinomycetes were 38% *Micromonosporaceae* (*Micromonospora* and *Actinoplanes*), 15% *Pseudonocardiaceae* (*Amycolatopsis* and *Saccharopolyspora*) and 14% *Thermomonosporaceae* (*Actinomadura*) (Lazzarini *et al.*, 2000).

The sequencing of the complete genome of *Streptomyces coelicolor* strain A3(2) revealed that 5% of its 8.7 Mb genome is dedicated to genes involved in secondary metabolite synthesis. It has been suggested that each actinomycete has the genetic potential to produce 10-20 different types of secondary metabolites (Bentley *et al.*, 2002; Donadio *et al.*, 2002). If one considers the number of yet undiscovered actinomycetes combined with their remarkable range of genetic diversity, it is obvious that they are still an important source of novel antibacterials. In fact, statistical modelling has estimated that the number of yet undiscovered antibiotics produced by the genus *Streptomyces* is 150 000 and some feel that this is a conservative estimate (Watve *et al.*, 2001; Donadio *et al.*, 2002). It is clear that the decline in novel antibiotic discoveries observed in the 1980s is a reflection of the decreased screening effort coupled with re-isolations of the known compounds, not a lack of novel antibiotics (Figure 1.4). If estimates are correct, less than 3% of natural products have been characterised thus far (Chopra *et al.*, 1997; Donadio *et al.*, 2002).

Figure 1.4 The best-fit logistic model for antibiotic discovery for the years 1947 to 1997.



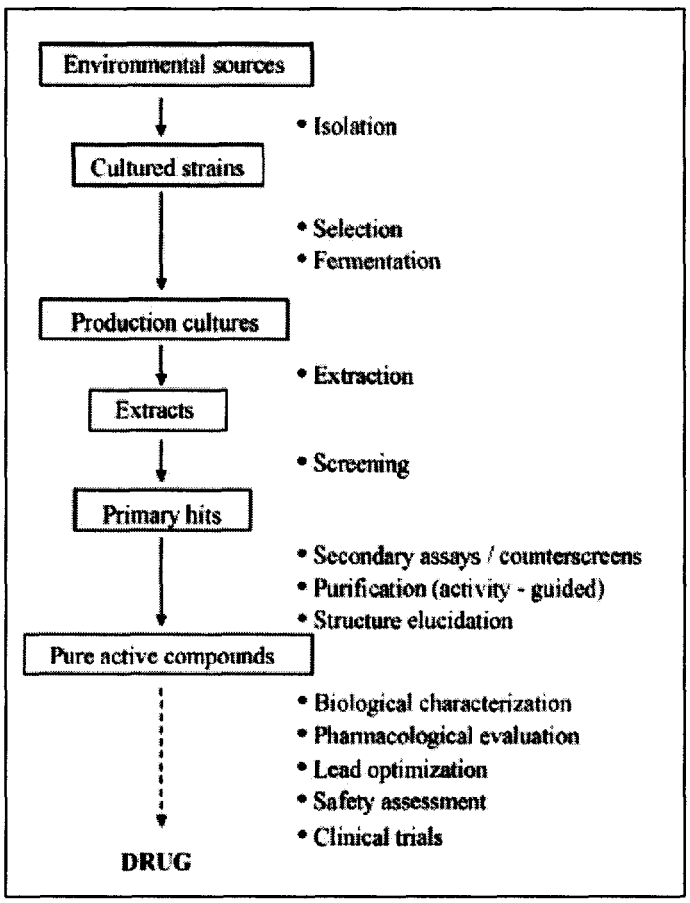
Symbols (•;▲) denote data points; the line is the fitted logistic curve. A – Discoveries per year; B – Cummulative trend (figure taken from Watve *et al.*, 2001).

It is felt that our best chance of success is to combine natural products with combinatorial chemistry. For this to be prosperous, screening for natural products should be focused on biologically diverse areas. This belief is underpinned by the fact that biodiversity is a marker of chemical diversity (Strobel & Daisy, 2003; Overbye & Barret, 2005). For natural product screens to take full advantage of microbial biodiversity, sampling can not be a randomised process. The intended target must first be identified, be it a new antibiotic or immunosuppressive,

as this will guide the search. It can then be decided which group of organisms is most likely to produce this product and which screening method to use (Bull *et al.*, 2000).

Once screening has identified compounds with antibacterial activity, combinatorial chemistry can be used to improve their pharmacokinetics and potency. The one advantage of this methodology is that natural products are usually more easily able to enter the cell; an inherent problem with many synthetic drugs is their inability to cross the cell wall/ membrane (Strobel & Daisy, 2003; Overbye & Barret, 2005). The diversity of natural products produced by microorganisms should not be underestimated, as the compounds they produce have evolved over the millennia to have enhanced antibacterial activity (Peláez, 2006). The development of a novel bioactive compound from isolation and screening, through to optimization and clinical trials, to final approval is represented in Figure 1.5. Although a large number of compounds are initially isolated, the numbers decrease with every step in the development process. Only a handful of compounds (represented by the dotted line in Figure 1.5) enter the final stages of clinical development. It is estimated that the success rate for antibacterials that enter clinical trials is 35%, which is four times higher than for any other type of therapeutic agent (Thomson *et al.*, 2004).

Figure 1.5 The development of a microbial natural product from isolation to final approval (Peláez, 2006).



1.4 Tuberculosis

1.4.1 The global incidence of TB

The ancient Greeks referred to phthisis, medieval England was cursed with the "Kings Evil", while in 18th century Western Europe consumption was feared by the poor. Today we refer to this disease as tuberculosis (TB). It has been a scourge on human health for centuries (tubercle bacilli have been detected in 1000 year old Peruvian mummies) and is likely to remain so for centuries to come. In the last century alone, TB caused 100 million deaths (Frieden *et al.*, 2003). In 1882 Robert Koch identified the "tubercle bacillis", and his experiments were the first to illustrate the microbial cause of an infection and demonstrate its infectious nature. His findings became known as "Koch's postulates" and still have relevance in modern epidemiological research. Research by Albert Calmette and Camille Guérin in 1908 identified an avirulent variant of *Mycobacterium bovis,* which they used to immunize a child whose mother died of TB. This attenuated strain of *M. bovis* was developed into the Bacille Calmette-Guérin (BCG) vaccine, which is still the most widely used vaccine worldwide (Bloom & Murray, 1992; Donoghue *et al.,* 2004; Murray, 2004).

TB is caused by *Mycobacterium tuberculosis* (MTB) and it is estimated that a third of the world's population harbours this organism, having a 10% lifetime risk of developing active TB. After the human immunodeficiency virus (HIV), TB has the second highest mortality rate from an infectious disease (Corbett *et al.*, 2003; Frieden *et al.*, 2003). MTB can survive in the host as dormant latent bacilli. This is the main problem in TB chemotherapy as most antibiotics are only active against actively replicating cells. In order to fully cure a patient, drugs that are effective against these persisters must be administered. Antibiotics which are active against non-replicating bacteria are said to have sterilising activity (Cohen, 2004; Duncan & Barry, 2004).

There are numerous studies reporting the worldwide incidence of TB, the most comprehensive published by the World Health Organization (WHO) in 2006 (WHO report 2006). Key findings published in this report included: a) in 2004, there were over 9 million new TB cases and 2 million TB deaths worldwide; b) >80% of all TB patients are living in sub-Saharan Africa and Asia; c) although the incidence of TB is declining in five of the six WHO regions, the global incidence is increasing by 0.6% annually; d) the prevalence of HIV in the WHO Africa region can account for the increase in the incidence of TB in this region. South Africa was fifth in the list of 22 high burden countries (HBC); however, it had the third highest TB incidence rate per capita. It is estimated that the cost of TB control in the 22 HBC was US$1.6 billion in 2004.

The current standard short course (SSC) chemotherapy recommended by the WHO and other medical bodies is a six-month regimen of the three first-line drugs, isoniazid, rifampicin and pyrazinamide (either daily or three times a week, depending on dosage), either with or without the addition of ethambutol/streptomycin. The current regime in South Africa for initial TB treatment is Rifater, which combines the three first-line drugs (Weyer *et al.*, 1995; Zhang *et al.*, 2006).

1.4.2 TB in South Africa

Of the estimated 2.5 million new TB cases reported in Africa in 2004, nearly 340 000 cases occurred in South Africa, with over 60% of these patients co-infected with HIV. Even though 93% of these patients were treated under the DOTS program (directly observed therapy, short-course), only 54% were cured in 2004. The WHO target cure rate under the DOTS system is 85% (WHO report 2006). The majority of the 63 000 TB deaths reported in 2004 occurred in young adults (15-49 years). This suggests that the high mortality rate in South Africa is due to co-infection with HIV. In fact, after Nigeria, South Africa has the second highest TB mortality rate in the WHO Africa region (WHO report 2006). Co-infection rates are greater than 60% in many sub-Saharan countries including Botswana, South Africa, Zambia and Zimbabwe.

The incidence rate of TB in South Africa is approximately 0.7% (718 per 100 000 population). However, the incidence rates vary between the nine provinces. The Western Cape Province accounts for over 20% of the cases, followed by the Eastern Cape Province and KwaZulu-Natal Province. Alarmingly, the highest infection rates in the world have been recorded in some communities of the Western Cape Province (Caelers, 2003; WHO report 2006).

When a person is co-infected with MTB and HIV, the risk of developing active TB increases to 10% per annum (Corbett *et al.*, 2003). The high incidence of TB and HIV co-infection can be attributed to a number of factors. As HIV depresses a person's immune response, when they are co-infected with TB their immune system is unable to destroy the bacilli and active primary TB develops. Many of the diseases that frequently occur in patients with acquired immune deficiency syndrome (AIDS), such as pneumonia, require hospitalisation where the immuno-compromised patients are exposed to TB, especially resistant strains. Many of the adverse side effects of anti-TB drugs are more severe in patients with HIV, this frequently leads to a lapse in treatment. Furthermore, some anti-retrovirals cause the anti-TB drugs to be malabsorbed (Harries *et al.*, 2001; Dye *et al.*, 2002). Thirty percent of HIV-positive patients co-infected with TB die in the first year of TB treatment (Frieden *et al.*, 2003).

1.4.3 Multi-drug resistant TB

Multidrug-resistant (MDR) tuberculosis is defined as a strain of MTB which is resistant to the two first-line drugs isoniazid and rifampicin. As MTB acquires resistance via mutations of the chromosome, MDR TB is the result of individual mutations in several genes, not the acquisition of large fragments carrying many resistance genes (Telenti & Iseman, 2000). The resistant mechanisms found in MTB include drug efflux systems, production of hydrolytic and modifying enzymes including β-lactamases and modifying of the antibiotic target site (mycobacteria also posses a type of 'natural resistance' in the form of a highly hydrophobic cell envelope, which is impermeable to many antibiotics). Resistance to the antibiotics used in SSC chemotherapy has been linked to the following genes: mutations in *katG, inhA, kasA* and *ahpC* are found in 62-90% of the isoniazid resistant strains; mutations in *pncA* occur in 72-97% of the pyrazinamide resistant isolates; and mutations in *rpoB* occur in >96% of the rifampicin resistant strains. As rifampicin resistance occurs in all cases of MDR TB and 96-100% of the rifampicin resistant strains have a mutation in *rpoB*, it can act as a marker for MDR TB. All the mutations in

the *rpoB* gene have been mapped to the rifampicin binding site between amino acids 511 to 533 (*E. coli* numbering), therefore a PCR based detection method that amplified an 80 bp fragment could potentially identify all mutations (Blanchard, 1996; Telenti & Iseman, 2000).

The full extent of MDR TB is not known, but it is estimated that of the 8.7 million new TB cases reported globally in 2000, over 273 000 were MDR (Raviglione & Smith, 2007). The emergence of MDR TB is likely to drain the health care budgets in the HBCs. The treatment of MDR infection requires longer treatment (from 6 months SSC to 18 months) with second-line drugs including capreomycin, cycloserine, ethionamide, fluoroquinolones, kanamycin and para-amino salicylate (PAS). These drugs are more costly, less effective or more toxic (including auditory-, renal- and hepatotoxicity) (Frieden *et al.*, 2003; Zhang *et al.*, 2006). The cost of treating a patient with MDR TB spirals from ZAR 200 for treatment of susceptible strains to ZAR 30 000 for MDR infections (Brümmer, 2003).

In late 2006, the South African public was shocked by newspaper headlines such as '*Death in the air as virulent strain takes hold*' (Makhaye, 2006). These articles reported the deaths of 52 of the 53 patients being treated for MDR TB at Tugela Ferry, KwaZulu-Natal Province (Salie & Smith, 2006). These deaths were caused by a new form of MDR TB, extensively drug-resistant (XDR) tuberculosis. XDR TB strains are resistant to isoniazid and rifampicin with the addition of resistance to at least three of the six second-line drugs (including any fluoroquinolone or any of the injectable antibiotics: capreomycin, kanamycin and amikacin) (World Health Organization, 2006; Raviglione & Smith, 2007). The rapid progression of this disease is striking. In the KwaZulu-Natal cohort, of the 53 patients, the median survival period was only 16 days from the time of the first sputum specimen collection; with 50% of the cases being primary infections. Of the 44 patients that were tested, all were found to be HIV positive (Raviglione & Smith, 2007). Cases of XDR TB have been confirmed in at least 17 countries, with the highest prevalence in Asia and countries belonging to the former Soviet Union. Some experts estimate that 10% of reported MDR TB infections are in fact caused by XDR strains. Special measures must be implemented when handling suspected XDR TB. These include rapid diagnosis and drug susceptibility testing, enforced supervision of treatment, isolation of hospitalised patients and best practice to prevent the spread to other patients and healthcare workers (Raviglione & Smith, 2007).

1.4.4 Future prospects

For future TB control programs to be effective, a three-pronged approach must be adopted, focusing on prevention, detection and cure.

Immunization with the BCG vaccine is still part of the national immunisation program in many HBCs including South Africa. As BCG only protects against pulmonary TB in children less than 15 years of age, there is a need to develop a new TB vaccine. A problem facing vaccine development is HIV co-infection. A new vaccine must be safe yet also able to stimulate an immune response in patients with reduced levels of CD4+ lymphocytes. (Brennan, 2005).

Most of the TB drugs currently used were released in the 1970s. In the last 30 years the only drugs that have been approved are modifications of existing drugs. A number of factors have limited the investment by the large pharmaceutical companies into TB drug development, including difficulties in testing new compounds, the belief that TB is a disease which affects third world countries (which will limit their return on investment) and a lack of incentives from government to develop new drugs (Cohen, 2004; Duncan & Barry, 2004).

The Global Alliance for TB Drug Development (GATB) prioritized the need to find new TB drugs. Ideally, a candidate drug must have improved sterilizing activity thereby shortening the treatment period (Cohen, 2004; Duncan & Barry, 2004). A number of new drugs are in the pipeline. Two quinolone antibiotics are of special interest. Moxifloxacin (Bayer) and the diarylquinoline R207910 (Johnson & Johnson) are both in early clinical trials. Moxifloxacin was approved for clinical trials against TB in SA in 2005 (Sapa-AP, 2005). The *in vitro* activity of R207910 against both drug-sensitive and drug-resistant MTB strains is MIC 0.06 μg/ml. The activity of this antibiotic is also specific to mycobacteria. Mutation studies identified the target as ATP synthase. Its mode of action is to inhibit the proton pump of MTB ATP synthase, thereby leading to a depletion of ATP. As the target is novel there is no cross-resistance with current TB-drugs (Andries *et al.*, 2005).

The July 2000 G8 summit identified HIV, TB and malaria as the "three priority diseases of poverty". A key decision of the summit was that a united global effort is required to eradicate these diseases. One of the targets that were agreed to at the summit was that by 2010 there must be a 50% reduction in both the death rate and prevalence of TB (Harries *et al.*, 2001; Frieden *et al*, 2003). With the rising levels of HIV-TB co-infection and an increasing number of MDR TB infections, this goal seem sadly out of reach.

1.5 Aims of the project

The four main aims of this study were:

- South Africa is known for its biological diversity, especially floral diversity, however, little research has been conducted on its microbial diversity. Therefore, the first aim of this study was to characterise actinomycetes isolated from three sources, the leaves of indigenous South African plants, aquatic sediment and soil. The potential of alternative isolation media and different sample pretreatment methods for the selective isolation of the rarer genera was also investigated. Actinomycete strains belonging to the rarer genera or those with antibiotic activity were selected for full characterisation.

- There are a number of antibiotics produced by actinomycetes that are effective against TB, including rifamycin and streptomycin. As actinomycetes are still considered a source of novel antitubercular compounds, the second aim of this study was to screen all actinomycete strains isolated from this study for the production of antibiotics that are effective against *Mycobacterium aurum* A+, a non-pathogenic mycobacterium with a similar antibiotic susceptibility to *M. tuberculosis*. All isolates were screened for antimicrobial activity by standard agar overlays, small scale fermentations and solvent extract, and a modified Eli Lilly method. Isolates were also PCR screened for the presence of antibiotic biosynthetic genes involved in the production of aminoglycosides, ansamycins, glycopeptides and Type II PKSs.

- *Streptomyces speibonae* PK-Blue^T has previously been reported to produce an antimycobacterial compound effective against *M. aurum* A+. The isolation and purification of this compound, PK-B, was optimised. Preliminary characterisation and structural determination was performed including elemental analysis, NMR and mass spectrometry.

- The 16S rRNA gene sequence similarity between members of the genus *Kribbella* ranges from 97.5 to 98.6%. Although *Kribbella* strains can be differentiated based on phenetic characteristics, DDH is usually required to distinguish them as unique species. Therefore, the aim of the last part of this study was to assess the potential of the *recN* and *gyrB* genes to resolve species relationships within the genus *Kribbella*.

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CHAPTER 2

Characterisation of actinomycetes isolated from soil, sediment and indigenous plant species

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2.1 Summary

The distribution of actinomycetes isolated from three different environmental niches was investigated using alternative isolation methods to increase the probability of isolating actinomycetes belonging to the rare genera. Initially 99 actinomycete strains were selected. Strains were identified to the genus level and preliminary phenetic testing was performed. Based on these findings 30 isolates were selected for further analysis. A polyphasic approach was employed which included phylogenetic analysis, phenetic characterisation and chemotaxonomy. Actinomycetes belonging to six genera were isolated, of which two, *Kribbella* and *Kineococcus* are considered rare. A total of 13 *Streptomyces*, eight *Micromonospora*, two *Kribbella*, four *Kineococcus*, two *Gordonia* and a single *Nocardia* species was isolated. Many of the streptomycetes isolated are likely to be strains of known species. However, three micromonosporae and at least two of the kineococci may be novel species and warrant further investigation. It was found that the selective isolation methods can be used successfully to isolate novel actinomycetes from terrestrial samples where streptomycetes dominate. Two novel *Kribbella* species were identified and the species descriptions of *Kribbella karoonensis* Q41^T and *Kribbella swartbergensis* HMC25^T have been published.

CHAPTER 2

Characterisation of actinomycetes isolated from soil, sediment and indigenous plant species

2.2 Introduction

Microorganisms adapt in response to changes in their environment. Ultimately, this will determine the types of secondary metabolites they produce. This idea forms the basis of many current isolation schemes. It is felt that the chance of isolating novel microorganisms is increased by sampling previously unexploited sources, especially those known to have a high biodiversity (Knight *et al.*, 2003). Two promising sources are plants and marine habitats. Novel actinomycetes have been successful isolated from both these sources. Most importantly, some of these novel species produce novel bioactive compounds.

Bacon and White defined endophytes as "microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects" (Bacon & White, 2000). Although the levels of these symbiotic interactions may vary, a true endophyte obtains either nutrients or other compounds from the host plant. This feature distinguishes them from epiphytes, which are microorganisms that live on plants but do not depend on them for nutrition (Strobel, 2002). Nearly all higher plants host at least one endophytic species and therefore represent a unique environmental niche. If one considers that there are potentially 300 000 plant species worldwide, plant endophytes represent an untapped source of novel species (Strobel & Daisy, 2003). When studying endophytes it may be difficult to prove that a relationship exists between the microorganism and the host plant, as the number of endophytes may be low and they are usually found in the intravascular spaces between cells (Strobel, 2002).

The study of plant associated actinomycetes has a long history. As early as 1890 *Streptomyces scabies* was shown to cause common scab on potatoes (Lambert & Loria, 1989). Although most research has focused on pathogenic *Streptomyces* species and nitrogen fixation by *Frankia* species, recent studies have shown that the actinobacteria may form complex relationships with plants (Miyajima *et al.*, 1998).

Studies have shown that a higher number of endophytes can be found in monocotyledonous plants such as corn (maize) than in dicotyledonous plants (Zinniel *et al.*, 2002). Most studies into endophytic actinomycetes and their potential as biological control agents have focused on this economically important crop, as well as bananas (Cao *et al.*, 2005), carrots (El-Tarabily *et al.*, 1997) and wheat (Anukool *et al.*, 2004), and have concentrated on the rhizosphere. The rhizosphere is a zone surrounding the roots and this root-soil interface is known to support large bacterial populations. Although *Frankia* species are frequently isolated from the rhizophere of healthy plants, other actinomycete genera have also been isolated. Coombs & Franco isolated a number of *Microbispora, Micromonospora, Nocardioides* and *Streptomyces* species from surface-sterilised wheat roots

(Coombs & Franco, 2003), while *Nocardia* and *Streptosporangium* species have been isolated from the roots of other crop plants (Cao *et al.*, 2005).

Recent studies have shown that many of the healing properties attributed to plants are in fact due to metabolites produced by endophytic microorganisms (Strobel, 2002). The diterpenoid taxol is a billion dollar grossing anticancer agent that is isolated from the yew (*Taxus*). Every yew species investigated has been found to produce at least one type of taxol. The large scale isolation of taxol has been limited by the slow growth rate and relative rarity of yew trees. A 1993 study by Strobel *et al.* isolated an endophytic fungus, *Taxomyces andreanae*, from the yew species *Taxus brevifolia* that produced a novel taxol. This finding was of special interest to the pharmaceutical industry as microbially derived compounds can readily be produced in large volumes by fermentation (Strobel *et al.,* 1993).

Most investigations into natural products produced by endophytes have focused on the abundant fungal species (Strobel, 2002). However, recent studies have found that endophytic bacteria, including actinomycetes, are a potential source of novel natural products. An endophytic *Streptomyces* sp. strain NRRL 30566 was isolated from a fern-leaved grevillea tree, *Grevillea pteridifolia,* growing in the Northern Territory of Australia. This isolate was found to produce novel quinoxaline type antibiotics called kakadumycins. These antibiotics have antimicrobial activity against a number of Gram-positive pathogens including strains of *Bacillus anthracis* and *Streptococcus pneumoniae*. The kakadumycins also have *in vitro* activity against *Plasmodium falciparum*, the causative agent of malaria (Castillo *et al.*, 2003).

In order to maximize the chances of isolating novel microorganisms, special consideration must be given to the sites chosen for the sampling of plants. The criteria highlighted by Strobel & Daisy that should be considered when selecting plant species include: plants which come from unique environments, selecting species with an ethnobotanical history, species that are endemic to a region, and plants that come from areas known for their biodiversity (Strobel & Daisy, 2003). The Cape Floral Region fulfils all these criteria.

South Africa has eight biomes which host more than 19 000 different flowering plant species. The Cape Floral Region encompasses the fynbos biome and spans an area from Nieuwoudtville, 360 km north of Cape Town, to Port Elizabeth 770 km eastwards along the coast. Although this biome accounts for only 6% (~90 000 km^2) of the total land area of Southern Africa, more than 50% of all the plant species found in Southern Africa are found in this region. The term fynbos is derived from the Dutch for fine-leaved shrubs, which are a characteristic feature of this biome. Many of these shrubs have leathery-leaves and are woody. The other dominant plant types are perennials and bulbous plants. Members of the genera *Erica*, *Protea* and *Restio* are the predominant plant species. Over 9 000 plant species from 950 different genera are found in the fynbos biome, of which 70% are endemic (Manning, 2003). The biodiversity of this region rivals that of the tropical rainforests.

Ethnobotany is defined as the use of plants in religion, folk medicine and traditional healing. South Africa has a long oral history of using plants for medicinal purposes. Over 3 000 plant species are used for various medical applications (Van Wyk *et al.*, 1997). The theory that endophytes isolated from plants with known medicinal applications may be the source of novel bioactive compounds has been proven to be correct. An endophytic

Streptomyces sp. strain NRRL 30562 isolated from the snakevine, *Kennedia nigriscans*, was found to produce the broad spectrum antibiotics munumbicins A, B, C and D. Traditionally, the snakevine has been used by Aboriginal groups to cure skin infections and promote healing. The munumbicins were found to be active against a number of Gram-positive pathogens including MDR *M. tuberculosis*, pathogenic fungi and *P. falciparum*. This study is also of interest as it is one of the first reports of endophytic actinomycetes being isolated from a dicotyledonous plant species (Castillo *et al.*, 2002).

There has been a long held belief that the actinomycetes isolated from aquatic environments, especially marine habitats, are due to terrestrial wash-ins and will result in the re-isolation of known species and, by default, known compounds. This fact, combined with the technical difficulties and increased cost of sampling from marine environments, especially the deep seafloors, has prevented the routine screening of these habitats (Ward & Bora, 2006).

For a microorganism to be described as indigenous to a marine environment there must be proof that it grows optimally at conditions which mimic those of the marine habitat including salinity, pressure, temperature and nutrient composition and that the metabolic signatures of marine organisms are present. *Rhodococcus marinonascens,* was the first 'true' marine actinomycete species to be described (Helmke & Weyland, 1984). A number of marine actinomycete species belonging to the genera *Dietzia, Streptomyces* and *'Marinomyces'* have subsequently been described (Bull *et al.*, 2005). *Salinospora* is the first obligate marine actinomycete genus to be identified. All *Salinospora* strains, originally designated MAR1 strains, require either seawater or medium supplemented with sodium for growth (Maldonado *et al.,* 2005). MAR1 strains have been isolated from near-shore sediment samples collected from geographically diverse sites including the Atlantic Ocean, the Red Sea in Egypt and Sea of Cortez, Mexico. Proof of the persistent occurrence of MAR1-type strains in the marine environment was confirmed by the re-isolation of these strains on several occasions between 1989 and 2000 (Mincer *et al.,* 2002).

Although actinobacteria make up a minor component of the total bacterial populations of many marine habitats, they have been isolated from diverse sources including near-shore sediments, tropical coral reefs and the deep seafloor (Lam, 2006; Ward & Bora, 2006). A study by Goodfellow and Haynes estimated that actinomycetes may account for less than 0.3% of the total bacterial population from near-shore marine sediments (reported in Goodfellow & Williams, 1983). However, other studies have found that actinomycetes can account for ~8% of the total microbial population in some locations (Takizawa *et al.*, 1993). *Streptomyces* and *Micromonospora* species are most frequently isolated from marine habitats. However, a number of studies using both culture-based and molecular techniques have identified strains most similar to *Actinomadura, Amycolatopsis, Gordonia, Nonomuraea* and *Verrucosispora* species from diverse marine environments (Ward & Bora, 2006). The distribution of actinomycete species in marine environments is not uniform. The near-shore regions are dominated by streptomycetes, many of which are likely to be of terrestrial origin. Actinoplanete species, mainly micromonosporae, are dominant from deep sampling sites (Jensen *et al.,* 1991). The dominance of actinoplanetes in deep sea samples and the near obligate requirement of seawater for growth by some *Micromonospora* isolates support the theory that these strains are true marine actinomycetes (Jensen *et al.,* 1991).

Actinomycetes have also been isolated from non-marine aquatic sources including freshwater streams and estuaries. These habitats must not be overlooked as sources of potentially novel species. A study investigating the distribution of bacteria in estuaries revealed that the actinomycetes present were a combination of marine and freshwater species. The mixing of fresh and seawater in an estuary generates a number of chemical and biological gradients including salinity, nutrient concentrations and organic matter, which alters the bacterial populations on a seasonal basis. Bacterial populations appear to be the most abundant in summer and autumn (Crump *et al.*, 2004).

As in marine habitats, *Micromonospora* and *Streptomyces* species appear to be dominant in freshwater environments. A study by Jiang and Xu investigated the distribution of actinomycetes in 12 lakes in China. They found that *Micromonospora* species accounted for 39 to 89% of the actinomycete populations isolated from freshwater sediment samples, followed by *Streptomyces* species. *Actinomadura, Nocardia, Rhodococcus* and *Saccharopolyspora* species were found at a number of locations with the complexity of the populations being the greatest in the dry seasons (Jiang & Xu, 1996). *Micromonospora* species are thought to play a key role in the degradation of cellulose and chitin in aquatic environments, while *Actinoplanes* are frequently isolated from decomposing leaf matter. Studies have shown that the number of actinomycetes found in foam from marine and freshwater settings is higher than that of the surrounding water column, which may be due to the hydrophobic spores concentrating at the air-water interface (Goodfellow & Williams, 1983).

It is estimated that in 1 g of soil there are over 10^6 actinomycetes. Although *Streptomyces* are the dominant species in the terrestrial environment, over 20 actinomycete genera have been isolated from soil. The dominance of streptomycetes may be due to their ability to survive as dormant spores which germinate when nutrients, in the form of root fragments and decaying fungal hyphae, become available. This feature is shared by many soil-dwelling organisms. These spores can be distributed by wind, water and by insects (Goodfellow & Williams, 1983). Many streptomycetes are unable to grow at high osmotic potentials and are likely to survive as dormant spores under these conditions (Williams *et al.*, 1989). Most soil actinomycetes are aerobic, however the isolation of *Micromonospora* species from moist soil, which is frequently poorly aerated, suggests that these species may be able to survive in microaerophilic environments. In terrestrial environments the main role of actinomycetes is likely to be the degradation of complex polymers, either natural in the form of plant litter or artificial polymers including petroleum and insecticides (Goodfellow & Williams, 1983).

Even though the terrestrial environment has been extensively screened for novel actinomycetes, its potential has not been exhausted. Novel microorganisms and natural products are still readily isolated from terrestrial environments (Lam, 2007), and the number of newly described actinomycete species isolated from soil samples far outweighs those isolated from other sources. By collecting samples from unique ecosystems and using selective culture based techniques, the likelihood of isolating novel actinomycetes from terrestrial settings is increased. Another potential source of novel terrestrial actinomycetes might be terrestrial animals, especially arthropods, many of which have close interactions with soil. The description of the '*Candidatus* Streptomyces philanthi' strains shows that insects may be a source of novel actinomycetes (Kaltenpoth *et al.*, 2006).

It is estimated that less than 1% of microorganisms from the natural environment are culturable under standard laboratory conditions. Recent studies have shown that a number of simple techniques can be applied to improve the isolation of actinomycetes from aquatic, plant and terrestrial samples. These include using alternative isolation media such as those containing chitin or soil extract (Goodfellow & Williams, 1983; Hamaki *et al.*, 2005). The contamination of isolation plates by Gram-negative microorganisms can be problematic, especially when isolating from marine and estuarine samples. It has been shown that a combination of mild heat treatment and the addition of nalidixic acid to the isolation medium reduces the number of Gram-negative organisms and increases the number of actinomycetes (Takizawa *et al.*, 1993), while the inclusion of cycloheximide in the isolation medium reduces the number of contaminating fungi (Ravel *et al.*, 1998).

The aim of this part of the study was to isolate novel actinomycetes from three sources: indigenous South African plants, aquatic sediment and terrestrial soil. The potential of alternative isolation media and different sample pretreatment methods for the selective isolation of the rarer genera was investigated. Comparisons could be made as to the optimal selection procedure to employ when screening for actinomycetes from these different sources. Actinomycete isolates were initially selected on colony morphology. All were screened for antimicrobial activity and identified to the genus level by a molecular method. Isolates with specific antimicrobial activity, those belonging to the rarer genera and actinomycetes with unusual morphologies were selected for full characterisation.

2.3 Materials & Methods

2.3.1 Isolation and strain cultivation

2.3.1.1 Isolation from sediment samples

Sediment samples were collected from five sites in the Western Cape Province, South Africa (Figure 2.1). Samples were collected from sites S1, S2, S3 and S5 in February 2004, while samples were collected from site S4 in May 2004. Site S1 was a beach between Muizenberg and St James along the False Bay coastline (Cape Town) and was the only truly marine sample site. Sites S2 to S4 are estuarine environments where the water would be defined as brackish with a salinity ranging from 0.05% to 3%. Samples for site S2 were collected off the shore of a small island in the Zandvlei Nature Reserve (Cape Town). Site S3 was a lagoon in the Rietvlei Wetlands Reserve (Cape Town). A freshwater vlei drains into this lagoon which connects to the ocean along the Table Bay coastline (Cape Town). Site S4 was the Hermanus Lagoon, approximately 150 km south-east of Cape Town. Two samples were collected from this site. Site S5 was the only freshwater sample site and a sample was collected from the banks of the Berg River, outside the town of Paarl. Samples were collected at a water depth of approximately 30 cm and were taken from approximately 5 cm below the sand bed in sterile glass universals. All samples were stored at 4°C and processed within 24 hours of collection. Samples from site S4 were collected by Candice Goodwin.

For each isolation site, a soil extract was prepared by adding 200 g of sediment to 750 ml distilled water and autoclaving. This was allowed to settle and the extract was poured off and stored in a sterile container at 4°C. The four isolation media used were starch-casein medium (SC) (10 g soluble starch, 1 g casein dissolved in 2 ml 0.3 M NaOH, 0.5 g K_2HPO_4, 15 g agar, distilled water to 1 l, pH 7-7.5); yeast extract-malt extract agar (ISP 2 or YEME) (Shirling & Gottlieb, 1966); Czapek solution agar (CZ) (Atlas, 1993); and PV8 agar (Tormo *et al.*, 2003). SC and YEME were made with distilled water supplemented with 250 ml soil extract per l. Sediment from sites S1 and S2 were also plated onto YEME and SC agar supplemented with 4% NaCl. All media were supplemented with 50 µg/ml cycloheximide and 10 µg/ml nalidixic acid.

Two pretreatment methods were used and for all five sites a heat treated, air dried and untreated sample were prepared. For the untreated sample, approximately 0.1 g sediment was added to 1 ml of sterile distilled water and vortexed for 1 min. The sample was serially diluted in sterile distilled water and 100 µl of each dilution was spread-plated on the various isolation media. Samples were heat treated by adding approximately 0.1 g soil to 1 ml of sterile distilled water and vortexing for 1 min. The sediment was allowed to settle for 10 min, before the liquid was decanted into a sterile glass petri dish, which was placed at 60°C for 1 h. The supernatant was serially diluted with sterile distilled water and 100 µl of each dilution was spread-plated onto the various isolation media. The air dried samples were prepared by placing approximately 10 g of sediment (wet weight) in a sterile glass petri dish at 28°C for 2 to 5 days (depending on sample type) until dry. Samples were then serially diluted with sterile distilled water and 100 µl of each dilution was spread-plated onto the different isolation media.

All isolation plates were incubated at 28°C for up to 21 days. Actinobacteria were selected based on their morphology. Selected isolates were sub-cultured onto the media from which they were isolated (without antibiotics) using a sterile toothpick and were incubated for 7 days at 30°C.

Figure 2.1 Location of the samping sites in the Western Cape Province.

Key. S, denotes a sediment sample; N soil sample; P, plant sample. **S1**, Muizenberg Beach; **S2**, Zandvlei Nature Reserve; **S3**, Rietvlei Wetlands Reserve. **S4**, Hermanus Lagoon; **S5**, Berg River, Paarl (red dot); **N1 & N2**, Karoo Desert National Botanical Garden Worcester; **N3**, Die Hel. Groot Swartberg Mountain range (green dot); **P1**, Fish Hoek, Cape Town; **P2**, Kirstenbosch National Botanical Garden, Cape Town (purple dot); **P3**, Wellington (yellow dot). Red arrows mark the approximate boundary of the Cape Floral Region.

Actinobacteria isolated from sediment samples were named as follows: the first part of the strain name refers to the origin of the sediment sample: *muiz*, isolated from site S1; *zand* or *vlei*, isolated from S2; *riet*, isolated from site S3; *NX03* and *NX01/CG* refer to the two samples from site S4; *berg*, isolated from site S5. Site designation is followed by a number which refers to the dilution factor of the isolation plate; *1-5*, isolated from the 10^{-2} dilution plates; *6-10*, isolated from the 10^{-3} or 10^{-4} dilution plates. The letter *A* before the dilution factor refers to the air drying pretreatment. The letter after the dilution factor refers to the isolation medium; *C*, CZ agar; *M*, YEME + 4% NaCl; *P*, PV8 agar; *S*, starch-casein agar; *Y*, YEME agar.

2.3.1.2 Isolation from soil samples

Soil from site N1 was collected from the base of a Giant quiver tree, *Aloe pillansii*, growing in the Karoo Desert National Botanical Garden, Worcester approximately 70 km from Cape Town. Site N2 was a dry riverbed in the Karoo Desert National Botanical Garden, Worcester, the sample collected from this site was defined as shale-like soil. The sample from site N3 was collected from the banks of the Gamka River, in Die Hel, situated in the Groot Swartberg mountain range, approximately 100 km north-west of Oudtshoorn. Samples from sites N1, N2 and N3 were obtained from Marilize le Roes and were collected between May and June 2004.

Two pretreatment methods were used, heat and air drying. For the heat treatment, the soil sample was initially heated at 120°C for 1 h. Approximately 0.1 g of soil was then added to 1 ml of sterile distilled water and vortexed for 1 min. The sample was then serially diluted in sterile distilled water and 100 µl of each dilution was spread-plated on the isolation media. For the air drying pretreatment, the soil sample was dried at 28°C for 24 to 72 h (depending on soil type). Approximately 0.1 g of soil was added to 1 ml of sterile distilled water and vortexed for 1 min. The soil suspension was serially diluted in sterile distilled water and 100 µl of each dilution was spread-plated on the isolation media.

The isolation media used were PV8 agar and Modified Czapek solution agar (MC) (Nonomura & Ohara, 1971). All isolation plates contained nalidixic acid (10 µg/ml) and cycloheximide (50 µg/ml) and were incubated at 28°C. PV8 plates were incubated for 5 days and MC plates for 21 days. Isolates were selected based on their morphology. Selected isolates were sub-cultured onto the media from which they were isolated (without antibiotics) using a sterile toothpick and incubated for 7 days at 30°C.

Actinobacteria isolated from soil samples were named as follows. The first part of the name refers to the origin of the soil sample; *Q*, isolated from site N1; *Shale*, isolated from site N2; *Hel*, isolated from site N3. Site designation is followed by a number (1-4) which refers to the dilution factor of the isolation plate; and the second number refers to the number of actinobacteria isolated from that plate. The letter *M* refers to the isolation medium MC agar, while the letter *P* refers to the isolation medium PV8 agar (the *P* in the strain names of some isolates was later omitted).

2.3.1.3 Isolation from the leaves of indigenous plants

Indigenous plants were collected from three sources. Samples collected from site P1 were collected from a river rehabilitation site in Fish Hoek (Cape Town); Site P2 samples were collected from Kirstenbosch National

Botanical Garden (Cape Town) and site P3 samples were collected from a fynbos garden (natural and cultivated) in Wellington, approximately 60 km from Cape Town. Samples from site P1 and P2 were collected in March 2004, while samples were collected from site P3 in October 2004. Samples were stored separately, at 4°C, in individual sterile plastic bags and were processed within 24 hours of collection. Table 2.1 lists the plants used for the isolations. The *Myrothamnus flabellifolius* sample was collected in Namibia and was obtained from John Moore; it had been allowed to dehydrate naturally over a period of three weeks. The identification of plant species was confirmed from Joffe (1993), Carruthers (2000) and Manning (2003).

Table 2.1 The 40 indigenous plants species screened for the presence of endophytic actinobacteria.

Plant species name	Common name	Biome [a]	Source of plant	Actinobacteria isolated [b]
Adiantum capillus-veneris	Maidenhair fern		Wellington	1 (7d)
Agapanthus africanus	Dwarf agapanthus	F	Wellington	/
Agapanthus praecox subsp. *orientalis*	Blue agapanthus	F	Fishhoek	/
Aloe arborescens	Krantz aloe	F	Fishhoek	/
Aptenia cordifolia	Aptenia		Wellington	/
Bulbine frutescens	Stalked bulbine		Wellington	1 (7d)
Carpobrotus edulis	Hottentot sourfig	F	Fishhoek	/
Clivia miniata	Bush / Fire lily	G	Wellington	2 (21d)
Colpoloma album	Cape May/Confetti bush	F	Wellington	1 (28d)
Dietes grandiflora	Wild iris		Wellington	1 (14d)
Elegia stipularis	None		Fishhoek	1 (7d), 1 (14d)
Erica nana	None	F	Kirstenbosch	/
Felicia bergerana	Kingfisher daisy		Wellington	1 (21d)
Geranium incanum	Carpet geranium	F	Wellington	3 (7d)
Gerbera jamesonii	Barberton Daisy	G	Fishhoek	/
Gloriosa superba	Flame lily	G	Fishhoek	/
Hemizygia transvaalensis	Pink salvia		Wellington	1 (7d)
Lampranthus coralliflorus	Mauve vygie	F	Wellington	1 (14d), 1 (21d)
Lampranthus sp.	Vygie	F	Fishhoek	/
Leonotis leonurus var. *leonurus*	Wild dagga	F	Fishhoek	/
Leucadendron argenteum	Silver tree	F	Kirstenbosch	/
Myrothamnus flabellifolius	Resurrection plant		c	1 (7d)
Othonna carnosa var. *carnosa*	Othonna	F	Wellington	1 (7d)
Pelargonium luridum	Waving pelargonium	G	Wellington	1 (7d)
Pentas lanceolata	Pentas		Wellington	/
Phoenix reclinata	Wild date palm		Wellington	1 (7d), 1 (14d), 1 (21d)
Plectranthus ecklonii	Mauve plectranthus		Wellington	/
Plectranthus saccatus var. *saccatus*	Stoep jacaranda		Kirstenbosch	/
Podocarpus falcatus	Outeniqua yellowwood		Wellington	/
Portulacaria afra	Elephant's foot	G	Fishhoek	/
Protea 'susara'	Hybrid	F	Kirstenbosch	/
Rumohra adiantiformis	Knysna / seven week fern		Wellington	1 (21d)
Scabiosa africana	Pincushion	F	Wellington	/
Senecio elegans	Wild cineraria		Fishhoek	/
Strelitzia reginae	Crane flower	G	Wellington	/
Streptocarpus rexii	Cape primrose	G	Wellington	/
Tecomaria capensis	Cape honeysuckle	G	Fishhoek	/
Tulbaghia violacea	Wild garlic	F	Wellington	1 (7d)
Veltheimia bracteata	Bush lily	G	Wellington	/
Zantedeschia aethiopica	Arum lily	F	Fishhoek	/

a, Data obtained from Manning (2003); b, the first number refers to the number of actinobacteria isolated, the number in parentheses refers to the isolation day e.g. 7d refers to isolation day seven; c, plant was obtained from Namibia, see text. Symbols: F, fynbos biome; G, Grassland and Savannah biomes, plant species that are highlighted in red are used medicinally (Van Wyk *et al.*, 1997), / no actinomycetes were isolated from these plant species. Plants were identified from Joffe (1993), Carruthers (2000), Manning (2003) and http://www.plantzafrica.com/search.htm

A direct and indirect isolation procedure was used for all plant samples. For both methods the leaves were initially surface sterilised by placing the whole leaf in 70% ethanol for 1 min, then soaking in 1% NaOCl for 3 min and rinsing twice in sterile distilled water. Surface sterilised leaves were cut into 1 cm by 1 cm blocks with sterile flamed scissors (Okazaki, 2003).

Three isolation media were used for plant isolations; PV8 agar, 0.8% tap water agar (8 g agar to 1 l tap water, pH 6) and Plant extract agar (PE) (400 ml plant extract, 8.5 g glucose, 4.5 g yeast extract, 17.5 g agar, water to 1 l, pH 6.6-6.8, autoclaved). A plant extract was prepared by placing 160 g cut up plant material in 1.2 l distilled water, bringing it to the boil and simmering for 30 min. The liquid was cooled, filtered through a double layer of coffee filters (House of Coffees, size 1X6, Perco) and stored in a sterile glass bottle at -20°C (method adapted from Demain & Davies, 1999). PV8 agar and PE agar were supplemented with nalidixic acid (10 µg/ml) and cycloheximide (50 µg/ml).

For the direct isolation procedure, the sterile leaf pieces were placed both axially and dorsally directly on the agar surface. For the indirect method leaf pieces were placed in a test tube containing 20 ml quarter-strength sterile phosphate buffer (full strength phosphate buffer 0.05 M: 5.62 g K_2HPO_4, 2.13 g KH_2PO_4, distilled water to 1 l, adjusted to pH 7, autoclaved) (Demain & Davies, 1999), vortexed briefly and incubated at 30°C for 30 min with agitation. The phosphate buffer was serially diluted with sterile distilled water and 100 µl of each dilution was spread plated onto PV8 agar and PE agar. For the indirect isolation on 0.8% tap water agar, 100 µl of undiluted buffer was spread-plated. Plates were incubated at 28°C for 28 days. Isolates were selected based on morphology and sub-cultured using a sterile toothpick onto PE agar (without antibiotics) and incubated at 28°C for 10 days.

Strain designation of actinobacteria isolated from plants was assigned as follows: the first two letters are the plant's generic and species name, followed by either a number *1-4*, which refers to the dilution factor or a *u*, which refers to undiluted sample. The last number in the strain name refers to the number of isolates from that plate.

2.3.1.4 Strain maintenance and cultivation

For short term maintenance (unless stated), all actinobacteria isolates were maintained on YEME or CZ at room temperature. For long term maintenance, stock cultures were stored at -70°C in 20% glycerol. Spore suspensions in 25% glycerol were also stored at -70°C. Table 2.2 lists the actinobacteria type strains used in this study. Unless stated, all type strains were maintained on YEME. Stock cultures were stored at -70°C in 20% glycerol.

Table 2.2 Actinobacteria type strains used in this study.

Species	Strain number	Source
Kribbella alba	DSM 15500^T	DSMZ, Germany
Kribbella antibiotica	DSM 15501^T	DSMZ, Germany
Kribbella flavida	CIP 107494^T	*Collection de l'Institut Pasteur*, France
Kribbella jejuensis	CIP 108509^T	*Collection de l'Institut Pasteur*, France
Kribbella koreensis	CIP 107494^T	*Collection de l'Institut Pasteur*, France
Kribbella lupini [a]	DSM 16683^T	M. Trujillo
Kribbella sandramycini	DSM 15626^T	DSMZ, Germany
Kribbella solani	CIP 108508^T	*Collection de l'Institut Pasteur*, France
Kribbella yunnanensis	DSM 15499^T	DSMZ, Germany
Micromonospora carbonacea	NRRL B-2972^T	D. Labeda [c]
Micromonospora echinospora [b]	DSM 43816^T	DSMZ, Germany
Micromonospora mirobrigensis [a]	DSM 44380^T	M. Trujillo
Micromonospora rosaria	NRRL 3718^T	D. Labeda [c]

DSMZ, *Deutsche Sammlung von Mikroorganismen und Zellkulturen* GmbH. a, strains were maintained on SA1 medium (Trujillo *et al.*, 2005); b, strain was maintained on DSM #553 (GPHF) medium; c, cultures supplied from the USDA, ARS, Actinobacterial Culture Collection.

2.3.2 Molecular identification

2.3.2.1 DNA extraction

One loopful of spores from a profusely sporulating culture was used to inoculate a 100 ml Erlenmeyer flask containing 10 ml YEME broth. Cultures were grown at 28°C for 18-72 h with constant shaking. Cultures were Gram stained to ensure their purity. Template DNA for 16S rRNA gene amplification was extracted using a modified boiling method. Up to 5 ml of culture was harvested at 14 000 x *g* for 2 min in a benchtop centrifuge to obtain a cell pellet of 500 µl. Cells were resuspended in 500 µl 10 mM Tris-HCl, 1 mM EDTA (TE) buffer, pH 7.8, vortexed for 30 s and pelleted by centrifugation at 14 000 x *g* for 2 min. The TE buffer was removed and the cells were resuspended in 200 µl TE buffer and vortexed briefly. Samples were placed in a boiling waterbath for 10 min, cooled for 5 min and centrifuged at 10 000 x *g* for 2 min. A 100 µl aliquot of supernatant was removed and stored at 4°C. For selected isolates, this template DNA failed to amplify, therefore DNA was extracted using a modified version of the method of Wang *et al.* (1996). The following modifications were made: the concentration of lysozyme was increased to 25 mg/ml, 0.2 mg/ml proteinase K was added to the lysozyme buffer and cells were incubated overnight in the lysozyme buffer. Following RNase A digestion, the DNA was redissolved in 50 µl TE buffer and stored at 4°C. DNA was quantitated on a Nanodrop™ spectrophotometer (model ND-1000).

2.3.2.2 16S rRNA gene amplification and phylogenetic analysis

The polymerase chain reaction (PCR) was carried out in 50 µl reaction volumes. Each reaction contained 2 or 4 mM $MgCl_2$, 0.5 U Super-Therm *Taq* polymerase (JMR Holdings, USA), 150 µM of each dNTP, 0.5 µM of each primer and 100-200 ng template DNA. The 16S rRNA gene was amplified using the universal bacterial primers F1 and R5, which were adapted from primers fD1 and rP2 respectively (Weisburg *et al.*, 1991) (See Table 2.3). PCR was performed using a Techne thermal cycler, model TC-512. The PCR programme used was an initial denaturation at 96°C for 2 min, followed by 30 cycles of denaturation (96°C for 45 s), annealing (48-60°C for 30 s), and extension (72°C for 2 min), and a final extension at 72°C for 5 min. An annealing temperature of 56°C was initially tested for all isolates. The PCR products were electrophoresed on 1% agarose gels containing

10 µg/ml ethidium bromide in 1 X TAE buffer (Sambrook *et al.*, 1989) at 100 V for 1 h and visualised on a GelDoc XR System (BioRad). A *Pst*I digestion of λ DNA was included on all gels as a molecular size marker.

For rapid genus identification, amplicons were initially digested singly with *Mbo*I and *Vsp*I (isoschizomers of *Sau3A*I and *Asn*I, respectively) according to the method of Cook & Meyers (2003). Tables 1 to 5 of Cook & Meyers (2003) were used to determine which restriction endonucleases to use for subsequent digestions. Restriction endonuclease digests were set up in 20 µl reaction volumes containing 6-10 µl PCR amplified DNA, 2 µl 10 x reaction buffer and 2 U restriction endonuclease. Restriction digests were incubated for 12 h at 37°C (Sambrook *et al.*, 1989). Two microlitres (2 µl) of 6 X tracking dye was added to each reaction and the digested DNA was electrophoresed on 1.5% agarose gels containing 10 µg/ml ethidium bromide in 1 X TAE at 100V for 1 h and visualised. An undigested control (4 µl of PCR product) was included on all gels. A *Pst*I digestion of λ DNA was included on all gels as a molecular size marker.

Amplified 16S rDNA was purified with either a Cleanmix kit (TA050CLN; Talent, Italy) or MSB® Spin PCRapace kit (Invitek). The purity and quantity of the cleaned PCR product was determined with a Nanodrop™ spectrophotometer. DNA (55 to 60 ng) was prepared for sequencing using a Big Dye terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and analysed on an Applied Biosystems 3130 Genetic Analyser. Electrophoresis was performed by GeneCare Genetics (Pty) Ltd, South Africa. Primers F1, F3, F5, R1, R3 and R5 (Table 2.3) were routinely used to obtain the almost full length of the 16S rRNA gene. Primers F2, F4, R2 and R4 were used if the sequence reads obtained from the other sequencing primers were short.

Table 2.3 Sequencing primers used to sequence the 16S rRNA gene.

Sequencing primer	Binding site	Primer sequence
F1	8-27	5'-AGAGTTTGATCITGGCTCAG-3'
R1	536-515	5'-GTATTACCGCGGCTGCTGGCAC-3'
F2	338-357	5'-ACTCCTACGGGAGGCAGCAG-3'
R2	806-785	5'-GGACTACCIGGGTATCTAATCC-3'
F3	517-536	5'-GCCAGCAGCCGCGGTAATAC-3'
R3	1074-1053	5'-CACGAGCTGACGACAICCAT-3'
F4	785-806	5'-GGATTAGATACCCIGGTAGTCC-3'
R4	1242-1220	5'-CCATTGTAGIACGTGIAGCCC-3'
F5	1053-1074	5'-GCATGGITGTCGTCAGCTCGTG-3'
R5	1512-1492	5'-ACGGITACCTTGTTACGACTT-3'

The binding site numbering system is that of *E. coli*. I, inosine.

Chromatograms were edited with Chromas version 2.01 (Technelysium Pty Ltd, Australia) and sequences were assembled in DNAMAN version 4.13; Lynnon BioSoft. Sequence similarities (global alignments) were determined using DNAMAN. Local alignments were obtained by performing a standard nucleotide-nucleotide BLAST search (*blastn*) (Altschul *et al.*, 1997) of the Genbank database. For phylogenetic analysis, reference strains (type strains only) identified from the BLAST search were selected for comparison. Isolates with similar BLAST results were grouped for phylogenetic analysis. A minimum of 1300 bp of confirmed sequence was

obtained for all isolates and sequences were aligned using CLUSTAL_X, version 1.81 (Thompson *et al.*, 1997). Phylogenetic analyses were conducted using MEGA version 3.1 (Kumar *et al.*, 2004). A neighbour-joining (Saitou & Nei, 1987); minimum evolution (Rzhetsky & Nei, 1993) and maximum parsimony (Fitch, 1971) tree was constructed for all isolates.

2.3.3 Chemotaxonomy

Actinobacteria isolates were grown in 100 ml YEME for 5 days at 30°C with shaking. The cell mass was collected by centrifugation at 10 000 x g for 10 min in a Beckman centrifuge and washed twice with 100 ml sterile distilled water. After the second wash, the cell pellet was resuspended in 20 ml sterile distilled water. Cells were placed in 1 l round-bottom flasks and frozen in an ethanol bath. Flasks were transferred to a freeze drier and left to sublime for 18 to 24 h.

For DAP analysis, the methods of Hasegawa *et al.* (1983) and Staneck & Roberts (1974) were modified as follows. Approximately 10 mg of freeze-dried cells were placed in a Pyrex test tube and 1 ml 6 M HCl was added, tubes were closed with a plastic lid. Samples were autoclaved for 20 min at 121°C. Samples were cooled and filtered through Whatman no. 1 paper. The filtrate was taken to dryness in a boiling waterbath, the residue was dissolved in 1 ml sterile distilled water and taken to dryness again in a boiling water bath. The resulting residue was resuspended in 200 µl sterile distilled water and stored at room temperature. For DAP analysis, 0.5 to 2 µl of the hydrolysate was spotted on cellulose TLC plates (Merck; 1.05552.0001). One microlitre (1 µl) of 1% commercial DAP standard (Sigma) and 1 µl 0.1% (v/v) glycine standard were included. The spots were allowed to dry and the TLC plates were placed in a glass TLC chamber and developed in a solvent system of methanol-distilled water-6 M HCl-pyridine (80:26:4:10; v/v). Chromatograms were dried in a fumehood and sprayed with 0.2% (w/v) acetonic ninhydrin (Merck). Plates were dried and heated at 110°C for approximately 5 min.

A modified version of Hasegawa *et al.* (1983) was used for sugar analysis. Approximately 100 mg of freeze-dried cells were placed in a Pyrex test tube and 1 ml 0.25 M HCl was added. Tubes were closed with a plastic lid and autoclaved at 121°C for 20 min. One to 5 µl of sugar hydrolysate were spotted on cellulose TLC plates. Two sugar standards were included on all plates: 0.5 µl 1% (w/v) of each of glucose, mannose, ribose and 0.5 µl 1% (w/v) of each of galactose, arabinose and xylose. The hydrolysates were allowed to dry and the TLC plate was placed in a glass TLC chamber. The solvent system used for sugar analysis was ethyl acetate-pyridine-water (100:35:25; v/v). Developed chromatograms were dried in a fumehood and developed for a second time in the same solvent system. Plates were dried and sprayed with a sugar detection reagent (2 ml aniline (Saarchem), 3.3 g phthalic acid (Sigma) and 100 ml water-saturated *n*-butanol). Plates were dried and heated at 110°C for 2-5 min.

For both DAP and sugar analysis, the TLC chamber contained 80 to 100 ml of the solvent system. An 8 cm X 12 cm piece of Whatman no. 1 paper was used as a wick and the atmosphere was allowed to become saturated with the solvent for 30 min prior to the development of the chromatogram.

2.3.4 Phenetic characterisation

2.3.4.1 Morphology and physiology

Morphological and physiological testing was performed according to the methods of Gordon *et al.* (1974), Shirling & Gottlieb (1966), and Williams *et al.* (1989). Unless stated, all plates were incubated at 30°C for the recommended period. Degradation of adenine, allantoin, casein, cellulose, gelatin, guanine, hypoxanthine, starch, Tween 80, L-tyrosine, urea, xanthine and xylan; hydrolysis of aesculin, arbutin and pectin; nitrate reduction; growth in the presence of inhibitory compounds; growth at different pH values and temperatures; and utilisation of different nitrogen sources was performed according to Williams *et al.* (1989). Growth at room temperature (RT) was determined at 20-22°C. For Bennett's and Modified Bennett's Medium, Lab Lemco powder (Oxoid) was substituted with beef extract powder (Biolab Diagnostics, SA). Acid production from carbon sources and acid fastness were tested according to the methods of Gordon *et al.* (1974). All ISP media were prepared according to the methods of Shirling & Gottlieb (1966). Unless stated, the colour of the substrate and aerial spore mass was determined on inorganic salts-starch agar (ISP 4). The production of diffusible pigments (DP) and pH sensitivity of substrate mycelium pigments and DP were determined on glycerol-asparagine agar (ISP 5) (Shirling & Gottlieb, 1966). All carbon sources were filter sterilised using AcetatePlus Cameo syringe filters, pore size 0.22 µm (OSMONICS) and tested at 1% (w/v), except sodium benzoate, sodium butyrate, sodium formate, sodium DL-malate, sodium oxalate, sodium salicylate, sodium succinate and sodium L(+) tartrate, which were tested at 0.1% (w/v). The recording of the carbon source results was modified from Shirling & Gottlieb as follows: ++, growth was greater than positive control; +, growth was equal to that of the positive control; +w, growth was weaker than the positive control but significantly more than the negative control; -, growth was less or equal to the negative control.

Antibiotic susceptibility was tested by incorporating the filter-sterilised antibiotics into sterile Bennett's Medium (Atlas, 1993) to the final concentration stated in the results tables. Growth was recorded as follows: ++, growth comparable to positive control; +, good growth but less than positive control; +w, weak growth; -, no growth. Antibiotic susceptibility testing was performed on two separate occasions. If a marked difference was observed between the two results, a third experiment was performed. The presence of three or less colonies on a plate was recorded as a negative result.

Proteolysis, lipolysis and lecithinase production on egg-yolk agar were determined as per the method of Nitsch & Kutzner (1969). Degradation of DNA was tested on DNase medium (Oxoid), plates were prepared according to the manufacturer's instructions. Activity was detected by flooding the plates with 1 M HCl for 1 min, pouring off the excess HCl and incubating the plates at room temperature for 20 min.

A standard spore suspension was used to inoculate all the physiological media, except for the carbon and nitrogen source plates and stated exceptions. Two to four loopfuls of spores from a sporulating culture were aseptically transferred to a sterile universal containing 3 ml sterile distilled water and vortexed vigorously for 30 s; 15 µl of this spore suspension was used to inoculate each plate. For the carbon and nitrogen source plates, washed inocula were prepared. Strains were grown in 100 ml of YEME for 4 days at 30°C with constant shaking. Cell mass was harvested by centrifugation at 10 000 x *g* for 10 min. The cells were resuspended in

100 ml of sterile distilled and pelleted at 10 000 x g for 10 min. The cells were resuspended in 100 ml sterile distilled water. The washing step was performed three times. After the final wash, the cells were resuspended in 5 ml sterile distilled water. The washed inocula were used within 2 h.

Gram stains was performed according to standard methods. For the catalase test, five drops of 3% (v/v) hydrogen peroxide solution were placed on a glass slide and mixed with a loopful of cells from a 7 day old culture grown on YEME agar. To detect the presence of motile spores, actinomycetes were inoculated onto YEME agar and incubated at 30°C for 5 days. Plates were flooded with a tepid phosphate buffer (0.05 M) or sterile water and viewed under a light microscope. Motile, flagellated cells were detected by performing a Ryu stain (Weyant *et al.*, 1996).

To test the growth on potato slices, one small potato was cut into 1 cm X 1 cm blocks and half of the blocks were placed in a glass bottle and autoclaved. The remaining blocks were placed in a bottle containing approximately 5 g of $CaCO_3$, shaken to mix and autoclaved. Potato slices were placed in sterile petri dishes and inoculated with 50 µl spore suspension. Plates were sealed with parafilm to prevent dehydration of the potato slices and incubated at 28°C for 14 days.

To determine the effect of light on pigment production, actinomycetes were inoculated onto YEME and Bennett's Medium agar. Inoculated plates were wrapped in aluminium foil and incubated at 30°C for 14 days.

For SEM, the isolates were grown on inorganic salts-starch agar (ISP 4) for 14 days at 30°C. A 1cm X 1cm plug of agar containing a sporulating colony was cut from the plate. Plugs were placed in 24-well cell culture plates. Samples were fixed in 100 mM sodium cacodylate buffer containing 2% (v/v) glutaraldehyde (pH 8.0) overnight at room temperature (RT, approximately 20°C). Samples were post-fixed in 100 mM sodium cacodylate buffer (pH 8.0) containing 1% (v/v) osmium tetroxide for 1 h at RT. Fixed specimens were serially dehydrated in an increasing ethanol gradient, critically point dried and sputter coated with gold-palladium.

2.3.4.2 Anaerobic growth

Growth under anaerobic conditions was tested on carbon utilisation agar (ISP 9) with glucose as the sole carbon source and ATCC medium 172 (http://www.atcc.org/common/documents/mediapdfs/172.pdf) with the exception that the N-Z amine type A was substituted with Casitone (Difco). When the plates were initially placed in the anaerobic chamber, they were each opened for 5 s to ensure all the residual O_2 was removed before being sealed in a plastic bag. Plates were incubated anaerobically at 25°C for 21 days (Model 1024 anaerobic chamber; Forma Scientific) in an atmosphere comprising of 5% H_2; 10% CO_2 and 85% N_2.

2.3.4.3 DNA-DNA Hybridization

For DDH, actinomycetes were grown in DSM #553 (GPHF) medium with the peptone from casein substituted with Casitone (Difco) (http://www.dsmz.de/microorganisms/html/media/medium000553.html). A 1 ml spore suspension was used to inoculate a 100 ml Erlenmeyer flask containing 10 ml of medium. This starter culture was grown for 18 h at 30°C with constant shaking. The starter culture was used to inoculate a 1 l Erlenmeyer

flask containing 90 ml of DSM #553 medium. This was incubated for 48 h at 30°C with constant shaking. This second (100 ml) culture was used to inoculate a 5 l Erlenmeyer flask containing 400 ml of DSM #553 medium. This was incubated for 4 days at 30°C with constant shaking. Cells were Gram stained and streaked for single colonies to ensure the cultures were not contaminated. Cell mass was harvested by centrifugation at 10 000 x *g* for 15 min. Cells were resuspended in 5 ml sterile distilled water and pelleted at 15 000 x *g* for 10 min. Cells were resuspended in 10 ml 50% isopropanol. DDH was performed as a service by the *Deutsche Sammlung von Mikroorganismen und Zellkulturen,* GmbH (DSMZ), Identification Service, using the spectrophotometric method of De Ley *et al.* (1970), incorporating the modifications described by Huss *et al.* (1983).

2.4 Results

Table 2.4 lists the sources of the 76 soil and sediment isolates selected for preliminary characterisation. Although 99 isolates were initially selected, 18 strains that appeared to be duplicated were excluded. Of the 36 isolates selected from the Zandvlei isolation plates, 14 isolates (all were isolated from the same pretreated sample on YEME) had identical morphological features on YEME and inorganic salts-starch agar (ISP 4), namely, yellow-brown substrate mycelium, grey spore mass and production of a yellow diffusible pigment; therefore only four of these strains were selected for characterisation. Likewise, 19 isolates with two common colony morphologies were initially selected from Die Hel, PV8 agar isolation plates, but eight were subsequently excluded. Table 2.1 lists the 40 plant species used in this study and the source of the 23 plant isolates initially selected. A number of the actinobacteria isolated from the plant samples either only grew on PE agar or grew very slowly on other media. Attempts to improve the growth of some of these isolates was successful, however, some isolates failed to grow and had to be excluded. All soil, sediment and plant isolates were screened for antimicrobial activity. Thirty isolates were selected for full characterisation based either on their unusual morphology or antimicrobial activity (Table 2.5).

Table 2.4 The source and isolation conditions of the 76 actinobacteria strains initially selected from the soil and sediment samples.

Sample source		**Isolation medium**				
		YEME	**SC**	**CZ**	**PV8**	**MC**
Sediment samples	Berg River	**3** (2 ht, 1 unt)	**5** (5 ht)	**1** (1 ht)		
	Hermanus estuary	**5** (5 ht)			**2** (2 ht)	
	Muizenberg beach	**2** (1 ad; 1 unt)	**1** (1 ad)		**1** (1 ht)	
	Rietvlei Wetlands Reserve	**3** (3 ht)	**3** (3 ht)	**2** (2 ht)	**1** (1 ht)	
	Zandvlei Nature Reserve	**16*** (10 ht; 5 unt; 1 ad)	**5** (4 ht; 1 unt)		**5** (5 ht)	
Soil samples	Die Hel				**7#** (5 ad; 2ht)	**4** (4 ad)
	Quiver, Worcester				**5** (3 ad; 2ht)	**3** (1 ad; 2 ht)
	Shale, Worcester				**2** (2 ad)	

Numbers in boldface refer to the total number of isolates selected, while numbers in parentheses refer to the number that was isolated from each pretreatment method. Symbols: ad, air drying; ht, heat treatment; unt, untreated. *, an additional ten isolates with identical morphology on YEME were excluded; #, an additional eight isolates with identical morphology on YEME were excluded.

Table 2.5 Isolation source of the 30 actinobacteria isolates selected for full characterisation.

Actinomycete isolate	Isolation source		Isolation medium	Pre-treatment	Identification
AC41	Plant P3	*Adiantum capillus-veneris*	PV8	Indirect	*Gordonia*
Berg1C	Sediment S5	Berg River	CZ	Heat	*Streptomyces*
Berg2S	Sediment S5	Berg River	Starch-casein	Heat	*Streptomyces*
Berg4Y	Sediment S5	Berg River	YEME	None	*Streptomyces*
CA21	Plant P3	*Coleonema album*	PE	Indirect	*Kineococcus*
CGM31	Sediment S4	Hermanus Lagoon	YEME + 4% NaCl	Heat	*Micromonospora*
DG41	Plant P3	*Dietes grandiflora*	PV8	Indirect	*Micromonospora*
GIU1	Plant P3	*Geranium incanum*	PE	Indirect	*Kineococcus*
GIU2	Plant P3	*Geranium incanum*	PE	Indirect	*Kineococcus*
GIU3	Plant P3	*Geranium incanum*	PE	Indirect	*Kineococcus*
Hel32	Soil N2	Die Hel	PV8	Air dry	*Streptomyces*
HMC25	Soil N2	Die Hel	MC	Heat	*Kribbella*
M25 *	Soil	Roodepoort	MC	Heat	*Nocardia*
MuizA5S	Sediment S1	Muizenberg	Starch casein	Air dry	*Micromonospora*
Muiz4Y	Sediment S1	Muizenberg	YEME	None	*Streptomyces*
NX0141	Sediment S4	Hermanus Lagoon	PV8	None	*Gordonia*
NX03U2	Sediment S4	Hermanus Lagoon	PV8	Heat	*Streptomyces*
PBPE §	Plant	*Myrothamnus flabellifolius*	PE	Indirect	*Micromonospora*
PHelU5	Soil N2	Die Hel	PV8	Air dry	*Streptomyces*
PLU1	Plant P3	*Pelargonium luridum*	PE	Indirect	*Micromonospora*
Q41	Soil N1	Worcester	PV8	Air dry	*Kribbella*
RAU1	Plant P3	*Rumohra adiantiformis*	PE	Indirect	*Micromonospora*
Riet1C	Sediment S3	Rietvlei	CZ	Heat	*Streptomyces*
ShaleUP	Soil N3	Worcester	PV8	Air dry	*Streptomyces*
TVU1	Plant P3	*Tulbaghia violacea*	PE	Indirect	*Micromonospora*
VleiA3C	Sediment S2	Zandvlei	Starch casein	Air dry	*Micromonospora*
Zand2Y	Sediment S2	Zandvlei	YEME	None	*Streptomyces*
Zand4Y	Sediment S2	Zandvlei	YEME	Heat	*Streptomyces*
Zand8Y	Sediment S2	Zandvlei	YEME	Heat	*Streptomyces*
Zand9Y	Sediment S2	Zandvlei	YEME	Heat	*Streptomyces*

Key: § Isolate PBPE was isolated from plant material obtained from Namibia, a full explanation is given in the text; * Isolate M25 was isolated from soil collected in Roodepoort, a full explanation is given in the text; none, sample was not pretreated prior to isolation

2.4.1 Characterisation of species belonging to the Suborder *Corynebacterineae*

2.4.1.1 Characterisation of members of the Family *Gordoniaceae*

Two isolates, AC41 and NX0141, were initially selected based on their bright orange, rubbery appearance. The rapid genus identification method identified both isolates as belonging to a group containing members of the genera *Gordonia*, *Nocardia* and *Skermania* (Table 1 in Cook & Meyers, 2003). Based on their appearance and lack of aerial mycelium, it was assumed that both isolates were *Gordonia* species. As of November 2006, four validly published species; *Gordonia amarae*, *Gordonia polyisoprenivorans*, *Gordonia rhizosphera* and *Gordonia terrae*, were classified as belonging to this group (A. Cook, personal communication).

A phylogenetic tree was constructed from 1361 bp of 16S rRNA gene sequence which compared the isolates to all of the type strains of the genus *Gordonia* (Figure 2.2). AC41 was found to cluster with *Gordonia sputi* DSM 43896^T, *Gordonia aichiensis* DSM 43978^T and *Gordonia otitidis* DSM 44809^T. All three of these species were isolated from clinical specimens (Arenskötter *et al.,* 2004; Iida *et al.,* 2005). NX0141 clustered with *Gordonia bronchialis* DSM 43247^T (which was isolated from a TB patient) and *Gordonia rhizophera* IFO 16068^T, which was isolated from a mangrove rhizosphere (Takeuchi & Hatano, 1998; Arenskötter *et al.,* 2004). A pairwise local alignment found the 16S rRNA gene sequence similarity between AC41 and NXO141 to be 97.25%.

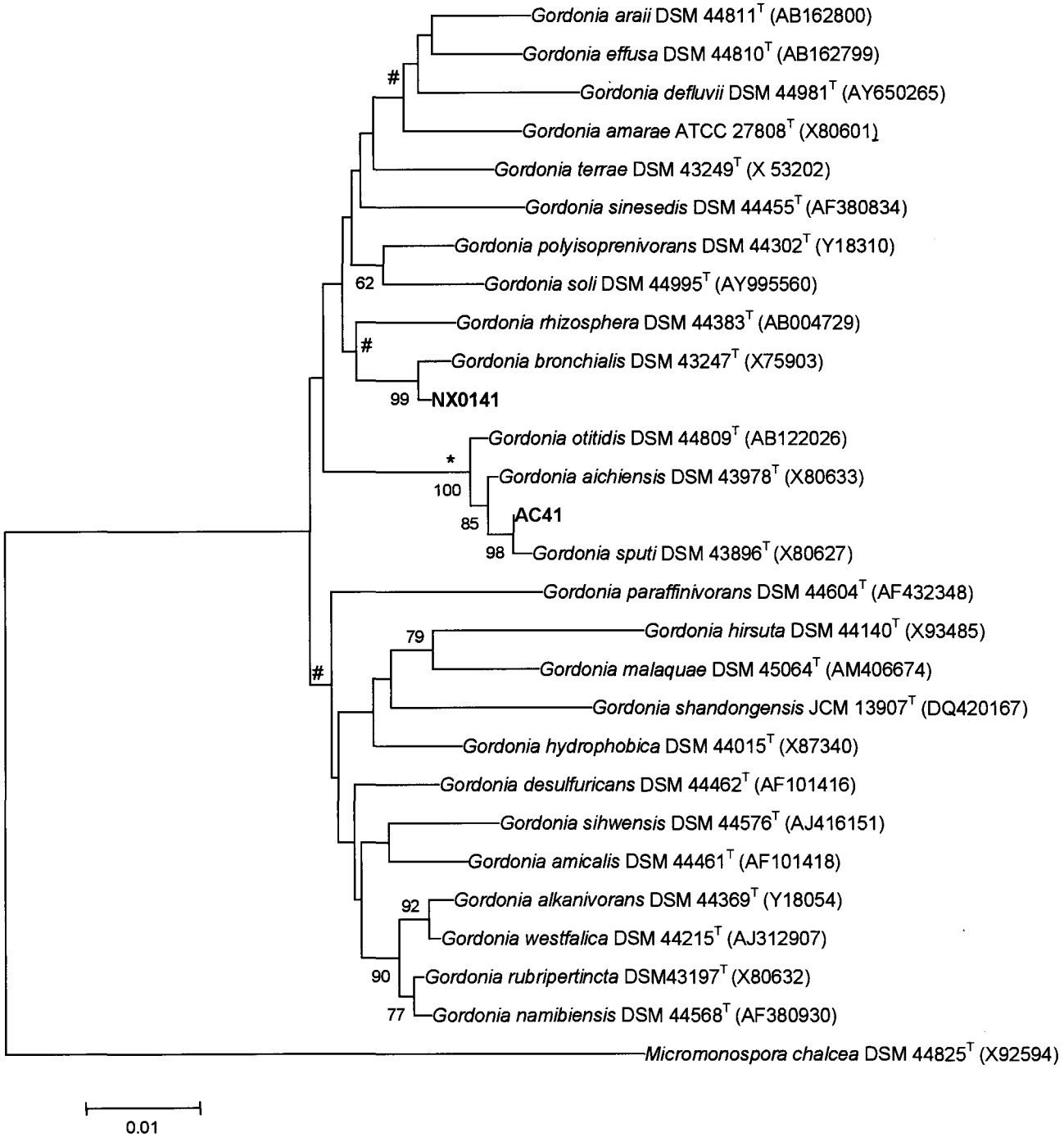
The morphological features of AC41 and NX0141 are similar. Both *Gordonia* strains are salmon orange in colour and have a rubber-like appearance, however, the colonies of AC41 darken with age to a red-orange. NX0141 is dependent on light to produce its pigments, as when it is incubated in the dark, the colonies appear pale peach in colour. The pigmentation of AC41 appears not to be affected by light. Both strains were catalase positive and slightly acid fast. AC41 is Gram-positive, while older cultures of NX0141 may appear Gram-variable. Both contain *meso*-DAP in their cell walls. The characteristic sugars for this genus are arabinose and galactose, however, only galactose was detected in the whole cell hydrolysates of AC41, while no sugars were detected in the hydrolysates of NX0141. Selected phenetic test results comparing AC41 and NX0141 to the closest related strains are represented in Table 2.6.

AC41 was isolated on PV8 agar from the leaves of the Maidenhair fern, *Adiantum capillus-veneris*, using the indirect isolation method. AC41 is able to grow from 20°C to 37°C, at pH 5, 7 and 9, and in the presence of 5% NaCl. AC41 degrades adenine, but is unable to degrade allantoin, casein, cellulose, gelatin, guanine, hypoxanthine, starch, urea and xylan. AC41 uses glycerol, D(+) mannose, D(-) mannitol, maltose and trehalose as sole carbon sources, but is unable to utilise D(-) lactose and D(+) raffinose. Diffusible pigments and melanin pigments are not produced. H_2S is produced and nitrate is not reduced. Phenetically, AC41 is similar to *G. sputi*, however, the ability of AC41 to degrade Tween 80 and grow at room temperature, as well as its inability to reduce nitrate does differentiate it from *G. sputi*.

NX0141 was isolated on PV8 agar from an untreated sediment sample collected from the Hermanus Lagoon. NX0141 grows from 20°C to 45°C, at pH 5, 7 and 9, and in the presence of 7% NaCl. NX0141 degrades gelatin and hydrolyses aesculin and arbutin. Adenine, allantoin, casein, cellulose, gelatin, guanine, hypoxanthine, starch, Tween 80, L-tyrosine, urea, xanthine and xylan are not degraded. NX0141 utilises glycerol, D(+) mannose, maltose and trehalose as sole carbon sources, but can not use D(-) lactose and D(-) mannitol. Growth on D(+) raffinose is weak at 21 days. H_2S is produced and nitrate is reduced (the reaction being very strong for both tests). There are five phenetic features that differ between NX0141 and its closest neighbour, *G. bronchialis*. These include colony colour, the inability of NX0141 to degrade Tween 80, as well as three differences in carbon source utilisation.

As both *G. bronchialis and G. sputi* are Risk Group 2 organisms and known human pathogens, after initial physiological tests were performed it was decided to not characterise isolates AC41 and NX0141 further.

Figure 2.2 Unrooted 16S rRNA gene sequence neighbour-joining phylogenetic tree comparing AC41 and NX0141 to the type strains of the genus *Gordonia*. The tree is based on 1361 bp of sequence and the bootstrap percentages are based on 1000 resampled datasets. Only bootstrap values above 50% are shown. Bar represents 0.01 nt substitutions per nt position. *Micromonospora chalcea* DSM 44825^T was set as the outgroup.



Asterisks (*) denote clusters that were conserved using the neighbour-joining, minimum evolution and maximum parsimony methods to construct the phylogenetic trees. A hash (#) denotes clades that were conserved between trees constructed from the neighbour-joining and minimum evolution methods only. The isolate in blue was isolated from an aquatic sediment sample, while the isolate in green was isolated from the leaves of an indigenous plant.

Table 2.6 Physiological results to differentiate AC41 and NX0141 from phylogenetically related *Gordonia* strains.

Physiological test	1	2	3	4	5	6	7
Colony colour	Salmon	Salmon	Pink to orange	Brown	Pale orange	Pink to orange	Salmon
Growth temperature (°C)	RT-37	RT-45	28-42	ND	ND	ND	28-37
Degradation of:							
Tween 80	+	-	-	+	v	-	-
L-Tyrosine	-	-	-	ND	ND	-	-
Xanthine	-	-	-	ND	ND	-	-
Nitrate reduction	-	+	+	+	ND	-	+
Carbon source utilisation:							
D(+) Galactose	+w	-	-	+	+	+	-
meso-Inositol	-	+	+	-	-	ND	-
D(-) Lactose	-	-	ND	+		ND	ND
D(-) Mannitol	+	-	ND	-	+	ND	ND
D(+) Mannose	+	+	ND	+	+	ND	ND
L(+) Rhamnose	-	-	v	-	-	v	+w
D(-) Ribose	+w	-	-	-	-	+	-
Trehalose	+	+	ND	+	+	ND	ND

1, AC41, **2**, NX0141; **3**, *G. aichiensis* DSM 43978[T] (Klatte *et al.*, 1994); **4**, *G. bronchialis* DSM 43247[T] (Shen *et al.*, 2006); **5**, *G. otitidis* IFO 10032[T] (Iida *et al.*, 2005); **6**, *G. rhizosphera* IFO 16068[T] (Takeuchi & Hatano, 1998); **7**, *G. sputi* ATCC 29627[T] (Riegel *et al.*, 1994). Symbols: +, positive reaction; -, negative reaction; +w, weakly positive reaction; v, results were variable; ND, not determined.

2.4.1.2 Characterisation of a strain belonging to the Family *Nocardiaceae*

M25 was isolated on MC agar from a heat treated soil sample collected in Roodepoort, Gauteng Province, South Africa. M25 was initially isolated by Marilize le Roes, but full characterisation of the strain was performed by the author. M25 initially grew with a yellow contaminant which was identified as a *Rhodococcus* species. M25 was serially subcultured on CZ in order to obtain it in pure culture.

For rapid genus identification, the amplified 16S *r*DNA was singly digested with *Mbo*I (*Sau*3AI), *Vsp*I (*Asn*I), *Sph*I, *Kpn*I and *Hind*III (Tables 1, 3 and 4 of Cook & Meyers, 2003), and the resulting restriction fragment pattern identified M25 as a *Nocardia* species, which was confirmed by 16S rRNA gene sequencing. A neighbour-joining phylogenetic tree based on 1303 bp of sequence was constructed and M25 was found to cluster with *N. nova* (Figure 2.3).

M25 is Gram-positive and readily fragments into long, narrow rods (Figure 2.7 A). In broth, M25 grows as fine, pale-peach growth that tends to float on the surface of the broth. M25 grows from 4°C to 37°C, at pH 5, 7 and 9, and in the presence of 7% NaCl. It does not produce diffusible pigments or melanin. M25 has fastidious growth requirements; growth was poor in all liquid media tested and it grows poorly on most physiological media. For routine maintenance M25 was grown on YEME with the glucose substituted with glycerol. M25 grows weakly on carbon utilization agar (ISP 9) with D(+) galactose, D(+) glucose, glycerol, D(+) mannose, maltose, D(-) ribose and trehalose as sole carbon sources, and can not utilise *meso*-inositol, D(-) lactose and D(+) raffinose. M25 produces H_2S. Cellulose, gelatin, guanine, L-tyrosine and xylan are not degraded. Based on initial phenetic

testing, M25 could not readily be differentiated from *N. nova*. As *N. nova* is a known human pathogen (Risk Group 2 strain), full physiological characterisation was not performed. Limited phenetic data are presented in Table 2.7.

Table 2.7 Selected phenetic characteristics to distinguish M25 from its closest phylogenetic neighbour *Nocardia nova*.

Physiological test	M25	*Nocardia nova* ATCC 33726^T
Degradation of:		
Adenine	NG	-
Casein	-	-
Hypoxanthine	NG	+
Starch	-	ND
Tween 80	+w	ND
L-Tyrosine	-	-
Xanthine	-	+
Utilisation of:		
D(-) Mannitol	+w	+
L(+) Rhamnose	-	-
Nitrate reduction	+	+
Growth at pH	5-9	ND
Colour of the substrate mycelium	Red-orange*	ND
Colour of the aerial mycelium	Peach*	ND

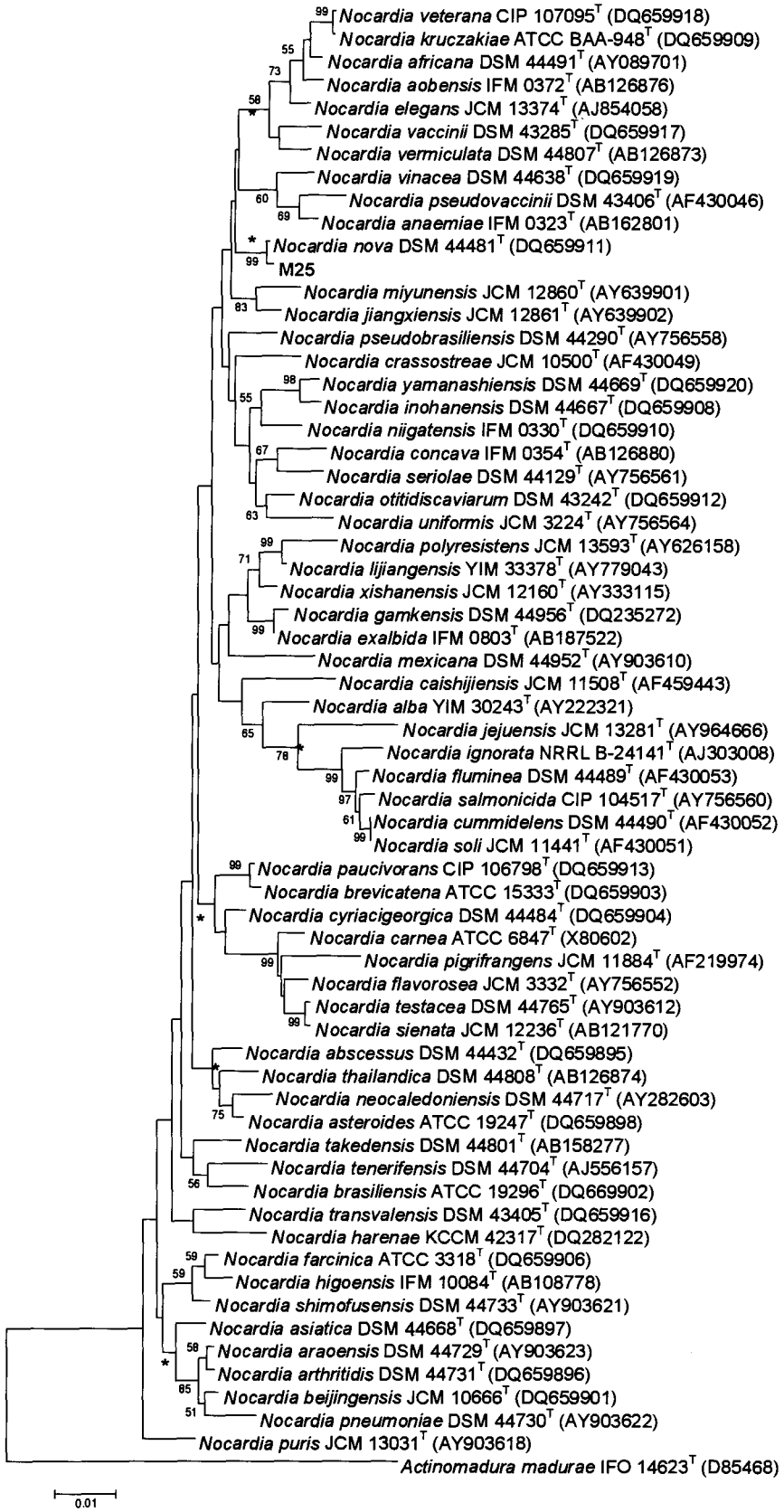
The data for *N. nova* was taken from Hamid *et al.*, 2001 and Cui *et al.*, 2005. Symbols: +, positive result/growth; +w, weak reaction/weak growth; -, negative result/no growth; ND, not determined; NG, no growth; *, colour was determined on YEME (glycerol).

2.4.2 The description of four plant isolates belonging to the Family "*Kineosporiaceae*"

Twenty three small, orange, dome-shaped colonies grew on the two PE agar plates which had been inoculated with the undiluted phosphate buffer from the *Geranium incanum* leaves. Five were initially subcultured onto PE agar, of which only three grew. These isolates were designated GIU1, GIU2 and GIU3. CA21 was isolated on PE agar after 28 days incubation using the indirect isolation method. This strain was isolated from leaves of the Cape May, *Coleonema album*. Both these plant samples were collected from a garden in Wellington.

For rapid genus identification, the initial *Mbo*I (*Sau*3AI) digestion of the 16S rDNA for all four isolates generated a large fragment of 990-1300 bp. Using the method of Cook & Meyers (2003), the 16S rDNA was singly digested with *Vsp*I (*Asn*I), *Sph*I, *Sna*BI, *Sal*I and *Age*I which identified the isolates as *Kineosporia* species (Table 2.8; the genus *Kineococcus* was not included in the method of Cook & Meyers, 2003). A standard nucleotide-nucleotide BLAST search of the GenBank database showed that both GIU1 (over 1353 bp) and GIU2 (over 1352 bp) are 96% similar to *Kineococcus aurantiacus* IFO 15268^T and 94% similar to *Kineosporia aurantiaca* ATCC 29727^T. The third isolate, GIU3, is 95% and 94% similar to *Kc. aurantiacus* and *Ks. aurantiaca* respectively, while CA21 was found to be 96% similar to *Kc. aurantiacus* and 93% similar to *Ks. aurantiaca*.

Figure 2.3 16S rRNA gene phylogenetic tree comparing isolate M25 to the type strains of the genus *Nocardia*. The tree is based on 1303bp of sequence and was constructed using the neighbour-joining method. Bootstrap values are based on 1000 replicates and only values greater than 50% are shown. Bar represents 0.01 nt substitutions per nt position. *Actinomadura madurae* IFO 14623^T was included as the outgroup.



Asterisks (*) denote clusters that were conserved using the neighbour-joining, minimum evolution and maximum parsimony methods to construct the phylogenetic trees. The isolate coded in purple was isolated from a soil sample.

Table 2.8 Identification of the genera in *Sau*3AI (*Mbo*I) Group 3 (largest DNA fragment: 980-135 bp). (Table adapted from Table 3 in Cook & Meyers, 2003).

Digest with	Relevant feature(s)	Go to:
(1) *Asn*I	Cut (470-590 bp and 900-960 bp	2
(2) *Sph*I	Cut (280-310 bp and 890-920 bp)	3
(3a) *Sna*BI	Not cut	4
(3b) *Sna*BI	Cut (470-510 bp and 970-1000 bp	6
(4a) *Sal*I	Not cut	5
(4b) *Sal*I	Cut (540-560 bp and 900-1040 bp): *Actinoplanes* (7/21); ***Micromonospora* (12/12);** *Couchioplanes* (1/1); *Pilimelia* (2/2); *Spirilliplanes* (1/1); *Verrucosispora* (1/1); *Virgisporangium* (2/2)	
(5a) *Pvu*II	Cut (560 bp and 970-980 bp): *Dactylosporangium* (6/6)	
(5b) *Pvu*II	Not cut: *Actinoplanes* (14/21); *Asanoa* (1/1); *Catellatospora* (3/3); *Catenuloplanes* (2/2)	
(6a) *Sal*I	Cut (235-265 bp and 1215-1245 bp)	7
(6b) *Sal*I	Not cut	9
(7a) *Age*I	Cut (660-680 bp and 910-930 bp)	8
(7b) *Age*I	Not cut: *Promicromonospora* (2/2)	
(8a) *Sst*I	Cut (585 bp and 930 bp): ***Hongia* (1/1)**	
(8b) *Sst*I	Not cut: ***Kribbella* (1/1)**; *Nocardioides* (1/5)	
9 *Age*I	Cut (670-680 bp and 750-830 bp): ***Kineosporia* (4/4)**	

The four genera isolated in this study are in boldface.

Phylogenetic analysis was performed over 1351 bp (Figure 2.4). All four isolates clustered with *Kineococcus radiotolerans* and *Kc. aurantiacus* in the neighbour-joining tree.

Most of the variability in the 16S rRNA gene sequence between the kineosporiae and kineococci appears to be confined to two regions between 118-218 bp and 975-1020 bp (Figure 2.5). From the multiple sequence alignment it can be seen that the 16S rRNA gene sequence of *Kineococcus marinus* is more similar to that of the kineosporiae, as reported by Lee (2006). GIU1, GIU2, GIU3 and CA21 all have a more *Kineococcus*-like sequence, including a TT insertion at 1010-1011 bp, and the C-G and A-T mutation at188 bp and 190 bp, respectively.

A comparison of the carbon and nitrogen source utilisation by GIU1, GIU2 and GIU3 are presented in Table 2.9. Physiological characteristics that differentiate the isolates from the type strains of the genera *Kineosporia* and *Kineococcus* are presented in Table 2.10. From Tables 2.9 and 2.10 the three *G. incanum* isolates can be differentiated. There are at least six clear phenetic differences between GIU1 and GIU2; seven between GIU1 and GIU3; and six between GIU2 and GIU3.

GIU1 and GIU3 degrade DNA within 7 days of incubation at 30°C, while GIU2 degrades DNA after 9 days. GIU1, GIU2 and GIU3 can not degrade guanine, hypoxanthine, starch, L-tyrosine and xanthine. These three isolates are unable to grow in the presence of adenine, aesculin and gelatin. GIU1 does not produce H_2S and grows between pH 5-9. H_2S production by GIU2 and GIU3 is weak at 14 days, positive at 21 days. GIU2 grows between pH 7 and 9, while GIU3 grows from pH 5 to 9. On most media, colonies of GIU1, GIU2 and GIU3 form bright orange, dome-like structures with a cauliflower-like appearance on agar. Spore formation is clustered around the central projection. Aerial mycelium is not formed. None of the strains produce diffusible pigments.

Under a light microscope they appear as spherical or club shaped cells. All produce an orange polysaccharide-like matrix surrounding the colonies. Figure 2.7 B shows the cauliflower-like appearance of GIU2 colonies.

Table 2.9 Comparison of sole carbon and nitrogen source utilisation by GIU1, GIU2 and GIU3.

Carbon source	GIU1	GIU2	GIU3	Nitrogen source	GIU1	GIU2	GIU3	Nitrogen source	GIU1	GIU2	GIU3
Adonitol	+	++	+	DL-α-ABA*	++	+	-	L-Isoleucine	-	+	+
D(+) Cellobiose	+	+	+	$(NH_4)_2HPO_4$	+	++	+	D-Leucine	-	+w	-
meso-Erythritol	++	++	++	DL-α-Alanine	++	+w	-	L-Leucine	-	+w	-
D(-) Fructose	+w	++	++	β-Alanine	-	+	-	L-Methionine	-	+w	+w
D(+) Melezitose	-	+w	+w	L-Arginine	++	+w	+	L-Phenylalanine	-	-	-
D(+) Raffinose	+w	+w	+w	L-Aspartic acid	-	+w	-	DL-β-Phenylalanine	-	-	+w
Salicin	+w	+	++	L-Cysteine	-	+w	-	KNO_3	+	++	-
L(-) Sorbose	++	++	+w	Gelatin	++	+	+	L-Proline	-	+	-
Starch	+	++	+	L-Glutamic acid	+w	+w	+w	L-Serine	+w	+w	+w
Xylitol	+	+	+	L-Glutamine	+	+w	-	$NaNO_3$	++	++	-
D(+) Xylose	+	++	++	L-Histidine	+w	+w	-	L-Valine	+	+w	++
				L-Hydroxyproline	-	+	+w				

Symbols: ++, growth better than positive control; +, growth equal to positive control; +w, growth weaker than positive control; -, no growth or comparable to negative control; ABA*, DL-α-amino-*n*-butyric acid.

CA21 does not grow on commercial DNase medium (Oxoid). CA21 degrades gelatin, Tween 80 and L-tyrosine. Cellulose, hypoxanthine and xanthine are not degraded. CA21 is unable to hydrolyse aesculin. Grows at pH 5, 7 and 9. Grows from 22°C to 30°C, with optimum growth at 22°C. Utilises D(-) lactose and D(+) mannose (weakly) as sole carbon sources, but does not use D(-) ribose.

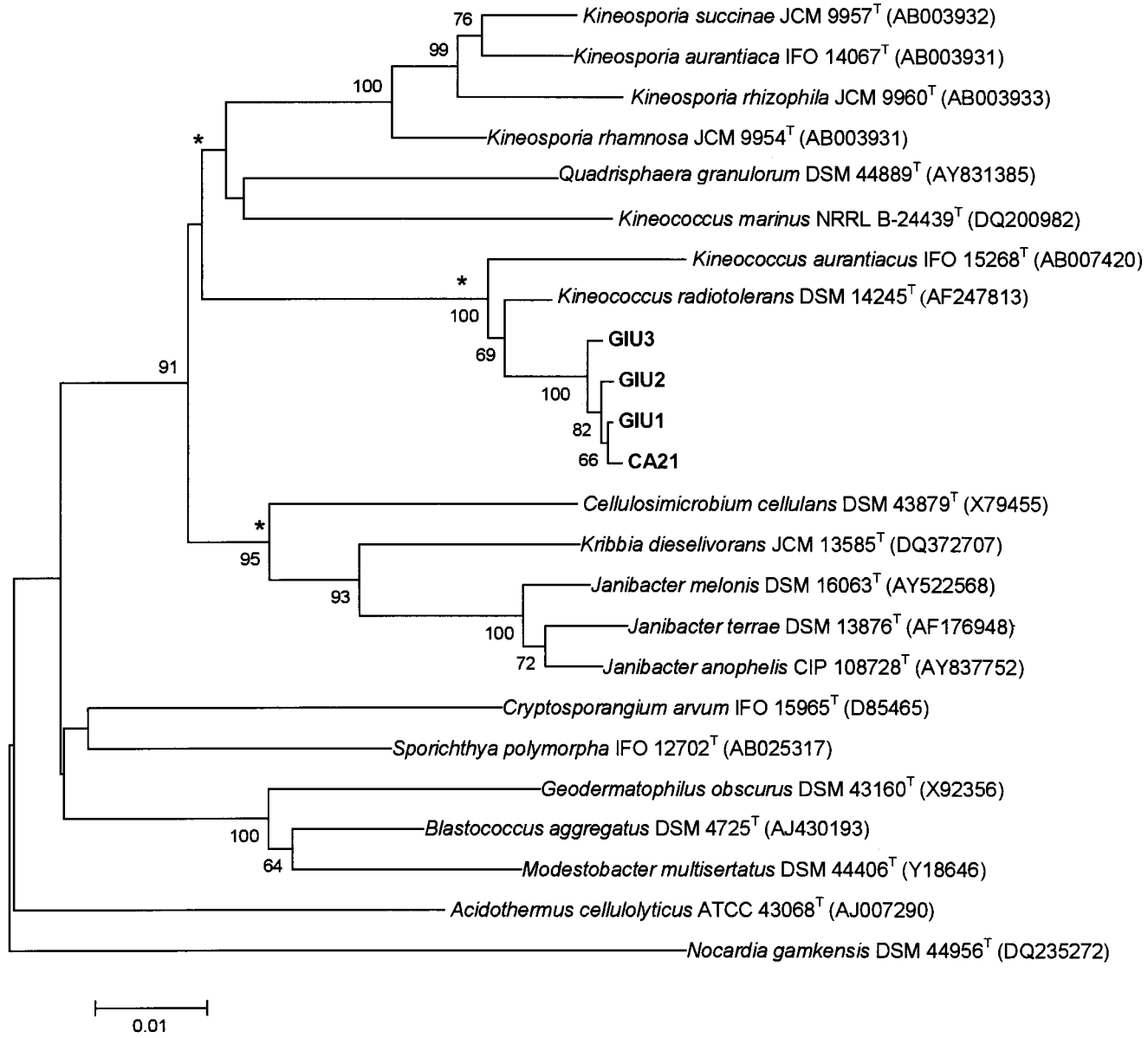
The morphological features of CA21 differ from those of the three *G. incanum* isolates. When viewed under a light microscope the cells of CA21 appear as large cocci either singly, in pairs or in tetrads. These cells are significantly larger than those of GIU1, GIU2 and GIU3. Figure 2.7 C shows the arrangement of CA21 cells in ordered tetrads encased in a polysaccharide-like layer, which resemble the published SEM pictures of *Kc. radiotolerans* (Philips *et al.*, 2002). CA21 grows slowly on most media, with colonies only forming after 7 days (at 20°C), however, an orange polysaccharide matrix is produced within two days of colony formation. Production of this substance is increased when incubated at room temperature.

Motile spores were detected for all four isolates and Ryu staining allowed for the detection of polar flagella, however, due to the lack of resolution under a light microscope it was not possible to determine with confidence whether a single flagellum or multiple flagella are formed.

Based on 16S rRNA gene sequence analysis, all four isolates are most closely related to *Kineococcus* species. However, the morphological features of GIU1, GIU2 and GIU3 are more similar to those reported for the five *Kineosporia* species (Itoh *et al.*,1989; Kudo *et al.*, 1998). All four isolates had chemotaxonomic features that

have been reported for both genera. The presence of ribose in whole cell hydrolysates has only been reported in the genus *Kineosporia*, while the presence of arabinose and galactose has been reported for the genus *Kineococcus*. Morphological features of CA21 are similar to those reported for the three published *Kineococcus* species (Yokota *et al.,* 1993; Lee, 2006). Based on phylogenetic (Figure 2.4) and phenetic characterisation (Tables 2.9 and 2.10) the four *Kineococcus* strains can be distinguished from the three *Kineococcus* type strains. Although the physiological features of these isolates differ, DDH is likely to be required to determine whether they are distinct genomic species.

Figure 2.4 Unrooted 16S rRNA gene phylogenetic tree showing the relationship of GIU1, GIU2, GIU3 and CA21 with members of the genera *Kineosporia* and *Kineococcus* and other related taxa of the suborder *Frankineae*. The tree was constructed using the neighbour-joining method and is based on 1351 bp of sequence. The bootstrap values are based on 1000 resampled datasets and only values above 50% are shown. Bar represents 0.01nt substitutions per nt position. *Nocardia gamkensis* DSM 44956^T was set as the outgroup.



Asterisks (*) denote clusters that were conserved using the neighbour-joining, minimum evolution and maximum parsimony methods to construct the phylogenetic trees. The isolates coded in green were isolated from the leaves of indigenous plants.

Figure 2.5 Multiple sequence alignment of the 16S rRNA gene sequence of the four *Kineococcus* isolates and the type strains of the genera *Kineococcus* and *Kineospora*.

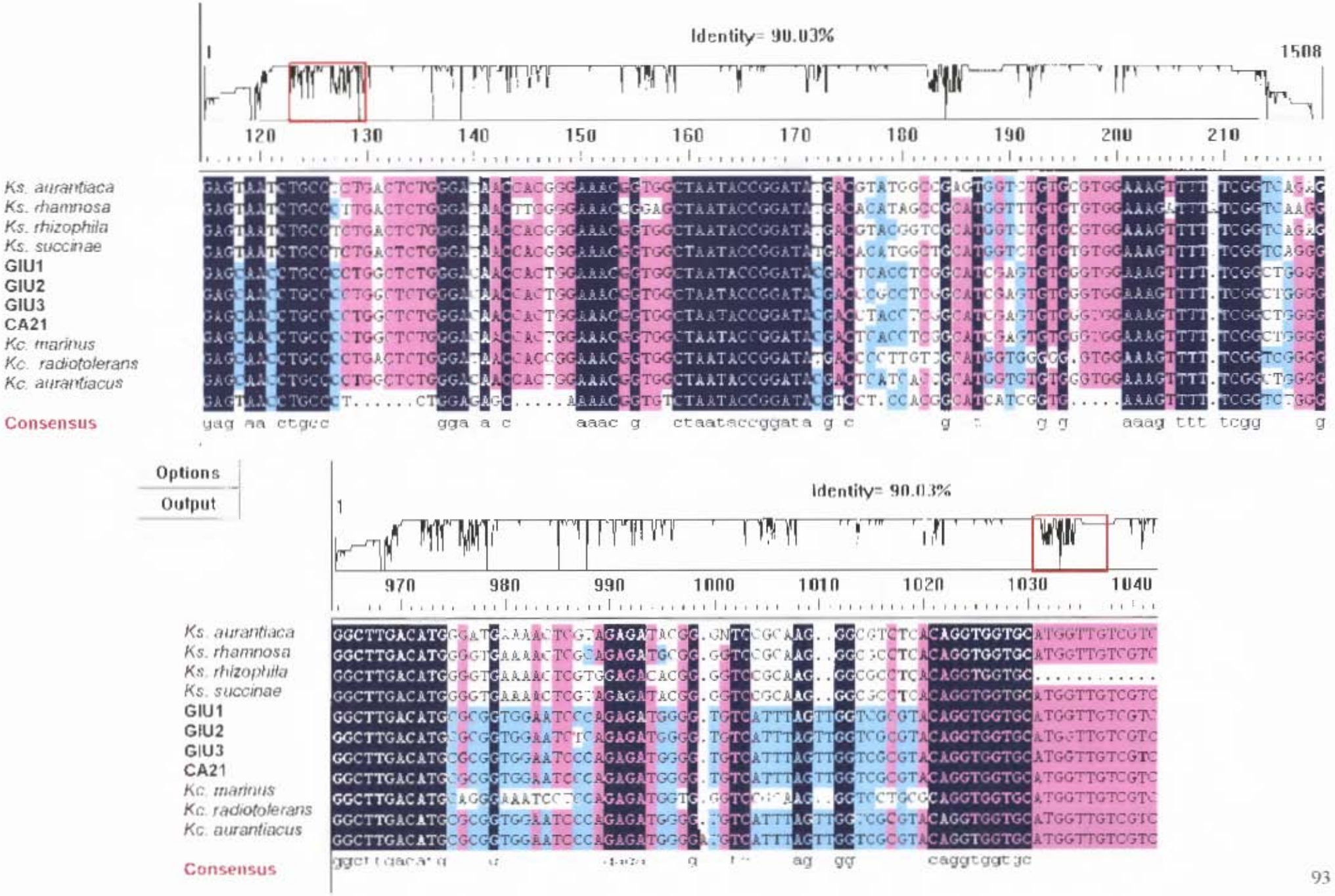


Table 2.10 Phenetic characteristics which differentiate GIU1, GIU2, GIU3 and CA21 from the type strains of the genera *Kineosporia* and *Kineococcus*.

Physiological test	1	2	3	4	5[a]	6[b]	7[c]	8[d]	9[e]	10[e]	11[e]	12[e]
Isomer of DAP	*meso*-DAP	*meso*-DAP	*meso*-DAP	*meso*-DAP	*meso*-DAP	*meso*-DAP	ND	LL-DAP *meso*-DAP	LL- DAP *meso*-DAP	*meso*-DAP	*meso*-DAP	*meso*-DAP
Whole cell sugars	Rib, Ara, Gal	Rib, Ara, Gal	Rib, Ara, Gal	Rib, Ara Gal	Ara, Gal	Ara, Gal	ND	Gal, Glc, Man, Rib	Gal, Glc, Man, Rib	Gal, Glc, Man, Rib	Gal, Glc, Man, Rib, Rha	Gal ,Glc, Man, Rib
Nitrate reduction	-	+ strong	+w	+w	-	-	ND	ND	ND	ND	ND	ND
Hydrolysis of:												
Casein	+w	NG	+w	NG	-	-	ND	+	+	+	+	+
Gelatin	NG	NG	NG	+	-	+	ND	ND	ND	ND	ND	ND
Starch	-	-	-	-	-	+	ND	ND	ND	ND	ND	ND
Temp range	20(+w)-30	4-30	20-30	20-30	9-37	4-37	11-41	20-30	ND	ND	ND	ND
Growth on:												
2% NaCl	+	+	+	+	+	+	+	+	+	+	-	-
5% NaCl	-	-	-	-	+	+	+	-	-	-	-	-
7% NaCl	-	-	-	-	-	+	-	-	-	-	-	-
Utilisation of:												
D(+) Galactose	+	+	+	+	-	+	+	+	+	+	+	+
Glycerol	ND	ND	ND	-	-	+	+					
meso-Inositol	+	+	+	+	-	+	+	+	+	-	+	-
Maltose	+	+	+	-	-	+	-	+	+	+	+	+
D(-) Mannitol	+	+	-	+	-	+	+	+	+	+	+	+
D(+) Raffinose	d	d	d	-	-	+	-	-	-	+	d	-
L(+) Rhamnose	+	+	+	+w	-	+	-	+	+	+	+	+
Trehalose	+	+	+	-	-	+	ND	+	+	+	-	+
Colony colour	Orange	Orange	Orange	Orange	Cream to orange	Deep orange	Orange	Cream to orange	Orange to light brown	Orange to light brown	Orange to light brown	Orange to light brown
Diffusible pigment	None	None	None	None	None	ND	ND	None	faint yellow on oatmeal	faint reddish on YE-starch	None	faint yellow on oatmeal
Polysaccharide matrix	Within 14 days	Within 7 days	Within 14 days	Within 14 days	ND	Prolonged incubation	ND	Prolonged incubation	ND	ND	ND	ND

1, GIU1; **2**, GIU2; **3**, GIU3; **4**, CA21; **5**, *Kineococcus aurantiacus* IFO 15268^T; **6**, *Kineococcus marinus* KCCM 42250^T; **7**, *Kineococcus radiotolerans* DSM 14245^T; **8**, *Kineosporia aurantiaca* ATCC 29727^T; **9**, *Kineosporia mikuniensis* JCM 9961^T; **10**, *Kineosporia rhamnosa* JCM 9954^T; **11**, *Kineosporia rhizophila* JCM 9960^T; **12**, *Kineosporia succinea* JCM 9957^T. Abbreviations: ara, arabinose; gal, galactose; glc, glucose; man, mannose; rha, rhamnose; rib, ribose; +, positive reaction/growth; -, negative reaction/no growth; +w, weak reaction/weak growth; d, doubtful growth; ND, not determined; NG, no growth on that medium. Data for type species from: a, Yokota *et al.*, 1993; b, Lee, 2006; c, Phillips *et al.*, 2002; d, Pagani & Parenti, 1978; Itoh *et al.*, 1989; e, Kudo *et al.*, 1998.

2.4.3 The identification of eight strains belonging to the Family *Micromonosporaceae*

Eight *Micromonospora* isolates were selected for full characterisation. All the strains were originally selected based on their colony morphology: small, wrinkled, bright orange or red colonies. MuizA5S, VleiA3C and CGM31 were isolated from sediment samples (either marine or brackish water); while isolates DG41, PLU1, PBPE, RAU1 and TVU1 were isolated from various plant species (Table 2.5).

For rapid genus identification, the amplified 16S rDNA was digested singly with *Mbo*I (*Sau*3A), *Vsp*I (*Asn*I), *Sph*I, *Sna*BI and *Sal*I. All eight isolates generated the same banding pattern and fell into the group containing the genera *Actinoplanes*, *Couchioplanes*, *Micromonospora*, *Pilimelia*, *Spirilliplanes*, *Verrucosispora* and *Virgisporangium* (Table 2.8). Based on their morphology it was assumed that five of the isolates were *Micromonospora* species, which was confirmed by 16S rRNA gene sequencing. The morphology of PLU1, RAU1 and VleiA3C was markedly different from that of 'typical' micromonosporae. The colonies of these isolates are bright orange and have a dry, crumbly appearance. Sporulation is sparse and the colonies neither darken nor become mucoid with age. However, from 16S rRNA gene sequence and phylogenetic analysis, these strains clearly belong to the genus *Micromonospora*.

Phylogenetic analysis was performed over 1318 bp (Figure 2.6). TVU1 and MuizA5S were found to be most closely related to *Micromonospora echinospora* subsp. *echinospora* ATCC 15837^T and *Micromonospora rosaria* ATCC 29337^T, while PLU1 and RAU1 formed a separate cluster, most closely related to the clade including TVU1 and MuizA5S. CGM31, DG41, PBPE and VleiA3C formed a monophyletic cluster which was most closely related to *Micromonospora mirobrigensis* DSM 44830^T. A comparison of the phenetic characteristics of the eight *Micromonospora* isolates to their closest phylogenetic neighbours is presented in Table 2.11.

All *Micromonospora* isolates are unable to degrade cellulose, guanine and xanthine. Only CGM31 can weakly produce melanin pigment on tyrosine agar (ISP 7); melanin pigment is not produced on peptone-yeast extract-iron agar (ISP 6) and tyrosine agar (ISP 7) by the other seven isolates. All are characterised by the presence of *meso*-DAP in their cell walls. Although arabinose and xylose are reported to be present in *Micromonospora* cell hydrolysates (Kawamoto, 1989), the only diagnostic whole cell sugar detected in all isolates was xylose. The eight strains are all catalase positive, however, DG41 is only weakly positive.

TVU1 was isolated on PE agar, using the indirect method, from the leaves of the bulbous plant *Tulbaghia violacea*, which is a member of the onion family (Manning, 2003). TVU1 is characterised by the formation of medium brown vegetative mycelium on YEME. Colonies become dark brown upon sporulation. Figure 2.7 D shows the smooth surfaced spores with a raisin-like appearance which are formed on sporophores borne on the branching hyphae. TVU1 degrades casein, gelatin, starch and Tween 80. Growth only occurs from pH 7 to 9. TVU1 can utilise D(-) fructose and D(+) glucose as sole carbon sources, but is unable to use L(+) arabinose, D(+) cellobiose, D(-) lactose, salicin and D(+) xylose. TVU1 is susceptible to cephaloridine (100 µg/ml) and vancomycin (50 µg/ml), and is resistant to kanamycin (10 µg/ml), lincomycin (100 µg/ml), oleandomycin (100 µg/ml), penicillin G (10 IU/ml) and rifampicin (50 µg/ml). $(NH_4)_2PO_4$, gelatin, L-glutamine, L-histidine, L-leucine, L-methionine and L-valine are utilised as sole nitrogen sources, while utilisation of DL-α-amino-*n*-butyric

acid, DL-α-alanine, β-alanine, L-arginine, L-glutamic acid, L-hydroxyproline, D-leucine, DL-β-phenylalanine, KNO_3, L-proline, $NaNO_3$ and L-serine is weak or doubtful. Weak growth occurs on m172F medium under anaerobic conditions over 14 days.

Phylogenetically, TVU1 is most closely related to *M. echinospora* and *M. rosaria*. From Table 2.11 it can be seen that there are eight phenetic differences between TVU1 and *M. echinospora*, including colony colour, the production of diffusible pigments and growth on potato slices. Similarly, there are ten phenetic differences between TVU1 and *M. rosaria*, including NaCl tolerance, colony colour and resistance to 50 µg/ml rifampicin. The DNA relatedness between TVU1 and *M. echinospora* DSM 43816^T was found to be 7.6% ± 4.5%, which confirms that TVU1 is a distinct specie. TVU1 therefore represents the type strain of a novel species, for which the name '*Micromonospora tulbaghiae*' sp. nov. is proposed (tul.ba' ghi.ae. N.L.gen. fem. n. *tulbaghiae* of/from *Tulbaghia*, the genus name of *T. violacea*, the host of the organism).

MuizA5S was isolated on SC agar from an air dried sediment sample collected from Muizenberg beach. The colonies of MuizA5S are dark brown on YEME and become black and very moist when sporulation occurs. The smooth, spherical spores form clusters (Figure 2.7 E). MuizA5S is able to degrade casein, gelatin, starch, Tween 80 and L-tyrosine, but is unable to degrade hypoxanthine and pectin. H_2S is produced and nitrate is reduced. Good growth occurs from pH 5 to 9. MuizA5S can utilise L(+) arabinose, D(+) cellobiose, D(+) glucose, glycerol, D(-) lactose, maltose, salicin, D(-) sorbitol and trehalose as sole carbon sources, with growth on D(+) xylose being weak at 21 days. MuizA5S can not utilise *meso*-inositol, D(+) melezitose and L(-) sorbose. MuizA5S is susceptible to cephaloridine (100 µg/ml), kanamycin (10 µg/ml) and vancomycin (50 µg/ml), and is resistant to lincomycin (100 µg/ml), oleandomycin (100 µg/ml), penicillin G (10 IU/ml) and rifampicin (50 µg/ml). MuizA5S is able to utilise DL-α-amino-*n*-butyric acid, $(NH_4)_2PO_4$, DL-α-alanine, L-arginine, L-cysteine, gelatin, L-histidine, L-isoleucine, L-methionine, DL-β-phenylalanine, L-serine and L-valine as sole nitrogen sources, with utilisation of L-hydroxyproline, L-phenylalanine, L-proline and $NaNO_3$ being weak or doubtful. Grows weakly on m172F medium under anaerobic conditions at 14 days.

From Table 2.11 it can be seen that MuizA5S can be differentiated from its closest relatives, *M. echinospora* and *M. rosaria*. There are ten phenetic differences between MuizA5S and *M. echinospora*, including colony colour, growth temperature, and use of glycerol and D(+)melibiose as sole carbon sources. There are eleven phenetic features that differ between MuizA5S and *M. rosaria*, including colony colour, NaCl tolerance and growth on potato slices. From Table 2.11 and Figure 2.6 both MuizA5S and TVU1 can be distinguished from their closest phylogenetic neighbours. A pairwise local alignment of the 16S rRNA gene sequences found that TVU1 and MuizA5S share a sequence similarity of 100% over 1368 bp. However, there are at least ten phenetic differences between these two isolates. The DNA relatedness between MuizA5S and *M. echinospora* DSM 43816^T is 36.35% ± 1.65%, while the DNA relatedness between MuizA5S and TVU1 is 13.75% ± 0.15%. MuizA5S therefore represents the type strain of a novel species, for which the name '*Micromonospora psammotica*' is proposed (psam.mo' ti.ca. Gr. n. psammos sand, N.L. fem. adj. *psammotica* pertaining to sand).

Phylogenetic analysis showed that two plant isolates, PLU1 and RAU1, formed a clade which branched off from the cluster containing MuizA5S, TVU1, *M. echinospora* and *M. rosaria*. Like VleiA3C, the colony morphology of

PLU1 and RAU1 is distinctly different from that of 'typical' micromonosporae, having a dry, crumbly appearance. Both PLU1 and RAU1 grew weakly with glucose as a sole carbon source and so were maintained on Modified Bennett's Medium or CZ.

PLU1 was isolated after 7 days incubation on PE agar using the indirect method. This strain was isolated from the leaves of the waving pelargonium, *Pelargonium luridum*. The colonies are bright orange on most media. PLU1 degrades casein, gelatin, starch and Tween 80, while adenine, guanine and xanthine are not degraded. Grows at pH 7 and 9. Lactose, D(+) mannose and trehalose are used as sole carbon sources, while D(+) galactose and maltose are not utilised. PLU1 is strictly aerobic. There are eight phenetic characteristics which differ between PLU1 and *M. rosaria*, including its inability to degrade L-tyrosine and xylan, and differences in colony morphology. *M rosaria* is also reported to produce tufts of white, sterile aerial mycelium (Horan & Brodsky, 1986), which none of the *Micromonospora* isolates produced. Likewise, there are eight features that distinguish PLU1 from *M. echinospora* which includes differences in colony colour and growth temperature. The phenetic features, especially the colony morphology, of PLU1 allow this strain to be distinguished from isolates MuizA5S and TVU1.

RAU1 was isolated from the leaves of the Knysna fern, *Rumohra adiantiformis*. It was isolated on PE agar after 21 days incubation using the indirect isolation method. RAU1 forms bright orange colonies on most media and diffusible pigments are not produced. RAU1 degrades casein, gelatin, starch and Tween 80, while adenine, guanine and xanthine are not degraded. Grows at pH 7 and 9. Utilises lactose, D(+) mannose, maltose and trehalose as sole carbon sources, with growth on D(+) galactose weak at 21 days. RAU1 is strictly aerobic. There are seven physiological characteristics that differ between RAU1 and *M. rosaria,* including its inability to degrade L-tyrosine and xylan, differences in growth temperature and differences in colony colour. Similarly, there are ten phenetic characteristics that differ between RAU1 and *M. echinospora*. These include the ability of RAU1 to use D(+) raffinose as a sole carbon source and grow at 45°C. From Table 2.11 and Figure 2.6 RAU1 can be distinguished from its closest neighbours. Like PLU1, RAU1 can clearly be distinguished from MuizA5S and TVU1.

CGM31 was isolated on YEME supplemented with 4% NaCl from a heat treated sediment sample collected in Hermanus Lagoon. On most media CGM31 forms dark red colonies and produces a red to orange diffusible pigment. CGM31 degrades casein, gelatin, starch and Tween 80, and grows from pH 7 to 9. CGM31 can utilise L(+) arabinose, D(-) fructose, D(+) galactose, D(+) mannose, maltose and D(-) sorbitol as sole carbon sources, with growth on D(-) lactose, D(+) melezitose and trehalose weak after 21 days. CGM31 is unable to use L(-) sorbose as a sole carbon source. CGM31 is strictly aerobic.

From the physiological data presented in Table 2.11 and those reported by Luedeman & Brodsky (1964), there are eight phenetic differences between CGM31 and *M. carbonacea* which includes the ability of CGM31 to use glycerol, D(-) mannitol, D(+) raffinose and D(-) sorbitol as sole carbon sources, as well as differences in colony pigmentation on YEME and Bennett's agar, the production of diffusible pigments and spore colour (data not shown). From Table 2.11 there are only five phenetic differences between CGM31 and *M. mirobrigensis,* including the ability of CGM31 to reduce nitrate, the utilisation of D(-) mannitol as a sole carbon source, and the

absence of glucose and mannose in whole cell sugar hydrolysates. However, a comparison with the characteristics reported by Trujillo *et al.* (2005) found additional differences including the ability of CGM31 to use D(-) sorbitol as a sole carbon source. *M. mirobrigensis* is also reported to grow slowly on Bennett's agar and nutrient agar, CGM31 readily grew on both these media. A local pairwise alignment found the 16S rRNA gene sequence similarity between CGM31 and *M. mirobrigensis* DSM 44830^T to be 98.28%. This is lower than the 98.5% similarity reported between *M. mirobrigensis* DSM 44830^T and *M. carbonacea* DSM 43168^T (Trujillo *et al.*, 2005). From phenetic and phylogenetic comparisons, CGM31 appears to be a novel species.

PBPE was isolated on PE agar from the dehydrated leaves of the resurrection plant, *Myrothamnus flabellifolius,* using the indirect method. On YEME the colonies of PBPE are brick red and a maroon diffusible pigment is produced. The vegetative mycelia branch infrequently and the spores are formed at the apical end of the hyphae (Figure 2.7 F, G). PBPE degrades casein, gelatin, starch and Tween 80, but can not degrade hypoxanthine. Growth occurs from pH 7 to 9. PBPE is able to utilise L(+) arabinose, D(+) cellobiose, D(-) fructose, D(+) glucose, D(-) lactose, salicin and D(+) xylose as sole carbon sources. PBPE is susceptible to cephaloridine (100 µg/ml), kanamycin (10 µg/ml) and vancomycin (50 µg/ml), and is resistant to oleandomycin (100 µg/ml) and rifampicin (50 µg/ml). Growth in the presence of lincomycin (100 µg/ml) and penicillin G (10 IU/ml) was weak after 14 days incubation. PBPE can utilise DL-α-alanine, L-arginine, gelatin, L-glutamic acid, L-proline and L-serine as sole nitrogen sources, with utilisation of $(NH_4)_2PO_4$, L-cysteine, L-histidine, L-isoleucine, L-leucine, DL-β-phenylalanine, L-phenylalanine, $NaNO_3$ and L-valine weak or doubtful at 21 days. PBPE is strictly aerobic.

Phylogenetic analysis found that PBPE is most closely related to *M. mirobrigensis* and *M. carbonacea* (Figure 2.6). There are six phenetic differences between PBPE and *M. carbonacea*, including the utilisation of glycerol, D(-) mannitol and D(+) raffinose as sole carbon sources, as well as the colour of the spore mass and diffusible pigments. Differences between PBPE and *M. mirobrigensis* include resistance to 50 µg/ml rifampicin and the ability of *M. mirobrigensis* to grow weakly on m172F under anaerobic conditions. From phylogenetic (Figure 2.6) and phenetic (Table 2.11) analyse, PBPE can be distinguished from its closest neighbours *M. mirobrigensis* and *M. carbonacea*, as well as DG41 and VleiA3C. However, further phenetic testing and DDH would probably be required in order to determine whether PBPE and CGM31 are separate species.

DG41 was isolated on PV8 agar, using the indirect method, from the leaves of the wild iris, *Dietes grandiflora*. Phylogenetic analysis found that DG41 is most similar to *M. mirobrigensis* and *M. carbonacea* (Figure 2.6). On most media the colonies of DG41 are wine-red to brown. A brownish-red diffusible pigment is produced on some media. Sporulation is sparse. DG41 degrades casein, gelatin, starch and Tween 80. Urea is not degraded and no growth occurs in the presence of adenine and allantoin. DG41 grows from pH 5 to 9. DG41 can utilise D(+) mannose and trehalose as sole carbon sources, with growth in the presence of D(+) galactose, D(-) lactose and maltose weak at 21 days. DG41 is strictly aerobic. A comparison of physiological features found that there are only three differences between this strain and *M. carbonacea* and nine differences between it and *M. mirobrigensis*.

VleiA3C was isolated on starch-casein agar from a sediment sample collected in Zandvlei Nature Reserve. Phylogenetic analysis found that VleiA3C is most closely related to *M. carbonacea* and *M. mirobrigensis*. The morphological features of VleiA3C differ from those of the other *Micromonospora* isolates and type strains. The colonies of VleiA3C are bright orange and do not change colour upon sporulation. VleiA3C degrades casein, gelatin, starch and Tween 80, but does not degrade hypoxanthine and pectin. VleiA3C grows from pH 7 to 9. H_2S is not produced and nitrate is not reduced. Can utilise L(+) arabinose, D(-) fructose, D(+) galactose, D(+) mannose, maltose and D(-) sorbitol as sole carbon sources, with growth on D(-) lactose, D(+) melezitose and trehalose weak at 21 days. VleiA3C is unable to utilise L(-) sorbose. VleiA3C is strictly aerobic. There are six phenetic features that differ between VleiA3C and *M. carbonacea*, including the utilisation of D(-) mannitol and D(+) raffinose as sole carbon sources, and the colour of the colonies. Although there are only five differences between VleiA3C and *M. mirobrigensis* (which includes growth under anaerobic conditions), the gross morphological features of these two strains are clearly different.

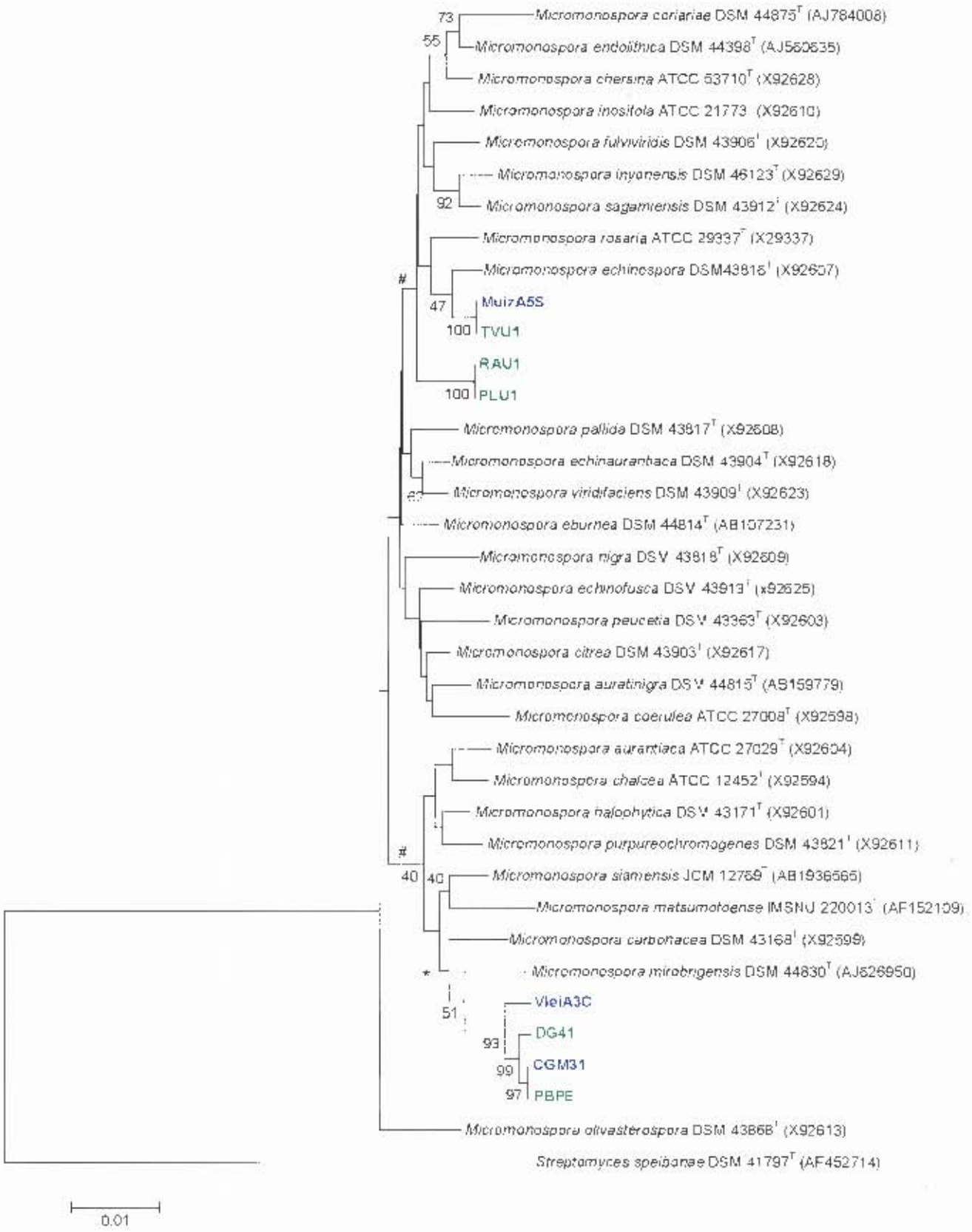
Although there are limited physiological differences between DG41, VleiA3C and the closest related *Micromonospora* species, *M. mirobrigensis* and *M. carbonacea*, these isolates can not confidently be distinguished as separate species. There are eight phenetic differences (Table 2.11) between DG41 and VleiA3C, as well as differences in colony morphology. Therefore, DG41 and VleiA3C can be distinguished from each other.

Footnote from Table 2.11 (on next page) **1,** CGM31; **2,** DG41; **3,** MuizA5S; **4,** PBPE; **5,** PLU1; **6,** RAU1; **7,** TVU1; **8,** VleiA3C; **9,** *M. carbonacea* ATCC 27114^T (data from Luedemann & Brodsky, 1964); **10,** *M. echinospora* subsp. *echinospora* ATCC 15837^T (Kawamoto, 1989); **11,** *M. mirobrigensis* DSM 44830^T (Trujillo *et al.*,2005); **12,** *M. rosaria* ATCC 29337^T (Horan & Brodsky, 1986). For colour determination isolates were grown on YEME for 14 days. * data determined in this study.; ** colour was determined on Modified Bennett's Medium. Symbols: +, good growth/positive reaction; +w, weak growth/reaction; d, growth is doubtful; -, no growth/reaction; ND, not determined; NG, no growth; gal, galactose; glu, glucose; man, mannose; ribo, ribose; xyl, xylose; /, no diffusible pigment produced.

Table 2.11 Physiological tests that distinguish the eight *Micromonospora* isolates from the phylogenetically related *Micromonospora* type strains.

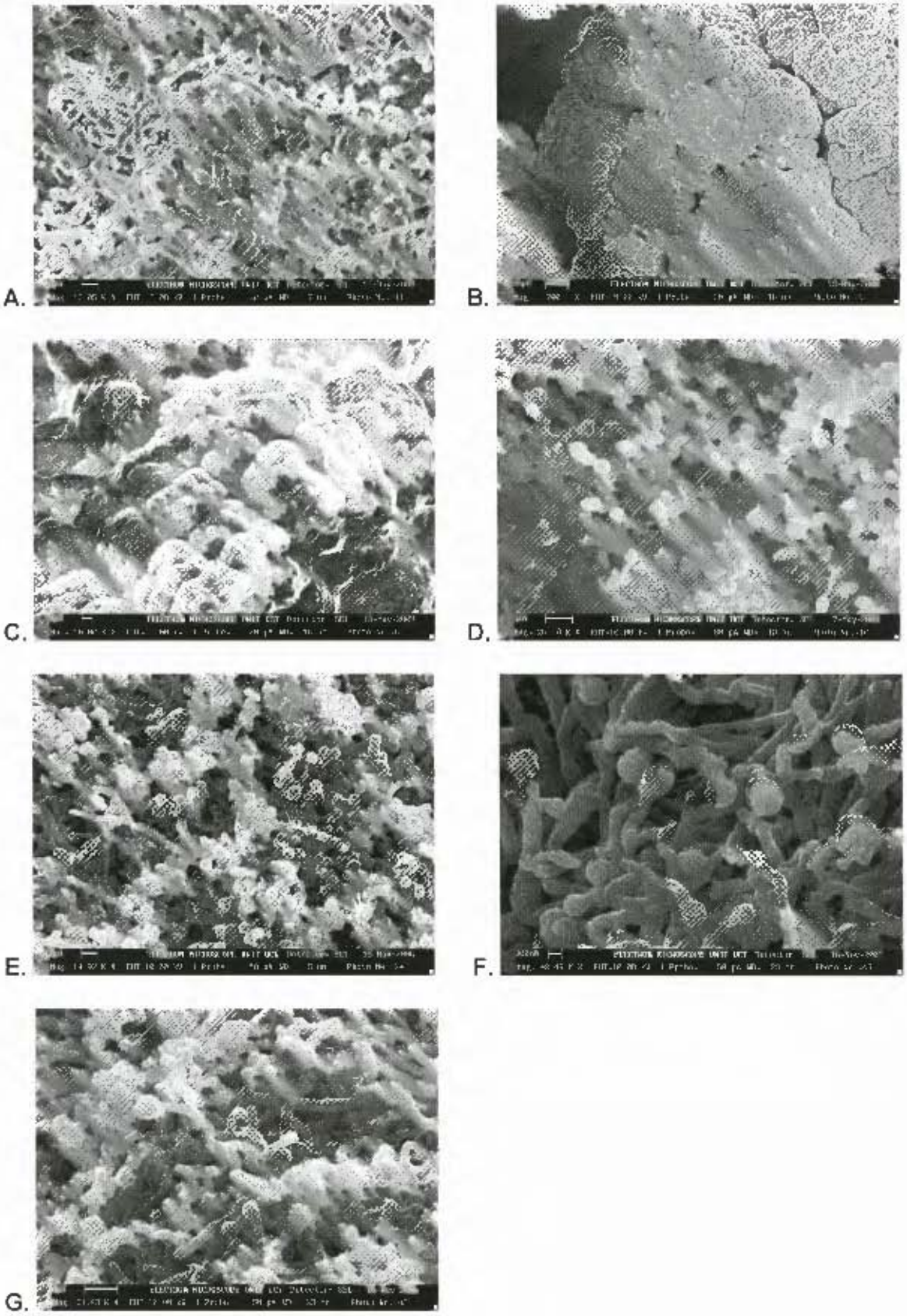
Phenetic characteristic	1	2	3	4	5	6	7	8	9	10	11	12
NaCl tolerance (% w/v)	<4	<4	5	4	<4	<4	5	<4	ND	ND	ND	3
Growth on potato	+w	+	+	+	-	-	+	-	+w	-	ND	-
Growth on potato + $CaCO_3$	+	+	-	+	-	-	-	-	+		ND	ND
H_2S production	+w	+w	+	+	-	+w	+	-	ND	-*	ND	-*
Nitrate reduction	+	+	+	-	-	+w	+w	-	+	-*	-	-
Egg yolk medium:												
Proteolysis	NG	+	+	-	NG	NG	+	NG	ND	ND	ND	ND
Lipolysis		-	-	-			-					
Lecithinase		-	-	-			-					
Degradation of:												
L-Tyrosine	+w	+w	+	+	-	-	+	-	ND	ND	ND	+
Xylan	+w	+w	+	+w	-	-	+w	+w	ND	ND	+	+
Growth temp (°C)	20-37	4-37	4-37	20-37	20-45	20-45	4-37	20-37	ND	27-37	20-37	10-42
Colony colour	Dark red	Wine red-brown	Dark brown	Brick red	Bright orange**	Bright orange**	Medium yellow-brown	Bright orange	Terracotta orange	Maroon-purple	Orange	Orange-brown to purple-black
Colour of diffusible pigment	Red-orange	Brownish-red	/	Maroon	/	/	/	/	/	Purple	/	Wine Red
Spore colour	Brownish-red	Brownish-red	Black mucoid	Dark brownish-red	Orange	Orange	Dark brown	Orange	Brown	Brown -black	Brown to black	Purple
Sole carbon source:												
Glycerol	+	+w	+	+	+w	-	d	-	-	-		-
meso-Inositol	-	-	-	-	-	+w	+w	-	-	-		
D(-) Mannitol	+	-	-	+	+w	-	+	+	-	-	+	-
D(+) Melibiose	ND	ND	+	+	ND	ND	-	+	+	-	-	
D(+) Raffinose	+	+	+w	+	-	+	-	+	-	-	-	+
L(+) Rhamnose	-	-	-	-	+	+	+	-	-	+	+	-
D(-) Ribose	+w	-	v	+w	-	-	++	+w	-	-	+	
Resistance to rifampicin (50 µg/ml)			+	+			+		+w*	-*	-*	-*
Anaerobic growth on m172F*	-	-	+w	-	-	-	+w	-	-	-	+w	-
Aerobic growth on m172F (control)*	+	+	+	+	+w	+w	+	+w	+	+	+	+
Whole cell wall sugars	Gal, xyl	Ribo, xyl	Glu, ribo, xyl	Gal, ribo, xyl	Ribo, xyl	Ribo, xyl	Glu, ribo, xyl	Ribo, xyl			Gal, glu, man, xyl	Gal, xyl

Figure 2.6 Unrooted 16S rRNA gene phylogenetic tree showing the position of the eight *Micromonospora* isolates within the genus. The phylogenetic tree is based on ~318 bp and was constructed using the neighbour-joining method. The bootstrap values are based on 1000 resampled datasets and only bootstrap values above 40% are shown. The bar represents 0.01 nt substitutions per nt site. *Streptomyces speibonae* DSM 41797^T was set as the outgroup.



An asterisk (*) denotes the cluster that was conserved using the neighbour-joining, minimum evolution and maximum parsimony methods to construct the phylogenetic trees. A hash (#) denotes clades that were conserved between trees constructed from the neighbour-joining and minimum evolution methods only. Strains coded in blue were isolated from aquatic sediment samples while strains coded in green were isolated from the leaves of indigenous plants.

Figure 2.7 Scanning electron micrographs of the *Kineococcus*, *Micromonospora* and *Nocardia* strains



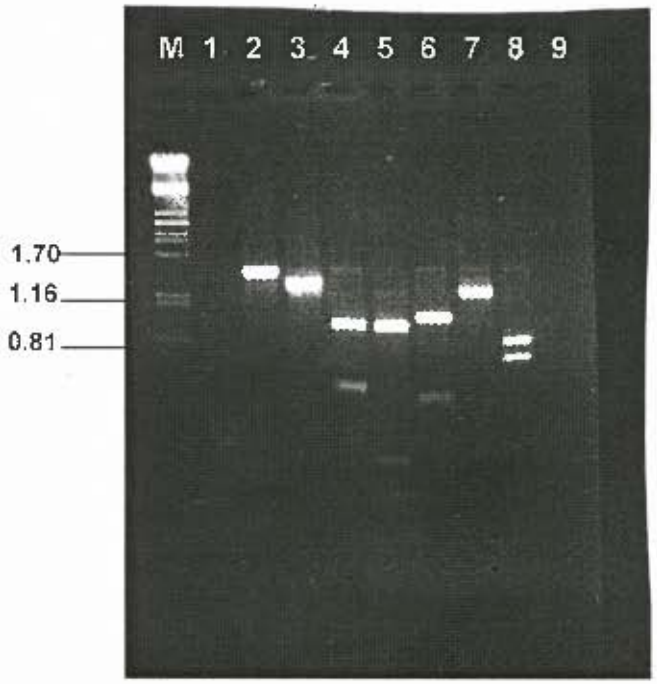
All isolates were grown on YEME at 30°C for 14 days, except for CA21 which was incubated at 22°C. Micrographs A-C were obtained by cryo-SEM. **A**, *Nocardia* strain M25, the fragmentation of the vegetative hyphae into rod-shaped elements is visible; **B**, *Kineococcus* species G-L2, the dome-shaped structure with a cauliflower-like appearance is clearly visible; **C**, *Kineococcus* species CA21, cells cluster in groups of either four or eight which are encased in a polysaccharide-like matrix; **D**, *Micromonospora* strain TVU1 produces spores with a raisin-like appearance on branching mycelium; **E**, the clustering of the spores of *Micromonospora* strain Mu/A5S; **F & G**, *Micromonospora* strain PBPE, forms long unbranched vegetative hyphae, with a single large, smooth surfaced spore at the end.

2.4.4 The Family *Nocardioidaceae* - The description of two novel *Kribbella* species, *Kribbella karoonensis* Q41^T and *Kribbella swartbergensis* HMC25^T

HMC25 and Q41 were selected based on their unusual appearance. Both formed small cream-coloured, wrinkled colonies with a cauliflower-like appearance. Q41 was isolated on PV8 agar from an air dried soil sample collected from the base of a giant quiver tree, *Aloe pillansii*, growing in the Karoo Desert National Botanical Garden. HMC25 was isolated on MC agar, by Mariize le Roes, from soil collected from the banks of the Gamka River, Die Hel. All characterisation of these strains were carried out by Bronwyn Kirby.

For rapid genus identification, the *Mbo*I (*Sau*3AI) digestion of the amplified 16S rDNA generated a large fragment of 990-1300 bp for both strains. According to the method of Cook & Meyers (2003), the 16S rDNA was digested singly with the *Vsp*I (*Asn*I), *Sph*I, *Sna*BI, *Sal*I and *Age*I. From Table 2.8 it can be seen that the isolates could either be *Kribbella* or *Hongia* species, however, as these two genera have subsequently been combined it was not necessary to perform an *Ssf*I digestion. An *in silco* digestion showed that only the 16S rRNA gene of *Kribbella alba* YIM 31075^T and *Kribbella koreensis* KACC 20250^T would be cut by *Ssf*I to generate two fragments of ~585 and 930 bp (data not shown). Figure 2.8 shows the resulting digestion pattern obtained for Q41.

Figure 2.8 Restriction analysis of the 16S rDNA of *Kribbella karoonensis* Q41^T.



Lanes: **M**, molecular size marker, λ DNA digested with *Pst*I (sizes marked in kb); **1**, empty lane; **2**, uncut Q41 16S rDNA (1.5 kb); **3**, Q41 *Mbo*I; **4**, Q41 *Vsp*I; **5**, Q41 *Sph*I; **6**, Q41 *Sna*BI; **7**, Q41 *Sal*I; **8**, Q41 *Age*I; **9**, empty lane.

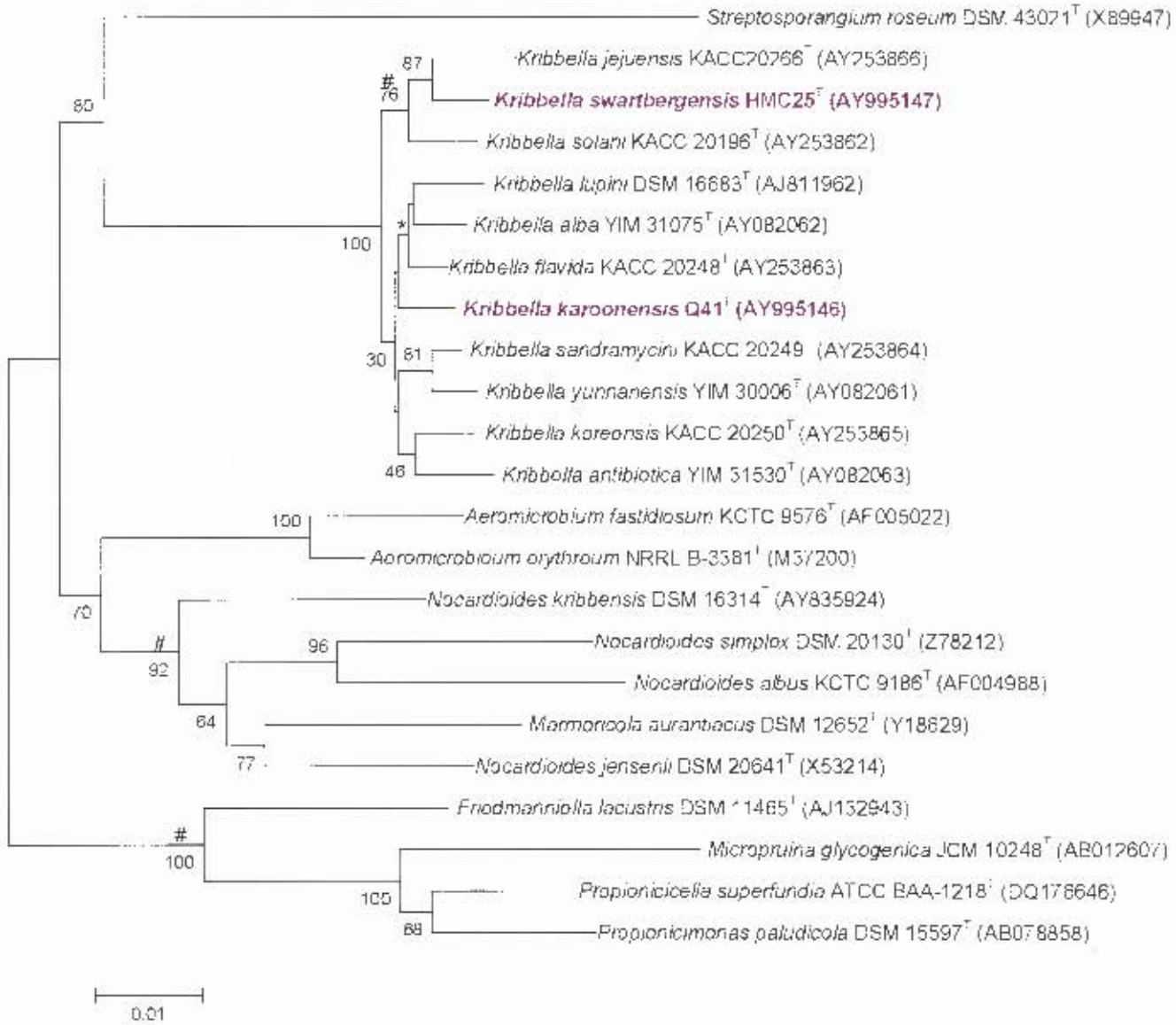
The phenetic characteristics of HMC25 and Q41 compared to the nine other validly published *Kribbella* type strains are presented in Table 2.12. Based on a large number of phenetic differences, both Q41 and HMC25 could be distinguished from the validly published *Kribbella* species. The 16S rRNA gene sequence similarity

between HMC25 and Q41 was 98.4%. Phylogenetic analysis revealed that HMC25 and Q41 are distinct species (Figure 2.9). The species description paper describing *Kribbella karoonensis* Q41^T (=DSM 17344^T) and *Kribbella swartbergensis* HMC25^T (=DSM 17345^T) was published in 2006 (Kirby *et al.*, 2006).

Morphologically, Q41 has pale cream to white aerial mycelium and cream to pale yellow branched vegetative mycelium on inorganic salts-starch agar (ISP 4). Hyphae fragment into rod-shaped elements in broth and on agar. Swelling at the apical tips of the hyphae to form bud-like structures may occur. Diffusible pigments are not produced on glycerol-asparagine agar (ISP 5) and melanin is not produced on peptone-yeast extract-iron agar (ISP 6) and tyrosine agar (ISP 7). Q41 degrades adenine (weakly), aesculin, arbutin, gelatin and hypoxanthine, while allantoin, cellulose and xanthine are not degraded. Glycerol, sodium succinate and sucrose are used as sole carbon sources. Growth on L(+) arabinose, adonitol, maltose, sodium acetate, sodium benzoate, sodium citrate and sodium lactate is weak at 21 days. Acid is produced from dulcitol, D(-) fructose, inulin, D(-) mannitol, D(+) raffinose, D(-) ribose, sodium citrate and trehalose. Acid is not produced from D(+) melibiose, D(+) melezitose and D(+) xylose. Q41 utilises DL-α-amino-*n*-butyric acid, DL-α-alanine, L-arginine, L-cysteine, gelatin, L-glutamic acid, L-glutamine, L-histidine, L-hydroxyproline, L-isoleucine, D-leucine L-leucine, KNO_3, L-proline, L-serine, $NaNO_3$ and L-valine as sole nitrogen sources, with growth on $(NH_4)_2HPO_4$, β-alanine, aspartic acid, L-methionine, L-phenylalanine and DL-β-phenylalanine weak at 21 days. Q41 grows from 20°C to 37°C, and at pH 5, 7 and 9. Q41 is susceptible to cephaloridine (100 µg/ml), penicillin G (10 IU/ml) and oleandomycin (100 µg/ml). A comparison of the antibiotic susceptibility profiles for all *Kribbella* type strains is presented in Table 2.13.

HMC25 produces white aerial mycelium and cream-coloured vegetative mycelium on inorganic salts-starch agar (ISP 4). Hyphae fragment into rod shaped elements in broth and on agar. Diffusible pigments are not produced on glycerol-asparagine agar (ISP 5) and melanin is not produced on peptone-yeast extract-iron agar (ISP 6) or tyrosine agar (ISP 7). Degrades adenine, aesculin, arbutin, gelatin and hypoxanthine, does not degrade xanthine and cellulose, and does not grow on allantoin-containing medium. Maltose, D(-) sorbitol and sucrose are used as sole carbon sources. Growth on L(+) arabinose, glycerol, sodium acetate, sodium citrate, sodium lactate and sodium succinate is weak at 21 days. HMC25 in unable to utilise adonitol, sodium benzoate and sodium oxalate as sole carbon sources. Acid is only weakly produced from D(+) melibiose and no acid is produced from dulcitol, D(-) fructose, inulin, D(-) mannitol, D(+) melezitose, D(+) raffinose, D(-) ribose, sodium citrate, trehalose and D(+) xylose. $(NH_4)_2HPO_4$, DL-α-amino-*n*-butyric acid, DL-α-alanine, L-arginine, gelatin, L-glutamic acid, L-glutamine, L-histidine, L-hydroxyproline, L-proline, $NaNO_3$, L-serine and L-valine are used as sole nitrogen sources. Growth on β-alanine, L-cysteine, L-isoleucine, D-leucine, L-leucine, L-methionine, L-phenylalanine, DL-β-phenylalanine and KNO_3 is weak at 21 days, with no growth on aspartic acid after 28 days. HMC25 grows from 20°C to 45°C, and at pH 7 and 9. HMC25 is the only *Kribbella* species known to grow at 45°C. HMC25 is resistant to oleandomycin (100 µg/ml) and penicillin G (10 IU/ml), but is susceptible to cephaloridine (100 µg/ml).

Figure 2.9 Unrooted 16S rRNA gene phylogenetic tree showing the relationship between HMC25, Q41 and other members of the family *Nocardioidaceae*. The tree was based on 1427 bp of sequence and was obtained using the neighbour-joining method. Bootstrap values are based on 1000 resampled datasets and only values above 40% are shown. Bar represents 0.01 nt substitutions per nt position. *Streptosporangium roseum* DSM 43021^{T} was set as the outgroup.



An asterisk (*) denotes the cluster that was conserved using the neighbour-joining, minimum evolution and maximum parsimony methods to construct the phylogenetic trees. A hash (#) denotes clusters that were conserved using the neighbour-joining and minimum evolution methods to construct the phylogenetic trees. Strains coded in purple were isolated from soil samples.

Table 2.12 Physiological tests that differentiate HMC25^T and Q41^T from the type strains of *Kribbella* species

Physiological characteristic	HMC25	Q41	1	2	3	4	5	6	7	8	9
Nitrate reduction	+	+	-	-	+[b]	-	-	-	-[b]	-	-
Degradation of:											
Casein	+	+		+[d]	+	-(+w)[d]	+	+	+	-	
Hypoxanthine	+	+		+	-	+	+	ND	-	+	
Tween 80	+	+w		ND	+	-	-[e]	ND	+	+	
L-Tyrosine	+	+		ND	ND	-	+	ND	ND	-	
Urea	NG	+		ND	+	+	+	-	+	-	
Hydrolysis of starch	+	+	+	+	-	-	+[d] (-)[ac]	-	+w(-)[ac]	-	+
Growth at:											
20°C	+	+		ND	+	ND	+	+	+	ND	
37°C	+	+		+[g]	+(-)[d]	+	+(-)[d]	+	+(-)[d]	-	
45°C	+	-		ND	-	-	-	-	-	-	
pH 5	+w	+		-[g]	+(-)[d]	-	-	-	+	-	
pH 7	+	+		ND	+	+	+	+	+	+	
pH 9	+	+		ND	+	-	+	+	+		
Growth in presence of NaCl at:											
2%	+	++	-	-	++	+	++	ND	++	++	++
3%	+	+w	-	-	-(+)[d]	-	-	+[a]	+	-	+w
4%	+w	+w	-	-	-	-	-	+[a]	+w	+	+w
5%	-	-	-	-	-	-	-	+[a]		+	-
Anaerobic growth on:											
ISP 9	-	-	-	-	-	-	+w	-	-	+w	-
ATCC 172	-	-	-	-	+w	+w	+w	+w	+w	+	-
Aerobic growth on (control):											
ISP 9	+	+	+w	+w	+w	+	++	+	+	++	+w
ATCC 172	++	++	+	+	++	++	++	+	++	++	+
Egg yolk medium											
Proteolysis	+	+		+	NG	+	+		+	+	
Lipolysis	-	-		-			-		-	+	
Lecithinase	+	+w		-		+	-		-	+	
Utilisation as sole carbon source:											
D(-) Fructose	+	+	+	+	-(+)[d]	ND	+	ND	+	ND	+
Inulin	-	-		ND	+	+	+	ND	+	-	
D(-) Lactose	+w	+	+	+	-(+)[ac]	+	+	ND	+(-)[ab]	+	+
D(+) Mannose	+w	+	+	+	-[cd](+)[ab]	-	+	+	+[ca](-)[ab]	+	+
D(+) Melibiose	+	-		ND	+	+	+	+	+	+	
D(+) Raffinose	+w	+	+	+	+(-)[ab]	+	+	+	+	+	+
L(+) Rhamnose	+w	+	+	+	-[cd](+)[a]	+	+	+	+	+	+
D(+) Xylose	+w	+	+	+	-	+	+	ND	+	+	+

1, *Kribbella alba* DSM 15500 (Li *et al*. 2006); **2**, *Kribbella antibiotica* DSM 15501^T (Li *et al*., 2004); **3**, *Kribbella flavida* KACC 20248^T (Park *et al*., 1999); **4**, *Kribbella jejuensis* KACC 20266^T (Song *et al*. 2004); **5**, *Kribbella koreensis* KACC 20250^T (Lee *et al*. 2000; Sohn *et al*., 2003); **6**, *Kribbella lupini* DSM 16683^T (Trujillo *et al*., 2006); **7**, *Kribbella sandramycini* KACC 20249 (Park et al., 1999); **8**, *Kribbella solani* KACC 20196^T (Song *et al*., 2004); **9**, *Kribbella yunnanensis* DSM 15499^T (Li *et al*., 2006). Results that differ from those in references are given in parentheses, with additional data taken from: a, Li *et al*., 2004; b, Li *et al*., 2006; c, Park *et al*., 1999; d, Trujillo *et al*., 2006; e, Song *et al*., 2004; f, Lee *et al*., 2000 and Sohn *et al*., 2003. Growth in the presence of NaCl and growth under anaerobic conditions were determined in this study. Symbols: ++, good growth; +, growth equal to positive control/growth; +w, weak reaction or growth weaker than positive control; -, no growth/reaction or comparable to negative control; ND, not determined; NG, no growth.

Table 2.13 Antibiotic susceptibility profiles of HMC25 and Q41 compared to other *Kribbella* species.

Antibiotic (µg/ml)	1	2	3	4	5	6	7	8	9	10	11
Carbenicillin, disodium salt (100)	++	++	++	++	+	++	+	+	+	++	+
Cefotaxime (10)	-	-	-	-	-	+	-	-	-	-	-
Cephaloridine (10)	+	-	-	+	++	-	+	-	-	-	-
Chloramphenicol (10)	++	+	+	++	++	++	++	+	++	++	++
Chloramphenicol (20)	+	+	-	+w	++	++	+w	+	++	++	++
Chloramphenicol (50)	-	-	-	-	++	+	-	+w	+	+	-
Cycloheximide (10)	++	++	+	++	++	+	++	+	++	++	++
Erythromycin (10)	+	+	++	-	+	+	+	+	+	+	++
Gentamicin sulphate (10)	++	-	-	-	+w	-	-	-	-	-	+w
Kanamycin (10)	+	++	+	-	-	+	-	-	-	++	-
Lincomycin (100)	++	+	+	-	++	+	++	+	+	+	++
Neomycin sulphate (10)	+w	-	-	-	-	+w	+w	+w	+	++	+
Neomycin sulphate (50)	-	-	-	-	-	+w	-	-	+w	++	-
Rifampicin (50)	-	++	++	+	+	+	++	+	++	++	++
Streptomycin sulphate (10)	+	+	-	+	++	+	++	+w	++	++	++
Streptomycin sulphate (100)	-	-	-	-	+w	-	-	-	-	++	++
Tobramycin sulphate (10)	-	-	-	-	-	-	-	+	-	+w	+w
Vancomycin hydrochloride (10)	-	-	-	+	-	++	++	-	++	++	++

1, *Kribbella swartbergensis* HMC25^T; **2,** *Kribbella karoonensis* Q41^T; **3,** *Kribbella alba* DSM 15500^T; **4,** *Kribbella antibiotica* DSM 15501^T; **5,** *Kribbella flavida* KACC 20248^T; **6,** *Kribbella jejuensis* KACC 20266^T; **7,** *Kribbella koreensis* KACC 20250^T; **8,** *Kribbella lupini* DSM 16683^T; **9,** *Kribbella sandramycini* KACC 20249^T ;**10,** *Kribbella solani* KACC 20196^T; **11,** *Kribbella yunnanensis* DSM 15499^T. Symbols: ++, good growth comparable to control; +, growth; +w, weak growth,; -, no growth. All *Kribbella* strains did not grow in the presence of 100 µg/ml ampicillin, sodium salt. All *Kribbella* strains were incubated at 30°C, except for *Kribbella antibiotica* which was incubated at room temperature ~22°C. All antibiotic susceptibility data was obtained in this study.

2.4.5 The characterisation of 13 species belonging to the Family *Streptomycetaceae*

Thirteen *Streptomyces* strains were selected for characterisation based either on their morphology or antimicrobial activity. Ten of these were from sediment samples and three were from terrestrial samples. Although a number of *Streptomyces* were initially isolated from plants, they grew very poorly on ISP media and therefore could not be characterised. As expected, streptomycetes were the dominant species isolated from the soil and sediment samples. All streptomycete isolates were Gram-positive, catalase positive and had LL-DAP in their cell walls.

2.4.5.1 Grey series – Strains Berg1C, Berg2S, Berg4Y, Muiz4Y and Zand4Y

The grey series includes species belonging to 11 numerically defined species clusters, namely, the *Streptomyces albidoflavus*, *Streptomyces atroolivaceus*, *Streptomyces chromofuscus*, *Streptomyces cyaneus*, *Streptomyces diastaticus*, *Streptomyces exfoliatus*, *Streptomyces griseoruber*, *Streptomyces lydicus*, *Streptomyces olivaceoviridis*, *Streptomyces rochei* and *Streptomyces violaceusniger* clusters (Williams *et al.*, 1983).

Five *Streptomyces* isolates produce a grey spore mass when grown on inorganic salts-starch agar (ISP 4) and were therefore classified as belonging to the grey series. Three of these species were obtained from the Berg River sediment sample, one was from the Zandvlei sediment sample and the fifth was isolated from the Muizenberg beach sediment sample. A phylogenetic tree based on 1363 bp of 16S rRNA gene sequence was constructed comparing these five grey series *Streptomyces* strains to the *Streptomyces* type strains identified from the BLAST results (Figure 2.10).

Berg2S was isolated on starch-casein agar from a heat treated sediment sample collected from the Berg River. A standard nucleotide-nucleotide BLAST search of the Genbank database of the partial 16S rRNA gene showed that Berg2S was most similar to *Streptomyces griseoaurantiacus* NBRC 15440^T and *Streptomyces jietasisiensis* JCM 12279^T, with a similarity of 100% and 99% over 1428 bp, respectively. Berg2S produces pinkish-brown substrate mycelium and dark grey aerial mycelium on inorganic salts-starch agar (ISP 4). The mature spore chains are *Retinaculiaperti*-type and form hooks with one or two turns. The spore surface ornamentation is smooth (Figure 2.12 A). A red/orange diffusible pigment is produced on most media, with a yellow diffusible pigment produced on glycerol-asparagine agar (ISP 5). Berg2S degrades casein, gelatin and L-tyrosine, but does not degrade allantoin and urea. H_2S is produced and nitrate is reduced. Pectin is not hydrolysed. Proteolytic activity occurs on egg yolk medium, while lecithinase and lipase activities are not detected. Grows well in the presence of 0.3% 2-phenylethanol and 0.0001% crystal violet, with weak growth in the presence of 0.01% NaN_3 and 0.1% phenol. Berg2S utilises glycerol and L(+) rhamnose as sole carbon sources, with weak growth on *meso*-inositol, maltose, D(-) sorbitol and trehalose at 21 days. D(+) Melibiose, D(+) melezitose and L(-) sorbose are not used. The whole cell sugars are ribose and galactose. From Table 2.14 it can be seen that Berg2S can be distinguished from *S. griseoaurantiacus* and *S. jietaisiensis.* There are six phenetic differences between Berg2S and *S. griseoaurantiacus* including the ability of Berg2S to grow at 8% NaCl and spore chain morphology, while there are eight differences between Berg2S and *S. jietaisiensis* including the colour of the substrate and aerial mycelium, the production of diffusible pigments and the inability of Berg2S to degrade adenine and xylan.

Berg4Y was isolated on YEME from an untreated sediment sample collected from the banks of the Berg River. A standard nucleotide-nucleotide BLAST search of the Genbank database using the partial 16S rRNA gene sequence of Berg4Y over 1366 bp, found that it was 100% and 99% homologous to *S. griseoaurantiacus* and *S. jietasisiensis*, respectively. Phylogenetic analysis found that Berg4Y clustered with Berg2S, *S. griseoaurantiacus* and *S. jietasisiensis* (Figure 2.10). A pairwise alignment showed that the 16S rRNA gene sequence similarity between Berg2S and Berg4Y over 1366 bp was 100%. Berg4Y produces dark grey aerial mycelium on most media and the colonies have a coral/sponge-like appearance. It produces a creamy-brown polysaccharide-like substance on complex media with the dark grey spores borne on sparse aerial mycelium around the edges of the colony. The mature spore chains are long and straight (*Rectiflexibiles*) with smooth ovoid spores (Figure 2.12 B, C). Berg4Y does not hydrolyse pectin. Proteolytic, lipolytic and lecithinase activities do not occur on egg yolk medium. H_2S is produced and nitrate is reduced. Casein, gelatin and L-tyrosine are degraded. Allantoin and urea are not degraded. Berg4Y grows in the presence of 0.3% 2-phenylethanol, with weak growth in the presence of 0.0001% crystal violet. Growth is inhibited by 0.01% NaN_3 and 0.1% phenol. Glycerol, *meso*-inositol, maltose, D(+) melibiose (weakly), L(+) rhamnose and trehalose are

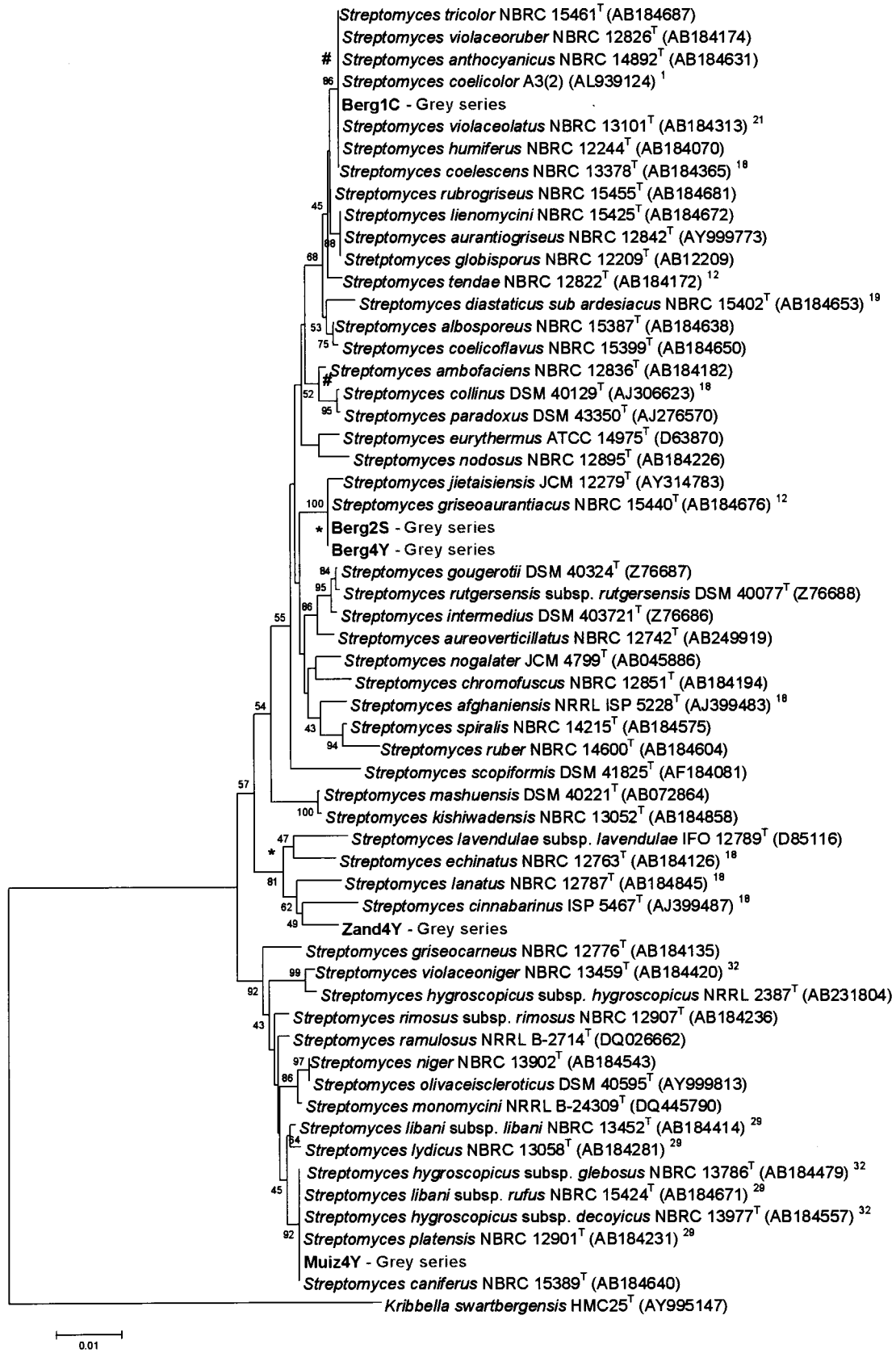
used as sole carbon sources. D(+) Melezitose and L(-)sorbose are not used. Based on the phenetic characteristics shown in Table 2.14 Berg4Y can be differentiated from its closest phylogenetic neighbours. There are seven phenetic differences between Berg4Y and *S. griseoaurantiacus* including the degradation of hypoxanthine, a NaCl tolerance of 7% and use of D(-) sorbitol as a sole carbon source by Berg4Y. Similarly, there are six physiological characteristics that differ between Berg4Y and *S. jietaisiensis.* The production of a yellow diffusible pigment by Berg4Y, its ability to degrade hypoxanthine and its inability to degrade adenine distinguish Berg4Y from *S. jietaisiensis.*

Although the phenetic characteristics of Berg2S and Berg4Y are similar, there are some clear differences including spore chain morphology, the inability of Berg2S to degrade hypoxanthine and xylan, as well as growth temperature range. Morphologically these two strains are distinct. Based on phenetic characteristics neither Berg2S nor Berg4Y can be classified as strains of *S. griseoaurantiacus* or *S. jietaisiensis*, but DDH would be required to confirm that they are distinct genomospecies.

Zand4Y was isolated on YEME from a heat treated sediment sample collected from Zandvlei Nature Reserve. Phylogenetic analysis found that Zand4Y is most closely related to *Streptomyces cinnabarinus* ISP 5467^T and *Streptomyces lanatus* NBRC 12787^T (Figure 2.10), and a comparison of phenetic characteristics is presented in Table 2.14. Zand4Y produces grey substrate and blue-grey aerial mycelium on inorganic salts-starch agar (ISP 4). The spore chain morphology is *Spirales* and the spores are spiny and ovoid (Figure 2.12 D). Zand4Y degrades casein, gelatin and L-tyrosine. Pectin is not hydrolysed. Protease and lecithinase activities occur on egg yolk medium. Produces H_2S but nitrate is not reduced. Allantoin is not degraded and no growth occurs in the presence of urea. Zand4Y grows weakly in the presence of 0.3% 2-phenylethanol and 0.0001% crystal violet, while growth is inhibited by 0.01% NaN_3 and 0.1% phenol. D(+) Galactose, glycerol, *meso*-inositol, D(-) lactose, D(-) mannitol, D(+) mannose, maltose, L(+) rhamnose, D(-) ribose and trehalose are used as sole carbon sources. There are ten phenetic differences between Zand4Y and *S. cinnabarinus*, including the inability of Zand4Y to produce melanin pigments, the morphology and ornamentation of the spore chains, as well as the colour of the substrate mycelium. Although the amount of phenetic data available for *S. lanatus* is limited, there are five differences between *S. lanatus* and Zand4Y, including the colour of the spore mass and the inability of Zand4Y to produce melanin. From Figure 2.10 and Table 2.14, Zand4Y can be distinguished as a unique species.

Berg1C was isolated on CZ agar from a heat treated sediment sample collected from the banks of the Berg River. BLAST analysis over 1367 bp of 16S rRNA gene sequence found that Berg1C was most similar to *Streptomyces violaceolatus* NBRC 13101^T and *Streptomyces humiferus* NBRC 12244^T (100% homology) and 99% homologous to *Streptomyces violaceoruber* NBRC 12826^T. Phylogenetic analysis showed that Berg1C belonged to a clade which contained seven streptomycete species with almost identical 16S rRNA gene sequences (Figure 2.10). A phenetic comparison between these strains is presented in Table 2.15. On inorganic salts-starch agar (ISP 4) Berg1C produces dark grey aerial mycelium and purple substrate mycelium. A purple diffusible pigment is produced on glycerol-asparagine agar (ISP 5). The spore chain morphology is *Rectiflexibiles* with relatively short chains of long, rectangular spores.

Figure 2.10 Unrooted 16S rRNA gene phylogenetic tree showing the relationship between the five grey series *Streptomyces* isolates and their closest phylogenetic neighbours. The tree was based on 1363 bp of conserved sequence and was obtained using the neighbour-joining method. Bootstrap values are based on 1000 resamplings and only values greater than 40% are shown. Bar represents 0.01 nt substitutions per nt position. *Kribbella swartbergensis* HMC25^T was set as the outgroup.



Asterisks (*) denote clusters that were conserved using the neighbour-joining, minimum evolution and maximum parsimony methods to construct the phylogenetic trees. A hash (#) denotes clades that were conserved between trees constructed from the neighbour-joining and minimum evolution methods only. Strains coded in blue were isolated from aquatic sediment samples. Superscript numbers refer to the numerically defined species clusters included in the grey series (Williams *et al.*, 1983).

Table 2.14 Phenetic characteristics that distinguish Berg2S, Berg4Y and Zand4Y from the type strains of *S. cinnabarinus*, *S. griseoaurantiacus*, *S. jietaisiensis* and *S. lanatus*.

Phenetic characteristic	Berg2S	Berg4Y	Zand4Y	*Streptomyces cinnabarinus* [a] ISP 5467^T	*Streptomyces griseoaurantiacus* [b] NBRC 15440^T	*Streptomyces jietaisiensis* [b] JCM 12279^T	*Streptomyces lanatus* [c] NBRC 12787^T
Colour of substrate mycelium	Pinkish-brown	Red to pinkish-brown	Grey	Greyish-red to reddish-brown	Reddish-brown	Orange-pink	ND
Colour of aerial mycelium	Dark grey	Grey (sparse)	Blue-grey	White (sparse)	Brown	Pale grey	ND
Colour of spore mass	Dark grey	Dark grey	Grey	Red	Smoky grey	Smoky grey	Blue
Spore chain morphology	RP	RF	SP	RF	SP	RF	SP
Spore chain ornamentation	Smooth	Smooth	Spiny	Smooth	Smooth	Smooth	Spiny
Production of DP (ISP 5)	Yellow	Yellow	Greenish-yellow	Red to lavender	-	-	-
pH sensitivity of pigments	+ (sub, DP)	+ (sub, DP)	+ (sub, DP)	ND	-	-	-
Melanin (ISP 6)	-	-	-	+	-	-	+
Melanin (ISP 7)	-	-	-	+	-	-	+
Degradation of:							
Adenine	-	-	+	+	-	+	+
Guanine	-	-	+	+w	-	-	+
Hypoxanthine	-	+	+	+	-	-	+
Starch	+w	-	-	+	ND	ND	ND
Tween 80	+	+	+	+	+	+	+
Xanthine	-	+w	+	-	-	-	ND
Xylan	-	+	ND	-	+	+	ND
Carbon source:							
L(+) Arabinose	+	+	ND	v	+	+	+
D(-) Fructose	+w	+	ND	+			v
D(+) Raffinose	+w	+w	+	+	+	+	+
D(-) Sorbitol	+w	+	ND	ND	-	+	ND
NaCl tolerance (%)	8	7	8	ND	<5	7	ND
Growth temperature (°C)	4-45	RT-37	4-37	ND	<45	10-40	ND

Data from: a, Shirling & Gottlieb, 1969; b, He *et al.*, 2005; c, Shirling & Gottlieb, 1968a. Symbols: +, good growth/positive reaction; +w, weak growth/reaction -, no growth/negative reaction; ND, not determined; sub, substrate mycelium; DP, diffusible pigment; RT- room temperature (22°C); RP, *Retinaculiaperti*; RF, *Rectiflexibiles*; SP, *Spirales*.

The spore surface ornamentation is smooth (Figure 2.12 E). Berg1C degrades adenine, casein, gelatin, guanine, hypoxanthine, starch, Tween 80, L-tyrosine and xylan, with degradation of adenine and xanthine weak at 21 days. Allantoin, cellulose and urea are not degraded. Pectin is hydrolysed. Produces H_2S and reduces nitrate. Berg1C is positive for proteolysis and lecithinase activities on egg yolk medium. Utilises glycerol, maltose, D(+) melibiose, D(+) melezitose (weakly) and trehalose (weakly) as sole carbon sources. Growth occurs in the presence of 0.3% 2-phenylethanol, 0.1% phenol and 0.0001% crystal violet, with weak growth in presence of 0.01% NaN_3. The whole cell sugars are ribose and glucose.

Based on the limited number of phenetic characteristics (Table 2.15), Berg1C is the most similar to *S. violaceoruber*. The only phenetic differences between these two strains are spore chain morphology and the inability of Berg1C to utilise D-mannitol. However, as some strains of *S. violaceoruber* are reported to have *Rectiflexibiles*-type spore chains (Shirling & Gottlieb, 1968a), it is possible that Berg1C is a strain of *S. violaceoruber*.

Table 2.15 Comparison of Berg1C physiological results with the seven phylogenetically related strains.

Phenetic characteristic	1	2	3	4	5	6	7	8
Colour of substrate mycelium	Purple	Blue	Grey to blackish-purple	Olive brown to dark brown	ND	Red to blue	Blue to red	Blue to violet
Colour of spore mass	Dark grey	Grey	Grey	Yellow	Grey	Grey	Grey	Grey
Spore chain morphology	RF	RP	SP	RF	SP	SP	SP	SP
Spore chain ornamentation	Smooth	Smooth	Smooth	Smooth	Smooth	ND	Smooth	Smooth
Production of DP (ISP 5)	Purple	Blue	Red to blue	Reddish-brown #	Red-orange	Blue	Blue to red	Violet
pH sensitivity of pigments	+ (sub, DP)	ND	+ (sub, DP)	+ (sub, DP) #	+ (sub, DP)	ND	+ (sub, DP)	+ (sub, DP)
Carbon source:								
L(+) Arabinose	+	+	+	+	+	*	+	+
D(-) Fructose	+	+	+	+	+	*	+	+
meso-Inositol	+	+	+	+	-	*	+	+
D(-) Mannitol	-	ND	+	+	+	*	+	+
D(+) Raffinose	-	+	d	+	-	*	+	ND
L(+) Rhamnose	+	+	+	+	-	*	+	+

1, Berg1C; **2**, *S. anthocyanicus* [a] NBRC 14892^T; **3**, *S. coelescens* [b] NBRC 13378^T; **4**, *S. coelicolor* [c] NBRC 12854^T; **5**, *S. humiferus* [d] NBRC 12244^T; **6**, *S. tricolor* [e] LMG 20328^T; **7**, *S. violaceolatus* [f] NBRC 13101^T; **8**, *S. violaceoruber* [g] NBRC 12826^T. Data from the reference strains obtained from: a, Williams *et al.*, 1989; b, Shirling & Gottlieb, 1972; c, Shirling & Gottlieb, 1968b; d, Goodfellow *et al.*, 1986a, e, Lanoot *et al.*, 2004; f, Shirling & Gottlieb, 1969; g, Shirling & Gottlieb, 1968a. *According to Williams *et al.* (1989) carbon source utilisation and phenetic data is not available for this species. # colour determination for *S. coelicolor* was on oatmeal agar (ISP 3). Symbols: +, growth/positive reaction; -, no growth; d, growth doubtful; ND, not determined; sub, substrate mycelium; DP, diffusible pigment; RP, *Retinaculaperti*; RF, *Rectiflexibiles*; SP, *Spirales*.

Muiz4Y was isolated on YEME from an untreated sediment sample collected from Muizenberg Beach. A BLAST search over 1363 bp of 16S rRNA gene sequence showed the homology between Muiz4Y and *Streptomyces caniferus* NBRC 15389^T, *Streptomyces platensis* NBRC 12901^T, *Streptomyces libani* subsp. *rufus* NBRC 15424^T

and *Streptomyces hygroscopicus* subsp. *glebosus* NBRC 13786^T to be 99%. Phylogenetic analysis (Figure 2.10) showed that Muiz4Y clustered with the type strains of *S. caniferus* and *S. platensis*. As no phenetic data for *S. caniferus* are available, a physiological comparison could only be made to *S. platensis* (Table 2.16).

Table 2.16 Physiological comparison of Muiz4Y and *S. platensis*.

Phenetic characteristic	Muiz4Y	*Streptomyces platensis* NBRC 12901^T
Colour of substrate mycelium	Grey and beige mottled	Grey-yellow
Colour of spore mass	Dark grey	Grey
Spore chain morphology	RF	SP
Spore chain ornamentation	Smooth	Smooth
Production of DP	None	Red to yellow (trace)
pH sensitivity of pigments	-	+ (sub)
Melanin (ISP 6)	-	-
Melanin (ISP 7)	-	-
Reduction of nitrate	+w	-
H_2S production	-	-
Degradation of:		
Guanine	-	ND
Xylan	-	-
Carbon source		
L(+) Arabinose	+	d
D(-) Fructose	+	+
meso-Inositol	+	+
D(+) Raffinose	+	+
L(+) Rhamnose	-	-
Trehalose	+	ND
NaCl tolerance (%)	10	<7
Growth temperature (°C)	20-37	ND

Data for *S. platensis* from Williams *et al.*, 1983. Symbols: +, growth; +w, weak growth; d, growth doubtful; -, no growth/negative reaction; DP, diffusible pigment; ND, not determined; sub, substrate mycelium; RF, *Rectiflexibiles*; SP, *Spirales*.

On most media Muiz4Y grows as a few large, single colonies which have a wrinkled, leathery appearance, with sparse black or grey aerial mycelium being produced. On inorganic salts-starch agar (ISP 4) the aerial mycelia are black, the spore mass is dark grey and the substrate mycelium has mottled grey and beige patches. Spore chain morphology is *Rectiflexibiles* and the spore surface is smooth (Figure 2.12 F). Diffusible pigments are not produced on glycerol-asparagine agar (ISP 5). Muiz4Y is able to degrade adenine, casein, gelatin (weakly), hypoxanthine, starch, Tween 80 and L-tyrosine, but is unable to degrade allantoin, cellulose, guanine, urea, xanthine and xylan. Grows from pH 5 to 9. Muiz4Y is unable to hydrolyse pectin, does not produce H_2S and can only weakly reduce nitrate. Muiz4Y is positive for proteolytic and lecithinase activities on egg yolk medium but negative for lipolysis. Utilises glycerol, maltose, D(+) melibiose, D(+) melezitose, D(-) sorbitol and trehalose as sole carbon sources, but is unable to use L(-) sorbose. Can grow in the presence of 0.3% 2-phenylethanol and 0.0001% crystal violet, but is inhibited by 0.01% NaN_3 and 0.1% phenol. Whole cell sugars are ribose and galactose. From Table 2.16 it can be seen that there are only three differences between Muiz4Y and

S. platensis; spore chain morphology, production of diffusible pigments and the ability of Muiz4Y to grow at 10% NaCl. As Muiz4Y can not be identified as a distinct species from phylogenetic analysis, further physiological testing would be required for it to be recognised as a unique species.

2.4.5.2 Red series – Strains NX03U2 and PHelU5

NX03U2 and PHelU5 are characterised by the formation of a pink to reddish-brown spore mass on inorganic salts-starch agar (ISP 4) and were thus identified as belonging to the red series. According to *Bergey's Manual of Systematic Bacteriology* (1989) the numerically defined species clusters that are included in the red series are the *Streptomyces cyaneus, Streptomyces diastaticus, Streptomyces exfoliatus, Streptomyces fulvissimus, Streptomyces griseoviridis* and *Streptomyces lavendulae* species clusters (Williams *et al.*, 1983). Species included in the red series are *Streptomyces albosporeus*, *Streptomyces erythrogriseus* and *Streptomyces ruber*, amongst others (Williams *et al.*, 1989).

PHelU5 was isolated on PV8 agar from an air dried soil sample collected from the banks of the Gamka River in Die Hel. BLAST analysis identified PHelU5 as being most closely related (99% homology over 1352 bp) to *Streptomyces asterosporus* NBRC 15872^T, *Streptomyces carpinensis* NBRC 14214^T and *Streptomyces purpurascens* NBRC 1307^T. Figure 2.11 shows the neighbour-joining phylogenetic tree comparing 1354 bp of 16S rRNA gene sequence. PHelU5 was found to be most closely related to *S. carpinensis* and a comparison of the phenetic characteristics is shown in Table 2.17. PHelU5 produces reddish-brown substrate mycelium and a sparse, pale pink spore mass on inorganic salts-starch agar (ISP 4). Due to the limited formation of aerial mycelium, the spore chain ornamentation could not be determined. PHelU5 degrades adenine, casein, gelatin, Tween 80 and L-tyrosine, and is unable to degrade allantoin, cellulose, guanine, starch, urea, xanthine and xylan. PHelU5 grows at pH 5, 7 and 9. Grows from 20°C to 45°C, with weak growth at 4°C. PHelU5 can utilise D(+) mannose, maltose and D(-) ribose as sole carbon sources. Growth occurs in the presence of 0.3% 2-phenylethanol, 0.0001% crystal violet and 0.1% phenol, with no growth detected in the presence of 0.01% NaN_3. There are seven phenetic differences between PHelU5 and *S. carpinensis* (Table 2.17), including the colour of the substrate mycelium, the ability of PHelU5 to use *meso*-inositol and D(-) lactose as sole carbon sources, and the production of melanin on peptone-yeast extract-iron agar (ISP 6) and tyrosine agar (ISP 7). Based on the physiological (Table 2.17) and phylogenetic analysis (Figure 2.11), PHelU5 can be distinguished from its closest phylogenetic neighbours.

NX03U2 was isolated on PV8 agar from a heat treated sediment sample collected from the Hermanus Lagoon. A standard nucleotide-nucleotide BLAST search of the Genbank database found NX03U2 to be most similar to *Streptomyces alboniger* NBRC 12738^T, *Streptomyces novaecaesareae* NBRC 13368^T and *Streptomyces resistomycificus* NBRC 12814^T (99% homology over 1361 bp). From Figure 2.11 it can be seen that NX03U2 is most closely related to *Streptomyces aureocirculatus* and *S. novaecaesareae*. NX03U2 forms violet substrate mycelium and pink aerial mycelium on inorganic salts-starch agar (ISP 4). The spore mass is pink and gives the mature colonies a chalky appearance. In broth (YEME and CZ), NX03U2 forms a mesh of pink filaments and produces purple spores on the surface of the culture. The spore chain morphology is *Spirales* with long, spiny spores (Figure 2.12 G, H). NX03U2 degrades adenine, casein, gelatin, Tween 80 and L-tyrosine, but is unable to

degrade cellulose, guanine, starch, xanthine and xylan. NX03U2 utilises D(+) mannose, maltose and D(-) ribose as sole carbon sources. NX03U2 grows well at pH 7 and 9, with weak growth at pH 5. Grows from 20°C to 45°C, with weak growth at 4°C at 14 days. NX03U2 does not grow in the presence of allantoin and urea. Growth is weak in the presence of 0.3% 2-phenylethanol and 0.0001% crystal violet, and is inhibited by 0.01% NaN_3 and 1% phenol.

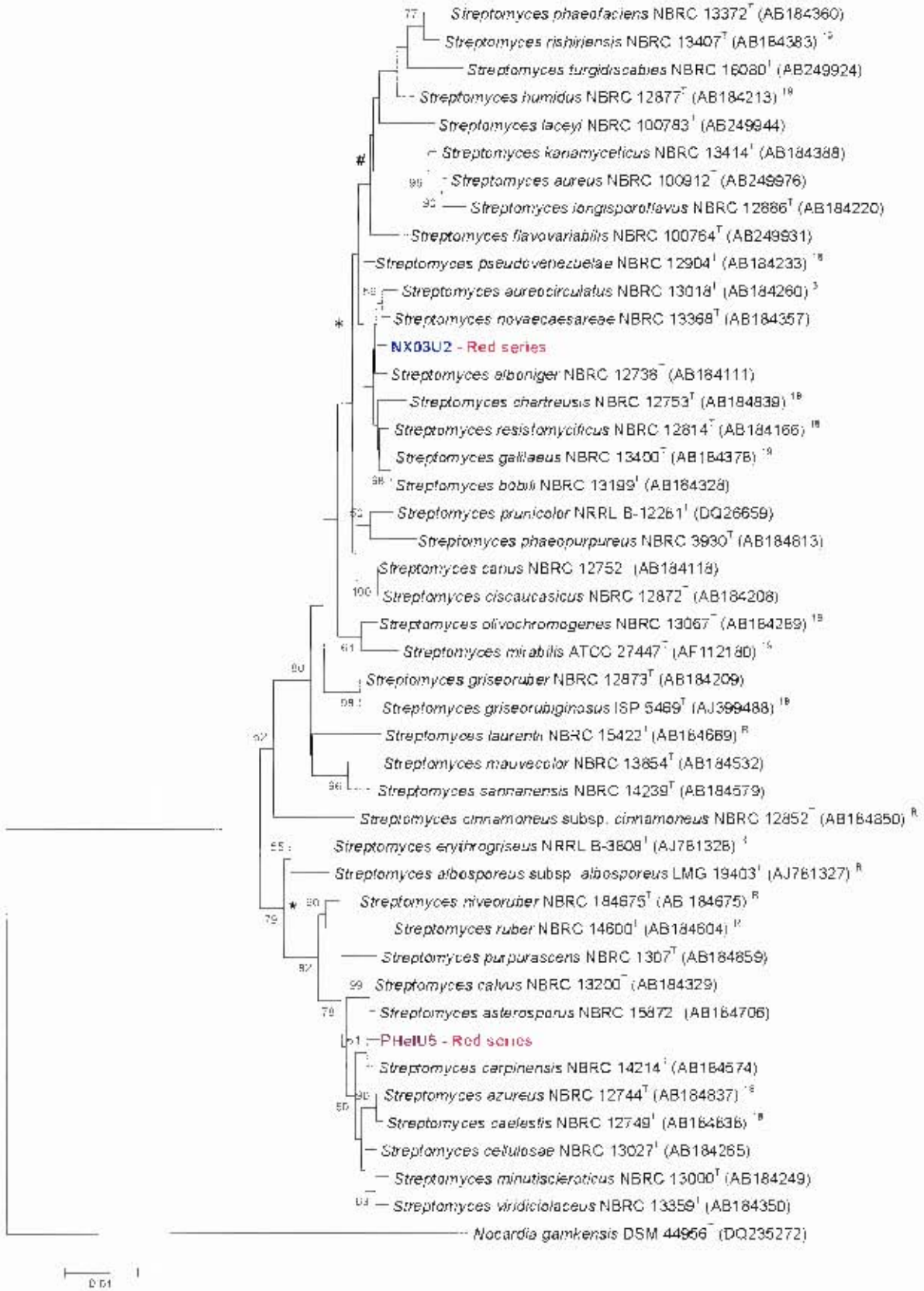
Table 2.17 Phenetic characteristics that distinguish NX03U2 and PHelU5 from their closest phylogenetic neighbours.

Phenetic characteristic	NX03U2	PHelU5	*Streptomyces aureocirculatus* NBRC 13018^T	*Streptomyces carpinensis* ATCC 27116^T	*Streptomyces novaecaesareae* NBRC 13368^T
Colour of substrate mycelium	Violet	Reddish-brown	Yellow to greyish-yellow	Black	Red-orange
Colour of spore mass	Pink	Pale pink	White	Dark grey	Sparse
Spore chain morphology	SP	SP	RF	SP	RP
Spore chain ornamentation	Spiny	ND	Smooth	Smooth	ND
Production of DP	Reddish-brown	Light brown	Yellow	ND	-
pH sensitivity of pigments	+ DP	+	-	-	Sub+
Melanin (ISP 6)	-	+	-	-	-
Melanin (ISP 7)	+	+	-	-	-
Production of H_2S	-	+	+	+	+
Nitrate reduction	+	-	ND	-	+
Utilization as sole carbon source:					
D(+) Galactose	++	++	v	+	-
Glycerol	++	++	+		
meso-Inositol	+	++	+	-	-
D(-) Lactose	+w	++	ND	-	-
D(-) Mannitol	++	+	+	+	+
D(+) Raffinose	+	+w	v	+	+
L(+) Rhamnose	-	++	v	+	-
Trehalose	++	+	ND	+	+
NaCl tolerance (%)	9	7	ND	7	<4

Data for strains were obtained from Williams *et al.*, 1983, 1989, except for *S. carpinensis*, for which information was obtained from Goodfellow *et al.*, 1986b. Symbols: ++, growth better than positive control; +, growth comparable to positive control or positive reaction; +w, growth less than positive control; -, no growth/negative reaction; DP, diffusible pigment; sub, substrate mycelium pigment; ND, not determined; v, variable results ; RP, *Retinaculiaperti*; RF, *Rectiflexibiles*; SP, *Spirales*

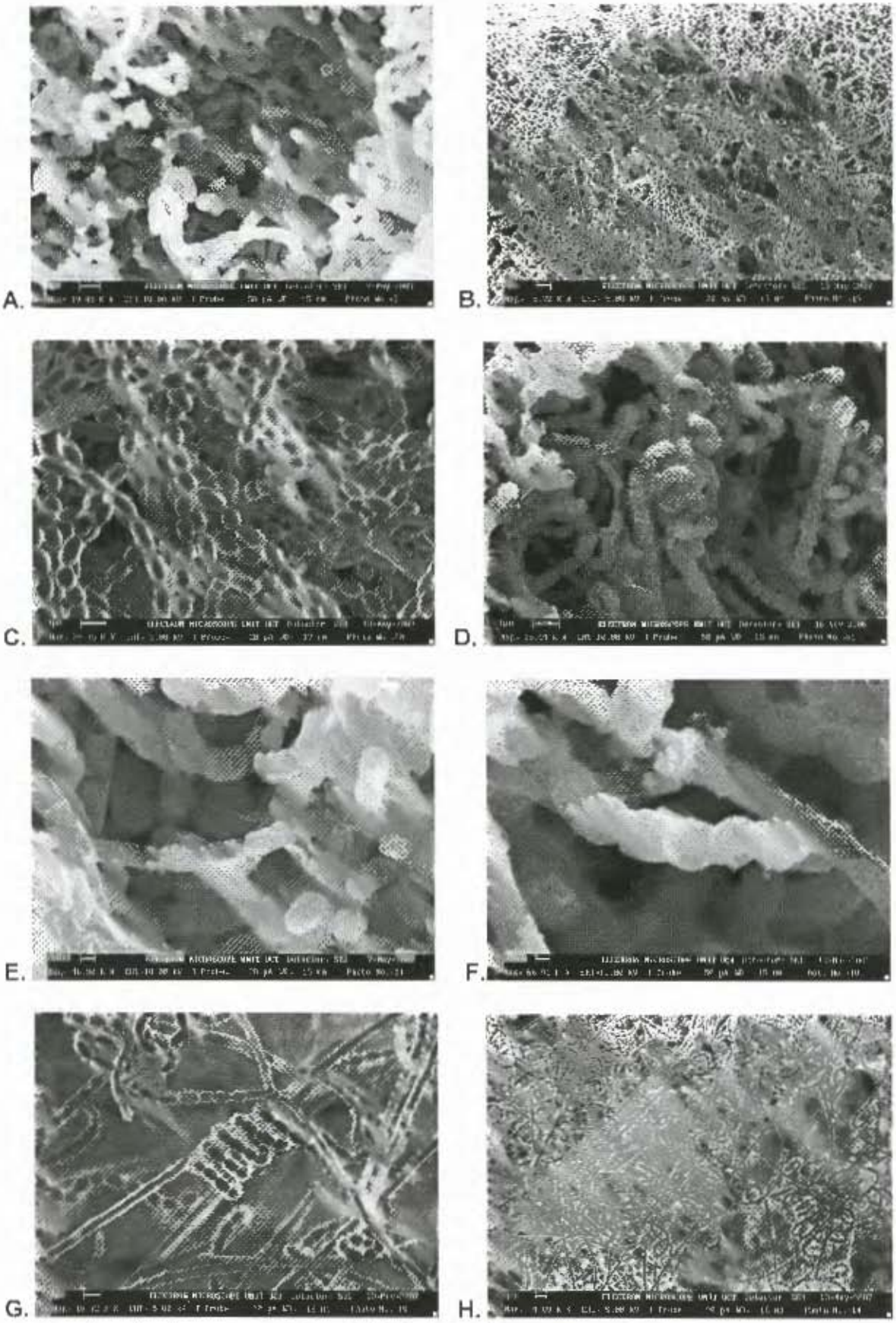
From the physiological features reported in Table 2.17, it can be seen that the morphological features of NX03U2 of pink spore mass, violet substrate mycelium and *Spirales*-type spore chains differentiate it from *S. aureocirculatus* and *S. novaecaesareae*. There are eight phenetic features that differ between NX03U2 and *S. aureocirculatus*, including the production of a pH sensitive diffusible pigment and melanin production on tyrosine agar (ISP 7) by NX03U2. Similarly, there are nine differences between NX03U2 and *S. novaecaesareae*, which include the ability of NX03U2 to grow at 9% NaCl and its utilisation of D(+) galactose and *meso*-inositol as sole carbon sources. Based on the phenetic and phylogenetic characterisation, NX03U2 can be distinguished from its closest phylogenetic neighbours.

Figure 2.11 Unrooted 16S rRNA gene phylogenetic tree showing the relationship between the two red series *Streptomyces* isolates, NX03U2 and PHelU5, and their closest phylogenetic neighbours. The tree was based on 1354 bp of conserved 16S rDNA sequence and was obtained using the neighbour-joining method. Bootstrap values are based on 1000 resamplings and values above 50% are shown. Bar represents 0.01 nt substitutions per nt site. *Nocardia gamkensis* DSM 44956^{T} was set as the outgroup.



Asterisks (*) denote clusters that were conserved using the neighbour-joining, minimum evolution and maximum parsimony methods to construct the phylogenetic trees. A hash (#) denotes the clade that was conserved between trees constructed from the neighbour-joining and minimum evolution methods only. Strains coded in purple were isolated from sediment samples, while strains coded in blue were isolated from aquatic sediment samples. Superscript numbers refer to the numerically defined species clusters included in the red series (Williams *et al.*, 1983). R. are strains that are included in the red series according to Williams *et al.*, 1989.

Figure 2.12 Scanning electron micrographs of *Streptomyces* species.



All isolates were grown on inorganic salts-starch agar (ISP 4) at 30°C for 14 days. Micrographs B, C, G & H were obtained by cryo-SEM. **A**, *Streptomyces* species Berg25 forms *Retinaculiaperti* spore chains with smooth spores; **B & C**, *Streptomyces* strain Berg4Y the long *Rectiflexibiles* spore chains with ovoid, smooth spores are visible; **D**, the ovoid spiny spores of *Streptomyces* Zand4Y are borne on *Spirales* spore chains; **E**, *Streptomyces* species Berg1C forms long *Rectiflexibiles* chains of smooth spores; **F**, *Streptomyces* strain Mu04Y has *Rectiflexibiles* type spore chains with smooth spores; **G & H**, the immature spore chains of *Streptomyces* species NX03U2 are long and thin, the mature chains are *Spirales* and the rectangular spores are spiny.

2.4.5.3 Blue series – Strains Riet1C, Zand2Y and Zand8Y

Based on the production of a blue aerial spore mass on inorganic salts-starch agar (ISP 4) strains Riet1C, Zand2Y and Zand8Y were classified as belonging to the blue series. Species belonging to the numerically defined *S. cyaneus* cluster are included in the blue series (Williams *et al.*, 1983). Other species that are included in the blue series are *Streptomyces inusitatus, Streptomyces ipomoeae* and *Streptomyces lomondensis* (Williams *et al.*, 1989).

A phylogenetic tree was constructed based on 1365 bp of conserved 16S rRNA gene sequence (Figure 2.13). Riet1C was found to be most closely related to *Streptomyces mirabilis* ATCC 27447^T and *Streptomyces olivochromogenes* NBRC 13067^T. Zand2Y and Zand8Y clustered with Zand9Y (classified as belonging to the green series), *Streptomyces viridochromogenes* subsp. *komabensis* NBRC 13859^T, *Streptomyces achromogenes* subsp. *tomaymyceticus* NBRC 13820^T and *S. cyaneus*.

Zand2Y was isolated on YEME from an untreated sediment sample collected from Zandvlei Nature Reserve. A standard nucleotide-nucleotide BLAST search using the partial 16S rRNA gene sequence for Zand2Y (over 1408 bp) showed that it is most closely related to *Streptomyces cyaneus* NBRC 13346^T and *Streptomyces coeruleorubidus* NBRC 12761^T (99% homology). Zand2Y produces dark blue substrate mycelium on inorganic salts-starch agar (ISP 4). A dark blue diffusible pigment is produced on many media. Growth is generally weak, with limited formation of aerial mycelium. This prevented the determination of spore chain morphology. Colonies generally have a wrinkled, leathery appearance. Weak growth occurs on ISP 9 with glucose as a sole carbon source. Optimal growth occurred on YEME-glycerol (glucose substituted with glycerol) at 20°C. D(-) Fructose, glycerol, maltose, D(+) melibiose, D(+) melezitose, D(-) sorbitol (weakly) and trehalose are used as sole carbon sources. L(-) Sorbose is not used. Protease and lecithinase activities occur on egg yolk medium. Degrades adenine, casein, gelatin, hypoxanthine, starch and Tween 80. No growth on allantoin, pectin, L-tyrosine, urea and xylan-containing media. As the growth was poor on Bennett's Medium, modified Bennett's Medium (glycerol) was used to determine growth parameters. Growth occurs at pH 6, 7 and 8, and at 20°C, with weak growth at 30°C. Growth is weak in the presence of 0.1% 2-phenylethanol and 0.0001% crystal violet, with no growth in presence of 0.01% NaN_3 and 0.1% phenol. H_2S is not produced. Nitrate is reduced.

From Table 2.18 it can be seen that there are four differences between Zand2Y and *S. cyaneus,* including the inability of Zand2Y to degrade guanine and the lack of melanin pigments on peptone-yeast extract-iron agar (ISP 6). The variability in the reported phenetic characteristics of *S. cyaneus* species prohibits a full comparison. However, morphologically, Zand2Y is an atypical *Streptomyces* species. The production of aerial mycelium is poor and is generally limited to the edges of the colonies when grown on most media, including minimal media. Zand2Y did not grow on some ISP media, as well as many of the media used for physiological testing.

Zand8Y was isolated on YEME from a heat treated sediment sample collected from Zandvlei Nature Reserve. A standard nucleotide-nucleotide BLAST search using the partial 16S rRNA gene sequence for Zand8Y (over 1375 bp) showed it is most closely related to *Streptomyces cyaneus* NBRC 13346^T and *Streptomyces coeruleorubidus* NBRC 12761^T (99% homology). From Figure 2.13 it can be seen that Zand8Y clusters with Zand2Y and Zand9Y. A pairwise local alignment of 1357 bp of conserved 16S rRNA gene sequence showed

that Zand8Y was 99.93% similar to both Zand2Y and Zand9Y. Zand8Y forms blue substrate mycelium and blue-grey aerial mycelium on inorganic salts-starch agar (ISP 4). A navy blue diffusible pigment is produced on most media (broth and solid medium), with a bluish-purple diffusible pigment being produced on glycerol-asparagine agar (ISP 5). The spore chains form relatively short spirals of three or four turns (*Spirales*). The spores are ovoid, with thick, blunt spines (Figure 2.15 A, B). Degrades gelatin, hypoxanthine, Tween 80 and urea (weakly). Degradation of xylan is variable and allantoin is not degraded. Pectin is hydrolysed. Protease, lipase and lecithinase activities occur on egg yolk medium. Utilises glycerol, maltose, D(+) melibiose, D(+) melezitose, D(-) sorbitol and trehalose, but not L(-) sorbose, as sole carbon sources. Grows in the presence of 0.3% 2-phenylethanol and 0.0001% crystal violet, with weak growth in the presence of 0.1% phenol, and no growth in the presence of 0.01% NaN_3. H_2S is not produced and nitrate reduction is weak at 14 days. Growth occurs from 4°C to 37°C and at pH 5, 7 and 9. The whole cell sugars are ribose and galactose. Due to the variability in the reported phenetic data for *S. cyaneus*, it is not possible to differentiate Zand8Y from *S. cyaneus*. From the results presented in Table 2.18, it is probable that Zand8Y is a strain of the *S. cyaneus* species group.

Riet1C was isolated on CZ agar from a heat treated sediment sample that was collected from the Rietvlei Wetlands Reserve. The aerial mycelium is blue-grey and the substrate mycelium is grey blue on inorganic salts-starch agar (ISP 4). On most other media the aerial mycelium is greyish green, the substrate mycelium is dark green and a green diffusible pigment is produced. The spore chains are *Spirales* and the ovoid to rectangular spores are spiny (Figure 2.15 C). Riet1C hydrolyses pectin and degrades adenine, casein, gelatin, guanine, hypoxanthine, starch, Tween 80, L-tyrosine, xanthine (weakly) and xylan. Positive for proteolytic and lecithinase activities on egg yolk medium, but does not produce lipases. Utilises D(-) fructose, glycerol, maltose, D(+) melibiose, D(+) melezitose, D(-) sorbitol and trehalose as sole carbon sources. L(-) Sorbose is not used. No growth in the presence of urea and allantoin. Grows in the presence of 0.3% 2-phenylethanol, 0.0001% crystal violet and 0.1% phenol, with growth inhibited by 0.01% NaN_3. Grows from 4°C to 37°C, and from pH 5 to 9. H_2S is not produced and nitrate is only weakly reduced. The whole cell sugars are ribose and glucose.

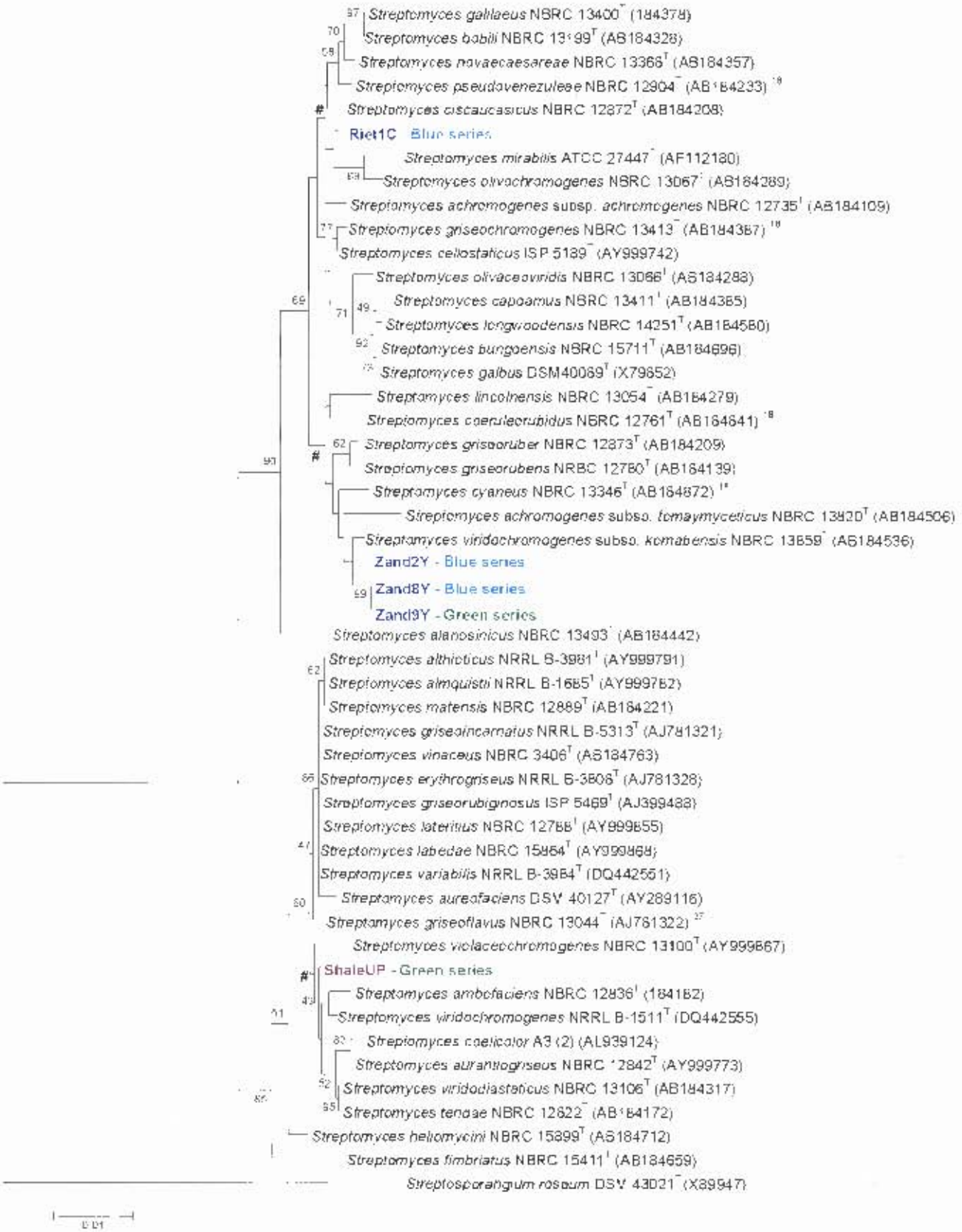
Phylogenetic analysis found that Riet1C clustered with *S. mirabilis* and *S. olivochromogenes*. From Table 2.18 it can be seen that there are a number of physiological features that differ between Riet1C and its closest phylogenetic neighbours, *S. mirabilis* (six differences) and *S. olivochromogenes* (four differences). The formation of grey blue substrate mycelium, the ability to use D(+) raffinose as a sole carbon source and spiny spore ornamentation differentiate Riet1C from *S. mirabilis*. Features that distinguish Riet1C from *S. olivochromogenes* include the formation of grey blue substrate mycelium, the lack of melanin pigments on peptone-yeast extract-iron agar (ISP 6) and the use of D(+) raffinose as a sole carbon source. From phylogenetic analysis (Figure 2.13) Riet1C appears to be a distinct species, however, further physiological testing would be required to distinguish it from its closest phylogenetic neighbours.

Table 2.18 Physiological characteristics to distinguish the blue and green series *Streptomyces* isolates from the type strains of phylogenetically related species.

Phenetic characteristic	1	2	3	4	5	6	7	8	9	10	11
Colour of substrate mycelium	Dark brown to grey	Grey blue	Dark green	Blue	Blue	Grey, purple at margins	Dark blue to dark brown	v	Grey yellow to olive brown	Indistinct	Olive brown to greenish yellow
Colour of spore mass	Bluish green	Blue grey	Dark green	Dark blue	Blue-grey	Grey green	Grey	Red, grey or blue	Grey	ND*	Blue
Spore chain morphology	SP	SP	RF	SP	SP	SP	SP	SP	SP	SP	SP
Spore chain ornamentation	Spiny	Spiny	Spiny	ND	Spiny	Spiny	Smooth to warty	Spiny or smooth	Smooth	Smooth	Spiny
Production DP (ISP 5)	Dark brown	Greenish yellow	Creamy yellow	Purple**	Bluish purple	Yellow-brown	-	v	-	v	Grey
pH sensitivity of pigments	-	+ (sub. DP)	+ (sub. DP)	-	+ (sub. DP)	-	+ (sub)	+	/	/	+ (sub)
Melanin (ISP 6)	+	-	+	-	+	+	-	+	+	+	-
Melanin (ISP 7)	+	+	+	-	+w	-	-	-	-	+	v
Degradation:											
Adenine	+	+	-	+	+	+	+	+	+	+	-
Casein	+	+	+	+	+	+	+	+	+	+	+
Guanine	-	d	-	-	+	+	+	+	+	+	+
Starch	+	+	+w	+	+	+	ND	ND	ND	ND	-
L-Tyrosine	+	+	+	NG	+	+	+	+	+	+	+
Xanthine	+w	+w	-	-	-	-					+
Xylan	-	+	-	NG	d	-					-
Carbon source:											
L(+) Arabinose	ND	+	ND	+w	+	ND	+	ND	+	+	+
meso-Inositol	+	+	+	+	+	+	+	+	+	+	+
D(-) Mannitol	+	ND	+	ND	ND	+	+	+	+		+
D(+) Raffinose	+	++	+	++	+	++	-	+	-	-	+
L(+) Rhamnose	+	+w	++	+	+	+	+	+	+	+	+
NaCl (%)	7	9	9	5	7	7	4	7	ND	ND	ND

1, Hel32; **2,** Riet1C; **3,** ShaleUP; **4,** Zand2Y; **5,** Zand8Y; **6,** Zand9Y; **7,** *Streptomyces ambofaciens* NBRC12836^T; **8,** *Streptomyces cyaneus* NBRC 13346^T; **9,** *Streptomyces mirabilis* ATCC 27447^T; **10,** *Streptomyces olivochromogenes* NBRC 13067^T; **11,** *Streptomyces viridochromogenes* NRRL B-1511^T. Symbols: ++, growth better than positive control; +, growth/positive reaction; +w, weak growth; -, no growth/negative reaction; d, growth doubtful; ND, not determined; NG, no growth; sub, substrate mycelium; DP, diffusible pigment; RF, *Rectiflexibiles*; SP, *Spirales*; * insufficient growth for colour determination. Data for the type strains obtained from Williams *et al.*, 1989; Shirling & Gottlieb, 1968b, 1969, 1972. ** The colour of diffusible pigment for Zand2Y was determined on ISP 4.

Figure 2.13 Unrooted 16S rDNA neighbour-joining phylogenetic tree showing the relationship between the isolates belonging to the green and blue series, and their closest phylogenetic neighbours. The tree was based on 1365 bp of conserved sequence and bootstrap values are based on 1000 resampled datasets. Only bootstrap values of greater than 40% are shown. The bar represents 0.01 nt substitutions per nt site. *Streptosporangium roseum* DSM 43021^T was set as the outgroup.



A hash (#) denotes clades that were conserved between trees constructed from the neighbour-joining and minimum evolution methods only. Strains coded in purple were isolated from soil samples while strains coded in blue were isolated from aquatic sediment samples. Superscript numbers refer to the numerically defined species clusters which are included in the blue and green series (Williams *et al.*, 1983).

2.4.5.4 Green series – Strains Hel32, ShaleUP and Zand9Y

Hel32, ShaleUP and Zand9Y all produce a green spore mass on inorganic salts-starch agar (ISP 4) and were grouped in the green series. Species included in the green series include *Streptomyces acrimycini*, *Streptomyces ghanaensis*, *Streptomyces griseostramineus* and *Streptomyces spinoverrucosus* (Williams *et al.*, 1989). For phylogenetic analysis ShaleUP and Zand9Y were included in a combined green- and blue series tree. ShaleUP was found to belong to a single membered clade which is most closely related to *Streptomyces violaceochromogenes* NBRC 13100^T, *Streptomyces viridochromogenes* NRRL B-1511^T and *Streptomyces ambofaciens* NBRC 12836^T (Figure 2.13). Zand9Y was found to cluster with Zand2Y and Zand8Y, with the most closely related type strain being *S. cyaneus*.

ShaleUP was isolated on PV8 agar from an air dried soil sample collected from a dry riverbed in the Karoo Desert National Botanical Garden, Worcester. A standard nucleotide-nucleotide BLAST search using the partial 16S rRNA gene sequence for ShaleUP (over 1340 bp) showed that it shared 98% homology with *S. ambofaciens*, *S. violaceochromogenes* and *S. viridochromogenes*. Both the aerial and substrate mycelia of ShaleUP are dark green on inorganic salts-starch agar (ISP 4). The spore chains are straight (*Rectiflexibiles*) and the spores are ovoid and spiny (Figure 2.15 D). ShaleUP produces a dark green pigment both in liquid and solid media, with a creamy yellow diffusible pigment produced on glycerol-asparagine agar (ISP 5). ShaleUP degrades gelatin and Tween 80. Cellulose and urea are not degraded. Grows in the presence of 0.3% 2-phenylethanol, 0.0001% crystal violet, 0.01% NaN_3 and 0.1% phenol. D(+) Galactose, glycerol, D(-) lactose, D(+) mannose, maltose, D(-) ribose and trehalose are used as sole carbon sources. H_2S is produced but nitrate is not reduced. Growth occurs from 4°C to 45°C and at pH 7 and 9. A comparison of physiological characteristics reveals that ShaleUP differs from the type strains of *S. ambofaciens* (11 differences) and *S. viridochromogenes* (seven differences) (Table 2.18). The ability of ShaleUP to grow at 9% NaCl, produce melanin pigments on peptone-yeast extract-iron agar (ISP 6) and tyrosine agar (ISP 7), and colony morphology distinguish this isolate from *S. ambofaciens*. Likewise, morphological features, the production of melanin pigments on peptone-yeast extract-iron agar (ISP 6), and its inability to degrade guanine differentiate ShaleUP from *S. viridochromogenes*. As comparisons were only made over a limited number of phenetic features, additional testing may allow this isolate to be differentiated with greater confidence from *S. viridochromogenes*, however, phylogenetically ShaleUP appears to be a unique species.

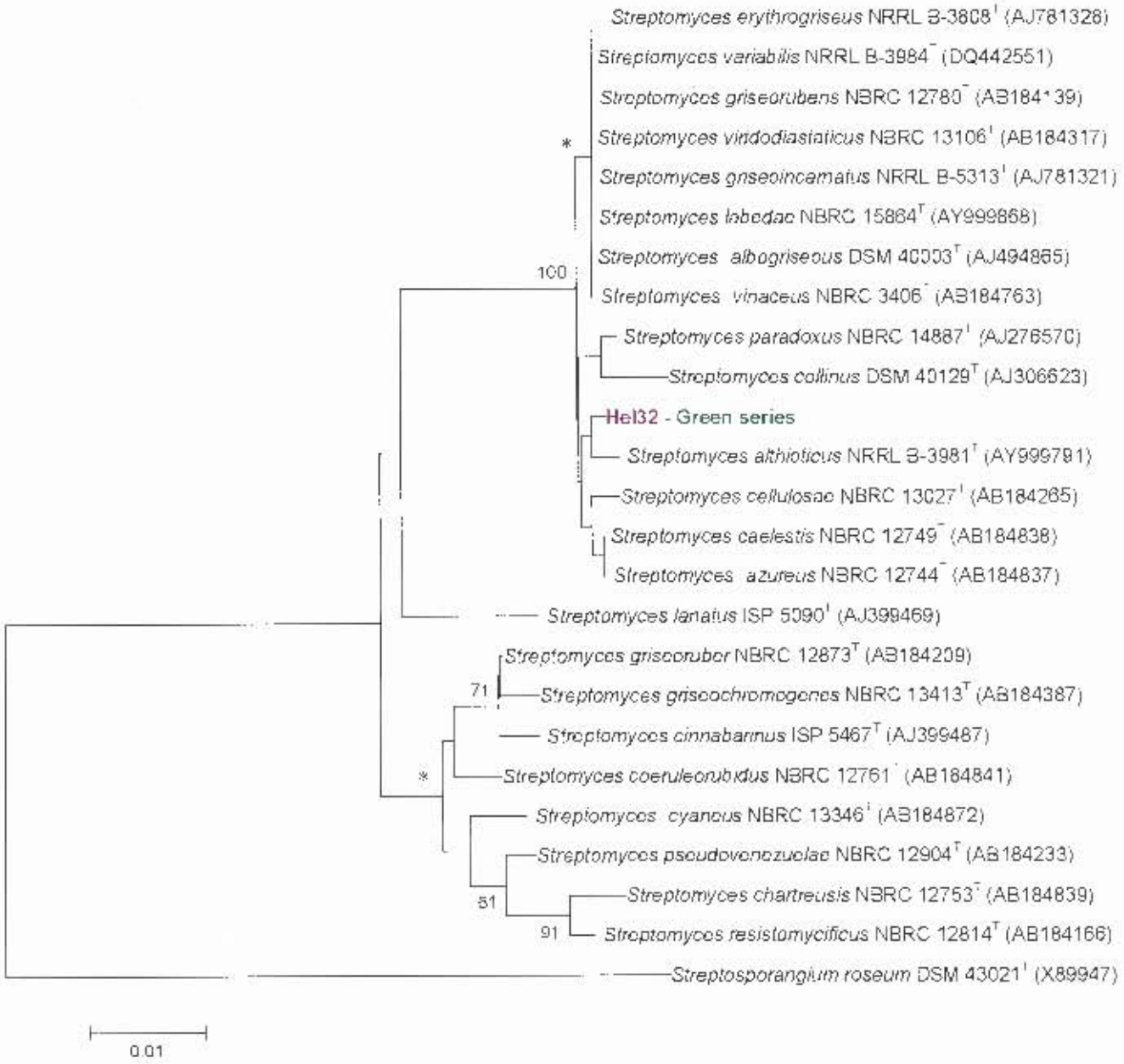
Zand9Y was isolated on YEME from a heat treated sediment sample collected from the Zandvlei Nature Reserve. BLAST analysis of the 16S rRNA gene sequence of Zand9Y (over 1354 bp) showed that it shared 99% homology to *S. cyaneus* and *S. coeruleorubidus*. On inorganic salts-starch agar (ISP 4), the substrate mycelium is grey, the aerial mycelium is purple-grey and only form at the edges of the colony, while the spore mass is grey-green. A yellow-brown diffusible pigment is produced on glycerol-asparagine agar (ISP 5), with a blue-black pigment produced on most other media. The spore chains form loose spirals of two to three turns and the spores are spiny (Figure 2.15 E). Zand9Y degrades gelatin, guanine, hypoxanthine and Tween 80. Allantoin, cellulose and urea are not degraded. Pectin is hydrolysed. Protease, lipase (weakly) and lecithinase production occurs on egg yolk medium. Growth occurs in the presence of 0.3% 2-phenylethanol and 0.0001% crystal violet, and is inhibited by 0.01% NaN_3 and 0.1% phenol. Zand9Y uses D(+) galactose, glycerol (weak), D(-) lactose, D(+) mannose, maltose, D(-) ribose and trehalose as sole carbon sources. H_2S is produced and

nitrate is reduced. Growth occurs from 4°C to 37°C, and at pH 5, 7 and 9. The whole cell sugars are galactose, ribose and xylose. The phenetic data presented in Table 2.18 show that there are only two differences between Zand9Y and *S. cyaneus,* the colour of the spore mass and the pH sensitivity of pigments. Like Zand8Y, it is possible that Zand9Y is a strain of the *S. cyaneus* species group.

Although numerous attempts were made to optimise the 16S rRNA gene PCR conditions for Hel32, amplification remained poor and only 579 bp of confirmed 16S rRNA gene sequence could be obtained for this strain. Sequencing primers F1 and R1 generated good sequence, however, only 300 bp of sequence was obtained from primer F3 and sequencing with primers R3 and R5 failed. BLAST analysis over 579 bp showed that Hel32 has a homology of 99% to *Streptomyces azureus* NBRC 12744^T and *Streptomyces caelestis* NBRC 12749^T. Phylogenetic analysis was limited to 579 bp at the 5' end of the 16S rRNA gene (less than 40% of the 16S rRNA gene) (Figure 2.14). Bootstrap values were low for most nodes (the clustering of Hel32 and *Streptomyces althioticus* NRRL B-3981^T had a bootstrap value of 2). Hel32 was included in Table 2.18 for phenetic comparison with the other green series strains.

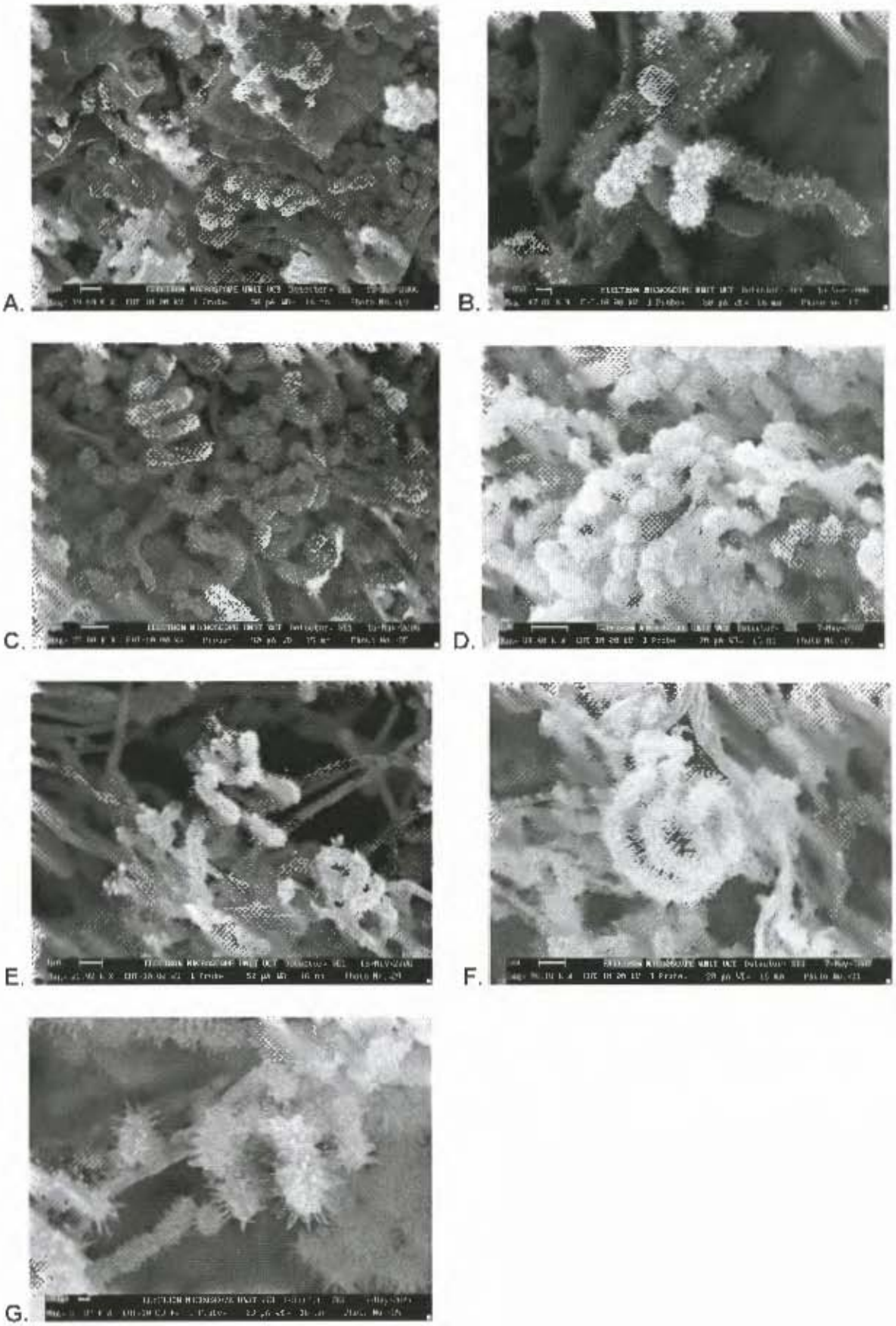
Hel32 was isolated on PV8 agar from an air dried soil sample collected from the banks of the Gamka River, Die Hel. The substrate mycelium is dark brown-grey and the aerial mycelium is bluish-green on inorganic salts-starch agar (ISP 4). The spore chain morphology is *Spirales* and the spores are long and thin with long spines (Figure 2.15 F, G). A brown pigment is produced on most media. In broth, Hel32 frequently grows as green balls with a fuzzy/spiky appearance on prolonged incubation. Tween 80 is degraded but cellulose and gelatin are not. Lecithinase, lipase and protease activities are not detected on egg yolk medium. Growth occurs in the presence of 0.0001% crystal violet, 0.3% 2-phenylethanol and 0.1% phenol, with weak growth in the presence of urea. Growth is inhibited by allantoin and 0.01% NaN_3. D(+) Galactose, glycerol, D(-) lactose, D(+) mannose, maltose, D(-) ribose and trehalose are all used as sole carbon sources. H_2S is produced and nitrate is reduced. Growth occurs from 4 to 45°C, and from pH 5 to 7.

Figure 2.14 Unrooted 16S rRNA gene phylogenetic tree showing the relationship between Hel32 and its closest neighbours identified by BLAST analysis. The tree was based on 579 bp of conserved sequence and was obtained using the neighbour-joining method. Bootstrap values are based on 1000 resampled datasets and only values greater than 40% are shown. The bar represents 0.01 nt substitutions per nt position. *Streptosporangium roseum* DSM 43021^T was set as the outgroup.



Asterisks (*) denote clusters that were conserved using the neighbour-joining, minimum evolution and maximum parsimony methods to construct the phylogenetic trees. The strain coded in purple was isolated from a soil sample.

Figure 2.15 Scanning electron micrographs of *Streptomyces* isolates.



All isolates were grown on inorganic salts-starch agar (ISP 4) at 30°C for 14 days. **A & B**, *Streptomyces* strain Zand8Y has *Spirales* type spore chains. The spores have short, blunt spines; **C,** the spore chains of *Streptomyces* species Kiet°C form long spirals with spiny spores; **D,** *Streptomyces* strain ShaleUP has *Rectiflexibiles* type spore chains with spiny spores; **E,** *Streptomyces* strain Zand9Y spore chains are *Spirales* forming loose spirals of three to four turns. The spores are spiny **F & G**, the spore chains of *Streptomyces* strain He32 are *Spirales* and the spore surface ornamentation is spiny.

2.5 Discussion

Thirty actinobacteria were chosen for full characterisation. Of these, ten were isolated from the leaves of indigenous plants, 14 were from aquatic and marine sediment, and six were isolated from soil.

Streptomyces are the dominant species in most terrestrial habitats. As the aim of this study was to isolate the rarer actinomycete genera, the ability of different techniques to increase the probability of isolating strains belonging to these genera from terrestrial environments was investigated. The Karoo Desert National Botanical Garden in Worcester would be described as an arid environment. Both samples collected from this site were dry, clay soil. The shale sample lacked visible organic matter and was probably nutrient poor. From Table 2.4 it can be seen that only two actinomycetes were isolated from this sample. Two selective media, PV8 and MC, were used in order to increase the chances of isolating the rarer actinomycetes. The six terrestrial isolates (Table 2.5) included three *Streptomyces*, two *Kribbella* and one *Nocardia* species.

The 14 actinobacteria isolated from the different sediment samples included ten *Streptomyces* and three *Micromonospora* species. Given the reported dominance of these genera in most aquatic habitats, this result was not surprising (Jiang & Xu, 1996; Ward & Bora, 2006). The only other actinobacteria species isolated from a sediment sample was a *Gordonia* species, NX0141, which was isolated from the Hermanus Lagoon. It is interesting to note that phylogenetic analysis (Figure 2.2) revealed that this isolate is related to *G. rhizosphera*, which was isolated from a Japanese mangrove forest (Takeuchi & Hatano, 1998).

It was found that actinobacteria were readily isolated from plants using the indirect isolation method. The direct method is likely to only isolate endophytes which occur at very high frequencies. As the leaves had been surface sterilised, the edges of the cut up leaves are the only sites which may contain actinobacteria that makes contact with the agar. The surface sterilisation method appeared to be successful as no contaminating non-actinomycete bacteria grew on the isolation plates. A number of bacteria grew on control plates with untreated leaves (as these plates were also overgrown with fungi it was not possible to accurately determine the number of bacteria on unsterilised leaves). Coombs & Franco reported that a similar sterilisation method successfully sterilised wheat roots (Coombs & Franco, 2003). Although contaminating fungi were still present, their numbers were greatly reduced. This may be due to the morphology of the leaves used. Many of the plants that are endemic to the fynbos biome have hairy leaves and it may be possible that fungi adhere to these hairs hindering the sterilisation of the leaf surface. The fungi that were present on the isolation plates may have also been endophytic.

It has been reported that a greater number of endophytes can be isolated on nutrient poor media (Zinniel *et al.*, 2002), however, no actinobacteria were isolated on tap water agar. Zinniel and collaborators found that the number of endophytes varies with plant source, the age of the plant, tissue type as well as the time of sampling, with increased numbers present in early summer (Zinniel *et al.*, 2002). From Table 2.1 it can be seen that only three of the 23 strains initially isolated from plants did not come from the Wellington sample site. The plant samples from Wellington were collected in early October which coincides with the beginning of the South African summer. It is also interesting to note that a number of the plant species from which actinobacteria were isolated

have known medicinal uses. *Tulbaghia violacea* is used for the treatment of asthma and tuberculosis, infusions prepared from the tubers of *Pelargonium luridum* are used to treat diarrhoea and dysentery, while *Myrothamnus flabellifolius* is used to treat a range of ailments and for the treatment of burns and for pain relief (Van Wyk *et al.*, 1997).

Both of the novel *Kribbella* species identified were isolated from soil samples. Of the eleven validly described species only *K. lupini* and *K. solani* were not isolated directly from soil (Song *et al.*, 2004; Trujillo *et al.*, 2006).

Eight isolates belong to the genus *Micromonospora*, of which three were isolated from sediment samples and five were isolated from leaves. On the soil isolation plates there were very few actinobacteria which had the typical *Micromonospora*-type appearance. The dominance of *Micromonospora* species in plants, compared to terrestrial habitats, has been reported by other authors (Coombs & Franco, 2003). Their prevalence in plants may indicate that they have formed a beneficial relationship with plants. It has been found that some *Micromonospora* species can parasitize fungal plant pathogens including *Pythium* species (El-Tarabily *et al.*, 1997). From Table 2.5 it can be seen that the three sediment *Micromonospora* strains came from pretreated sediment samples. As micromonosporae are resistant to heat, the use of mild heating methods can be employed for the selective isolation of members of this genus (Jensen *et al.*, 1991).

Although there is a close phylogenetic relationship between *Micromonospora* strains PLU1 and RAU1, they were isolated from different plant species. Likewise, DG41 and PBPE are phylogenetically closely related but were isolated from different plant species. DG41, PLU1 and RAU1 were all isolated from leaves collected from a garden in Wellington, while PBPE was isolated from a leaf sample collected in Namibia. As this study focused on plants that are indigenous to the fynbos biome, further studies should be contacted on plants collected from other regions of South Africa to determine whether actinomycete species are endemic or are widely distributed.

The four kineococci identified were all isolated from plants. GIU1, GIU2 and GIU3, which are morphologically more like kineosporiae, were isolated from the leaves of *G. incanum*. Given that four of the five validly published *Kineosporia* species were isolated from plant samples, it is possible that kineosporiae have adapted to survive in this environmental niche (Kudo *et al.*, 1998). Interestingly, *Micromonospora* strain PLU1 was isolated from the leaves of *P. luridum* which, like *G. incanum*, is a member of the Family *Geraniaceae*. Both these plant species are used medicinally (Van Wyk *et al.*, 1997) and it is possible that some of these curative properties may in fact be attributed to endophytic actinomycetes. Similarly, *Micromonospora* strains TVU1 and PBPE were both isolated from medicinal plants.

It can be concluded that screening for actinomycetes from different aquatic environments and indigenous plants is rewarding. By collecting samples from unique habitats and using a number of selective techniques a number of actinomycetes belonging to the rarer genera were isolated. More than half of the actinobacteria isolated in this study belong to the rarer genera. The application of these methods to selectively isolate actinobacteria belonging to these genera from terrestrial environments was confirmed.

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CHAPTER 3

Screening of actinomycete isolates for antimicrobial compounds with emphasis on antimycobacterial activities

CHAPTER 3

Screening of actinomycete isolates for antimicrobial compounds with emphasis on antimycobacterial activities

3.1 Summary

Actinomycetes isolated from the leaves of indigenous plants, sediment and soil samples were screened for the ability to produce antimicrobial compounds. As the aim of this part of the study was to discover novel antibiotics that are effective against *M. tuberculosis*, emphasis was placed on those strains that exhibited antibiosis against *Mycobacterium aurum* A+, a non-pathogenic strain with a similar antibiotic susceptibility profile to *M. tuberculosis*. Of the 30 actinomycete isolates that were initially selected for characterisation, 24 produced antimicrobial compounds that inhibited the growth of *M. aurum* A+. Antibiotic production was also found to be the most prevalent in strains isolated from soil samples. Members of the genus *Streptomyces* were found to be the most prolific antibiotic producers and all *Streptomyces* strains produced antimicrobial compounds. Antibiotic production was enhanced in strains belonging to the genera *Kribbella* and *Micromonospora* when cultured in specially formulated production media. The feasibility of different methods to detect antimicrobial activity was investigated, including small scale fermentations and PCR screening with primers that target antibiotic biosynthetic genes. The antimycobacterial compound produced by *Streptomyces speibonae* strain PK-Blue^T was characterised. Based on preliminary structural and physical characterisation, it is probable that this compound is a tetramic acid type antibiotic.

CHAPTER 3

Screening of actinomycete isolates for antimicrobial compounds with emphasis on antimycobacterial activities

3.2 Introduction

Worldwide, the rising mortality rates due to a number of infectious diseases can be attributed to an increase in antibiotic resistance. The emergence of drug resistance strains of *M. tuberculosis*, especially MDR strains, is of growing public concern (World Health Organization, 2002). Although there are a few promising antibiotics in the pipeline, such as the quinolone moxifloxacin and the ATP synthase inhibitor diarylquinoline R207910, no new class of antitubercular drug has been developed in the last thirty years. There is an urgent need to discover new compounds, which not only have improved sterilising activity, but also have unique targets (Duncan & Barry, 2004; Andries *et al.*, 2005).

It is estimated that more than 20 000 microbial secondary metabolites have been identified. If one considers that only 1% of bacterial species have been described, it is clear that microorganisms are still a bountiful source of novel compounds (Knight *et al.*, 2003). As many terrestrial environments have been exhaustively sampled, many natural product screens are now focused on previously unexplored environments, such as plants and marine habitats (including marine invertebrates) (Knight *et al.*, 2003; Peláez, 2006). Despite the potential of actinomycetes (especially the genus *Streptomyces*) to be a source of new compounds, the 1980s witnessed a decline in the number of antibiotics isolated from these microorganisms (Watve *et al.*, 2001).

The recent advances in combinatorial chemistry, molecular biology and HTS have been coupled with a decline in the screening for novel natural products. However, with so few targets identified by these modern methods reaching clinical trials, it has become apparent that there is still a need to isolate novel compounds using traditional means. Thus, natural product screening is back in *vogue* (Lawrence, 1999). A recent review by Lam reported that of the 23 new drugs derived from natural products that were approved between 2001 and 2005, all eight novel antibacterial and antifungal agents were of microbial origin (Lam, 2007).

Traditionally, natural product screening has been seen as being complicated, labour intensive and time consuming. However, recent developments such as culturing the test organisms in microtitre plates has allowed for the simultaneous screening of a large number of strains, cultured under different conditions (Peláez, 2006). For a natural product screen to be successful, it is important to culture the strains under different conditions, as altering the growth conditions can be used to increase yields and improve the range of compounds produced (Tormo *et al.*, 2003). A number of parameters can affect the production of secondary metabolites including aeration, incubation temperature and media components (Knight *et al.*, 2003). However, the number of parameters tested is generally inversely proportional to the number of microorganisms that can be screened. In order to optimise screening, it is better to screen a larger number of strains grown in three to five media, than it is

to test a limited subset of strains grown under numerous conditions. The type of bacteria being screened should also be considered when selecting media and growth conditions (Tormo *et al.*, 2003; Peláez, 2006).

A number of recent studies have reported the use of PCR primers to screen isolates for the presence of different antibiotic biosynthetic gene clusters. Many antibiotics that are produced by actinomycetes are synthesized by nonribosomal peptide synthetases (NRPS) and modular Type-I polyketide synthases (PKS-I) including vancomycin (NRPS) and erythromycin (PKS-I) (Ayuso-Sacido & Genilloud, 2005). Other studies have focused on Type-II polyketide synthases (PKS-II) which, unlike the multifunctional PKS-I, are iterative and involve complexes of monofunctional proteins. Aromatic compounds such as angucyclines, actinorhodins and tetracyclines are synthesized by PKS-II (Hutchinson, 1999; Metsä-Ketelä *et al.*, 2002). Many of the studies that have investigated the feasibility of PCR screening have focused on PKS-I, PKS-II and NRPSs, as they contain conserved domains that can be used for the design of PCR primers. The main advantage of PCR screening, prior to small scale fermentations, is that it can be used as a guide to determine what growth conditions (such as growth medium and incubation temperature) and extraction methods to use for an isolate with the known potential to produce a specific class of antibiotic (Wood *et al.*, 2007). Ayuso and co-workers combined PCR screening using primers that target bacterial NRPSs and PKSs with restriction analysis. The resulting fingerprints could be used for strain dereplication when screening environmental isolates (Ayuso *et al.,* 2005). One important criterion to consider when selecting which genes to target is the frequency at which they occur within the genome. This is exemplified by NRPSs, which are involved in the synthesis of multiple oligopeptides from either amino or hydroxy-acid monomers. A study by Sosio *et al.* revealed the abundance of these gene clusters in the genomes of a number of antibiotic-producing actinomycetes. It was found that almost 2% of the genome of a thiazolylpeptide-producing strain of *Planobispora rosea* could potentially code for genes which encode an NRPS (Sosio *et al.*, 2000). Similarly, Ōmura and co-workers identified eight gene clusters for peptides that are synthesized by NRPSs in the almost complete genome of *Streptomyces avermitilis* (sequences covered >99% of the genome) (Ōmura *et al.*, 2001). As NRPSs are also involved in the production of secondary metabolites other than antibiotics, they are widely distributed in actinomycete genomes. Therefore, it is likely that PCR screening with primers that target these gene clusters will be positive for most actinomycetes screened and one can thus not assume that a strain found to contain these gene clusters will produce oligopeptide-type antibiotics.

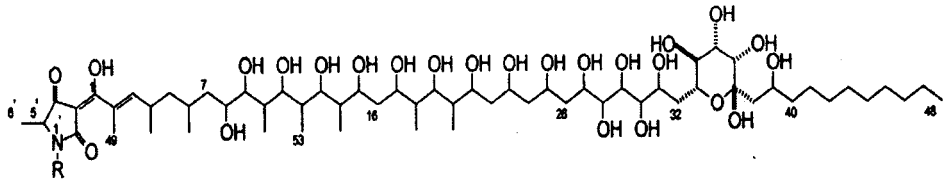
It is estimated that more than 45% of all known microbial bioactive compounds are produced by the actinomycetes (Bérdy, 2005). Actinomycetes can synthesize a seemingly limitless array of chemical structures, from simple molecules such as fosfomycin (Hendlin *et al.,* 1969) to complex glycopeptides such as bleomycin, a potent antitumor agent (Lehmann *et al.*, 1997). Many antibiotics of microbial origin share numerous structural features, such as ring systems and aromatic atoms, which are rare in synthetic compound libraries (Koehn & Carter, 2005)

Tetramic acids are an example of natural products that contain a ring system. This family of antibiotics is characterised by the presence of a 2,4-pyrrolidine-dione subunit (the tetramic acid moiety). Secondary metabolites that contain this moiety have frequently been isolated from fungi and marine invertebrates (many sponge pigments contain this moiety) (Royles, 1995; Segeth *et al.*, 2003). However, a number of tetramic acid

type antibiotics have been isolated from actinomycetes, including lydicamycin from *Streptomyces lydicus* strain 224-S3 (Hayakawa *et al.*, 1991), the antibacterial tirandamycin from '*Streptomyces tirandis*' strain NRRL 3689 (Karwowski *et al.,* 1992) and vancoresmycin from *Amycolatopsis vancoresmycina* ST 101170^T (Hopmann *et al.*, 2002). Although a number of tetramic acid antibiotics share a 2,6-dioxabicyclononane skeleton, this family of antibiotics is structurally diverse (Figure 3.1) (Iwata *et al.*, 2005). Many tetramic acids are potent antivirals and antibacterial agents, however, the range of their biological activity is broad. Geodin A is a nematocide (Capon *et al.*, 1999), dihydromaltophilin has fungicidal activity (Graupner *et al.*, 1997), while tirandalydigin is an antianaerobe antibiotic (Karwowski *et al.*, 1992).

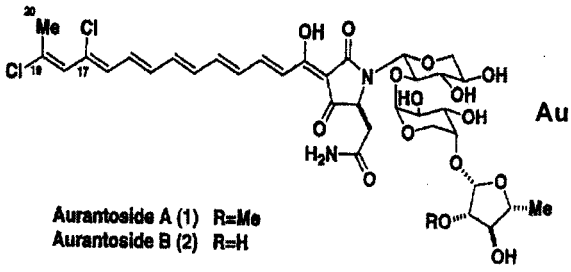
There were two main aims to this part of the study. The first aim was to identify novel actinomycetes that produce antibiotics that are effective against *M. aurum* A+ (as a substitute for *M. tuberculosis*), based on the premise that a novel producing strain may produce uncharacterised antibiotics. Although many of the drugs that form the basis of SSC are semi- or fully synthetic, there are a number of antibiotics produced by actinomycetes that are effective against TB, including rifamycin (rifampicin is a derivative of this compound) and streptomycin (Zhang *et al.*, 2006). Therefore, it is probable that actinomycetes will be a source of other, novel antitubercular compounds. The actinomycetes isolated in this study were isolated from three diverse sources. As many natural product screens now focus on unexplored areas of known biodiversity (Peláez, 2006), the feasibility of isolating antibiotic producing strains from these unique environments was also investigated. The second aim of this study was to characterise the antimicrobial compound that is produced by *Streptomyces speibonae* strain PK-Blue^T (Meyers *et al.*, 2003).

Figure 3.1 The structures of biologically active tetramic acids (figure continued on next page).



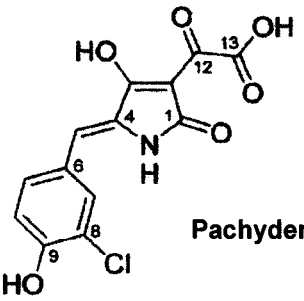
Aflastatin A (1) R=CH_3
Aflastatin B (5) R=H

Aflastatins [1]



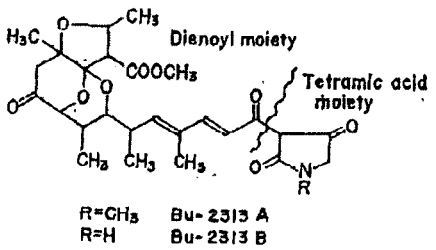
Aurantoside A (1) R=Me
Aurantoside B (2) R=H

Aurantosides [2]



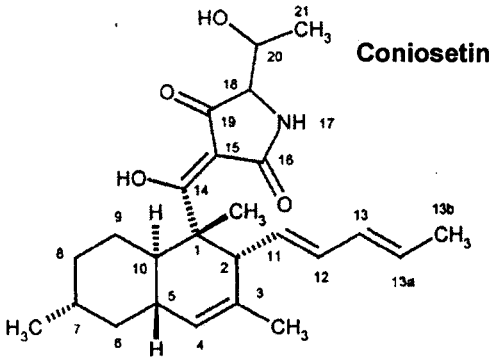
Pachydermin [3]

Bu-2313 [4]

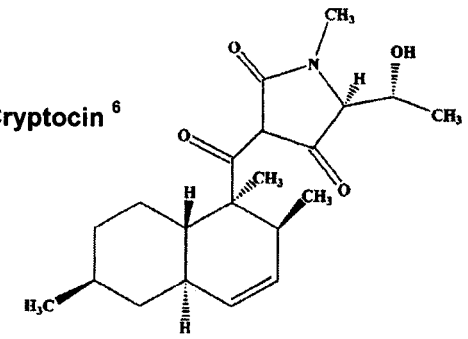


Dienoyl moiety
Tetramic acid moiety

R=CH_3 Bu-2313 A
R=H Bu-2313 B

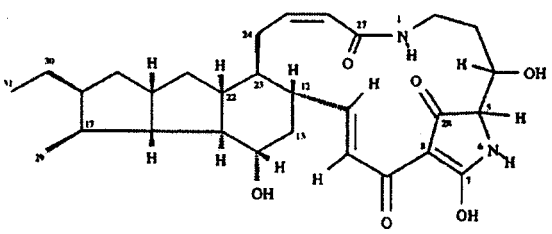


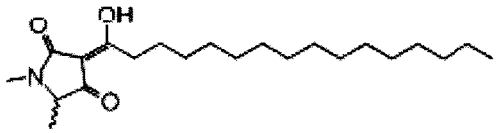
Coniosetin [5]



Cryptocin [6]

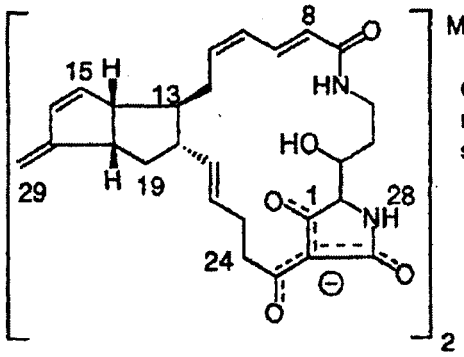
Dihydromaltophilin [7]



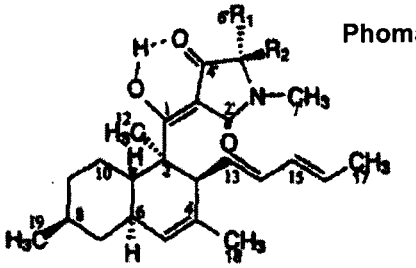


Melophlins P [8]

Figures taken from: **1,** Ono *et al.*, 1998; **2,** Sata *et al.*, 1999a; **3,** Lang *et al.*, 2006; **4,** Toda *et al.*, 1980; **5,** Segeth *et al.*, 2003; **6,** Li *et al.*, 2000; **7,** Graupner *et al.*, 1997; **8,** Xu *et al.*, 2006.

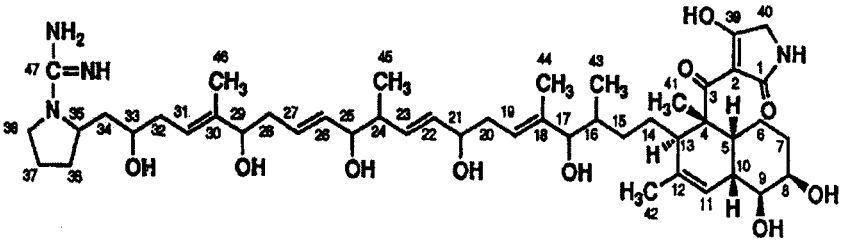


Geodin A magnesium salt [9]

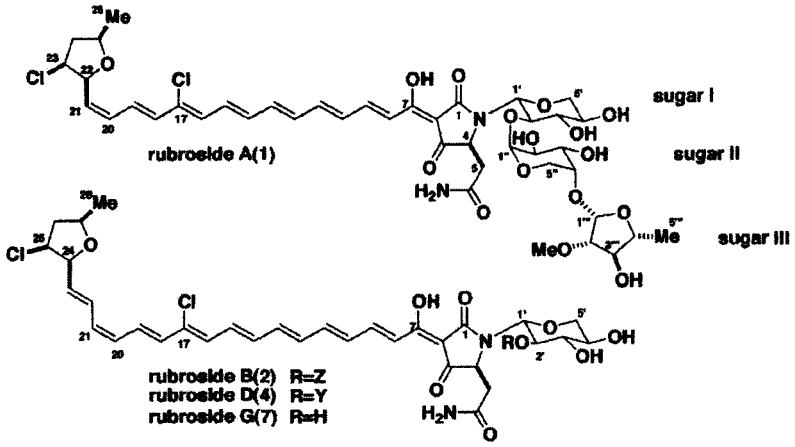


Phomasetin [10]

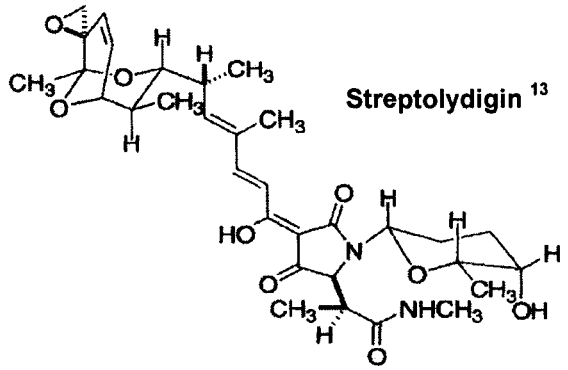
2a: R_1=H, R_2=CH_2OH



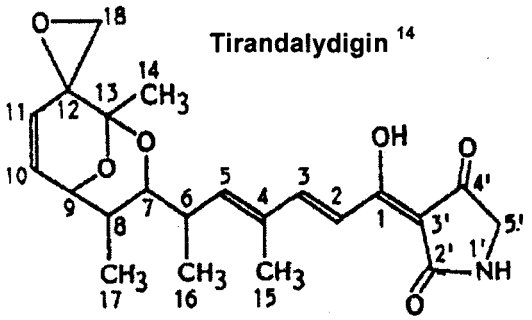
Lydicamycin [11]



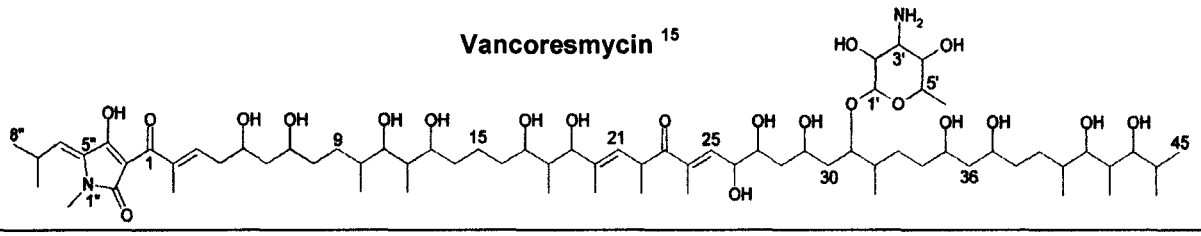
Rubrosides [12]



Streptolydigin [13]



Tirandalydigin [14]



Vancoresmycin [15]

Figures taken from: **9,** Capon *et al.*, 1999; **10,** Singh *et al.*, 1998; **11,** Hayakawa *et al.*, 1991; **12,** Sata *et al.*, 1999b; **13,** Iwata *et al.*, 2005; **14,** Karwowski *et al.*, 1992; **15,** Hopmann *et al.*, 2002.

3.3 Materials and Methods

3.3.1 Antibacterial testing

3.3.1.1 Bacterial strains and cultivation

Unless stated, all test bacteria for antimicrobial testing were maintained on Luria agar (Sambrook *et al.*, 1989) and as 20% glycerol stock cultures at -20°C (Table 3.1). *Amycolatopsis lurida* NRRL 2430^T and *Amycolatopsis mediterranei* NRRL B-3240^T were obtained from the Actinobacterial Culture Collection of the Agricultural Research Service, Department of Agriculture, Peoria, IL, USA and were maintained on YEME. *Micromonospora echinospora* subsp. *echinospora* DSM 43816^T was obtained from the DSMZ. *'Streptomyces polyantibioticus'* strain SPR and *Streptomyces speibonae* PK-Blue^T (= ATCC BAA-411^T) are lab isolates. SPR was maintained on Difco Middlebrook 7H9 containing 10 mM glucose (ADC supplement omitted) (Becton Dickinson) and PK-Blue was maintained on Hacène's Medium (HM) (5 g glucose; 10 g malt extract; 2 g yeast extract; 1 g NaCl; water to 1 l; pH 7.0) (Hacène & Lefebvre, 1995). All actinomycete type strains were grown at 30°C. The actinomycete isolates screened in this study are those that were described in Chapter 2 and these strains were cultured on YEME at 30°C, unless alternative culture conditions were stated in the species description.

Table 3.1 Bacterial strains used for antimicrobial testing.

Microorganism and strain number	Culture conditions [a]	Source
Citrobacter braaki strain 90 (clinical isolate)		V. Davids, GSH
Enterobacter cloacae strain 67 (clinical isolate)		V. Davids, GSH
Enterococcus faecium (VanA) clinical isolate[b]		V. Davids, GSH
Escherichia coli ATCC 25922*		V. Davids, GSH
Klebsiella pneumoniae strain 66 (clinical isolate)		V. Davids, GSH
Micrococcus sp. clinical isolate		V. Davids, GSH
Mycobacterium aurum strain A+		Med Micro
Mycobacterium bovis BCG (Tokyo)	7H9, SC	Med Micro
Mycobacterium smegmatis LR 222	7H9, SC	Med Micro
Mycobacterium tuberculosis H37Rv^T(=ATCC 27294^T)	7H9, SC	Med Micro
Proteus mirabilis strain 87 (clinical isolate)		V. Davids, GSH
Pseudomonas aeruginosa ATCC 27853*		V. Davids, GSH
Serratia sp. strain 83 (clinical isolate)		V. Davids, GSH
Staphylococcus aureus ATCC 25923*		V. Davids, GSH
Streptococcus sp. (clinical isolate)		V. Davids, GSH

Key: a, unless stated otherwise, all strains were cultured in Luria broth (Sambrook *et al.*, 1989) and incubated at 37°C for 18-24 h with constant shaking; 7H9, mycobacteria were cultured in Difco Middlebrook 7H9 broth with the addition of the AD supplement and 0.05% Tween 80; SC, cultures were incubated as standing cultures (full description in text); b, vancomycin resistant strain; GSH, Groote Schuur Hospital, Cape Town; Med Micro; Division of Medical Microbiology, Department of Clinical Laboratory Sciences, Faculty of Health Sciences at UCT; *, standard antimicrobial susceptibility test strains.

3.3.1.2 Antibacterial testing by standard agar overlays

Actinomycete isolates were stab-inoculated into YEME, CZ and Difco Middlebrook 7H9 agar supplemented with 10 mM glucose (ADC supplement omitted) (Becton Dickinson) using sterile toothpicks. *Nocardia* sp. M25 was stab inoculated into *Streptosporangium* medium (SM) (10 g glucose; 10 g starch; 10 g glycerol; 2.5 g tryptone; 5 g peptone; 2 g yeast extract; 1 g NaCl; 3 g $CaCO_3$; tap water to 1 l; pH 7.3; Pfefferle *et al.*, 2000). Four isolates were inoculated per plate. Plates were incubated for 11 days at 30°C. For all isolates, antimicrobial activity was determined against *Ent. faecium* VanA, *E. coli* ATCC 25922, *M. aurum* A+ and *S. aureus* ATCC 25923. Test bacteria were inoculated into 10 ml Luria broth (Sambrook *et al.*, 1989) and incubated at 37°C for 18 h with constant shaking. Cultures were Gram stained to check for contamination. The optical density was determined at 600 nm (OD_{600}) using a Beckman DU-530 spectrophotometer. Plates were overlaid with 6 ml Luria sloppy agar (Sambrook *et al.*, 1989) containing the test bacterium. To ensure the reproducibility of the overlay experiments, the amount of test culture to add to each 6 ml sloppy agar was determined as follows: OD_{600} x A µl = 160, where A is the volume of test culture (for *E. coli* the formula OD_{600} x A µl = 4 was used). The determined volume of test culture was added to the molten sloppy agar, vortexed and poured around the stab inoculated actinomycete colonies, ensuring not to pour the sloppy agar directly on top of the colony. The agar was allowed to solidify before the plates were inverted and incubated at 37°C for 24 h (*Ent. faecium* and *M. aurum* were incubated at 37°C for 48 h). The area of the zone of activity in mm^2 (Z) was determined as follows: Z = the total area of the inhibition zone (mm^2) - the area of the actinomycete colony (mm^2). All overlay plates were duplicated so results presented are the average of a minimum of two experiments. The overlay experiments were repeated on all isolates which had activity against *M. aurum,* however, the actinomycete cultures were only incubated for 7 days at 30°C for the second experiment.

3.3.1.3 Small scale solvent extraction

A spore suspension or a loopful of cell mass from an agar plate was used to inoculate a 100 ml Erlenmeyer flask containing 10 ml YEME broth. The culture was incubated at 30°C for 48 h with constant shaking. This 10 ml starter culture was used to inoculate a 1 l Erlenmeyer flask containing 100 ml YEME and the culture was incubated at 30°C for 7 days with constant shaking. The pH of the culture filtrate was recorded prior to solvent extraction. The culture was filtered through a coffee filter (size 102, House of Coffees) to collect the cell mass, which was washed with 200 ml (two times culture volume) of sterile distilled water. Ethyl acetate (EtAc) and chloroform ($CHCl_3$) were used to extract the antimicrobial compounds from the culture filtrate (CF), while methanol (MeOH) was used to extract compounds from the cell mass. The volume of the CF after the cells were washed was 300 ml (filtrate combined with the sterile water wash), which was divided equally between two 250 ml glass blue-topped bottles. Approximately 45 ml of each solvent was added to the separate bottles containing the CF. The liquids were mixed by vigorous shaking for 10 minutes and poured into 250 ml separating funnels. The phases were allowed to settle for 15 min, if phase separation was not complete at this time, an additional 10 ml of the relevant solvent was added. The aqueous layer was removed and discarded while the organic solvent layer was collected in a glass beaker. The solvent was allowed to evaporate overnight in a fumehood and the remaining residue was redissolved in 400 µl of the same solvent used for extraction. For solvent extraction from the cells, the filter paper containing the cells was torn open and placed on paper towel for approximately 10 min to allow the excess water to be absorbed by the paper towel. The cell mass was transferred to a sterile universal and 10 ml of methanol was added. The cells were mixed with the methanol by

vortexing vigorously for 2 min. The cells were allowed to settle and the methanol was removed with an automatic pipette and placed in a glass beaker. The solvent was allowed to evaporate and the residue was redissolved in 400 µl methanol. All solvent extracts were stored at -20°C in Eppendorf tubes. All methanol extracts from the cells were diluted either 1 in 500 or 1 in 1000 (in sterile H_20) and scanned from 200 to 700 nm on a Beckman DU-64 spectrophotometer.

3.3.1.4 Thin layer chromatography

All solvent extracts were spot tested for antimycobacterial activity prior to thin layer chromatography (TLC). For spot testing, silica TLC plates (Merck 1.05554.0001) were divided into 1.5 cm by 1.5 cm squares and 5 µl of extract was spotted per square (each extract was spotted in duplicate). Plates were placed in a fumehood to allow the solvent to evaporate. Antibacterial activity was determined by bioautography. Extracts which were found to have activity were subsequently tested by TLC. For TLC, 10 µl of the extract was spotted on 6 cm (width) by 12 cm (length) silica TLC plates. The spots were allowed to dry and the plates were placed in either a TLC chamber or glass beakers covered with aluminium foil, containing the solvent system. The solvent was added to the chamber or beaker 30 min prior to chromatography to allow the atmosphere to become saturated with solvent. Sufficient solvent was used to allow approximately 0.5 cm of the plate to be immersed in the solvent (but without the solvent coming in direct contact with the origin). The plates were chromatographed for 30 to 45 min, until the solvent front was 1 cm from the top of the TLC plate. Developed chromatograms were placed in a fumehood to allow the solvent to evaporate and antibacterial activity was detected by bioautography. For some extracts, the antimicrobial activity was weak and TLC was repeated, spotting 20 µl of the extract.

3.3.1.5 Bioautography

One loopful of *M. aurum* A+ was used to inoculate a universal containing 10 ml Luria broth. The culture was vortexed briefly and incubated at 37°C for 18-24 h with constant shaking. *Mycobacterium bovis* BCG (Tokyo), *Mycobacterium smegmatis* (ATCC 19420) and *M. tuberculosis* $H37Rv^T$ were inoculated from -70°C stocks and grown in 10 ml Difco Middlebrook 7H9 broth, containing 0.05% Tween 80 (Sigma) and AD supplement (final concentration in culture of 0.5% bovine serum albumin, 0.2% glucose), and were grown as standing cultures (with intermittent agitation) at 37°C for 5 days for *M. bovis* and *M. smegmatis* and 7 days for *M. tuberculosis*. The OD_{600} of all cultures was determined and the cultures were diluted to OD_{600}=0.5 with either sterile Luria broth or 7H9 broth. As it was not possible to determine the optical density of the *M. tuberculosis* culture using a spectrophotometer, the turbidity was estimated by direct comparison to *E. coli* cultures of known optical densities (grown in 7H9). *M. aurum* cultures were Gram stained to ensure they were not contaminated. A Ziehl-Neelsen stain was performed on *M. bovis*, *M. smegmatis* and *M. tuberculosis*. Sterile non-absorbent cotton wool was used to dab the diluted culture on the prepared TLC plates. The plates were incubated in sealed plastic containers, containing moistened paper towel, at 37°C for 24 h. Following incubation, the plates were dabbed with 0.25% thiazolyl blue tetrazolium bromide (MTT) (Sigma) in phosphate buffered saline (1.78 g Na_2HPO_4; 8.50 g NaCl; 1 l distilled water; pH 7.3) and incubated at 37°C for 1 h for testing against *M. aurum* and 24 h for bioautography against *M. bovis*, *M. smegmatis* and *M. tuberculosis* before the results were read. All the experiments with *M. tuberculosis* were conducted in a Biosafety Level 3 laboratory.

3.3.1.6 Modified Eli Lilly method

Antimicrobial activity was tested using a method adapted from that used in industrial screening programs (http://www.accessexcellence.org/AE/AEC/AEF/1995/goudie_soil.htm). Actinomycetes were inoculated onto YEME agar and incubated at 30°C for 11 days. The back end of a sterile glass Pasteur pipette was used to bore an agar plug containing a sporulating (if spores were present) actinomycete colony. Silica TLC plates were divided into 3 cm by 3 cm squares and one agar plug was placed in the centre of each square. Three drops (~ 50 µl) of solvent (chloroform, ethyl acetate, hexane, methanol or toluene) were slowly dropped on top of the agar plug. The TLC plates were placed at 4°C for 15 min. The agar plugs were removed with forceps and the plates were placed in a fumehood to allow the solvent to evaporate. Bioautography was subsequently performed by the standard method (Section 3.3.1.5).

3.3.2 The effects of alternative growth media on antibiotic production

3.3.2.1 Production of antibiotics by *Kribbella* strains HMC25^T and Q41^T

A loopful of spores was used to inoculate a 1 l Erlenmeyer flask containing 100 ml *Nocardioides* production medium, adapted from Matson & Bush (1989) (2 g sucrose; 1 g soy flour; 1 g freshly ground linseeds; 0.5 g $CaCO_3$; distilled water to 1 l; linseed meal was substituted with freshly ground linseeds, the pH of the medium was not adjusted and ranged from pH 7.0 to pH 7.3). Four flasks were inoculated per isolate to test for antibiotic production at two incubation temperatures, 30°C and 37°C, and two incubation periods, 5 and 10 days. Antimicrobial compounds were extracted from the cell mass as described for standard solvent extraction (Section 3.3.1.3). For solvent extraction from the CF, the standard method was employed except only ethyl acetate was used: 90 ml of EtAc was added to the 300 ml CF (100 ml culture filtrate and 200 ml sterile distilled water wash). Extracts were tested by standard TLC and bioautography. For testing against *M. tuberculosis* H37Rv^T, the compounds extracted from the cells and CF were spotted in a concentration range of 5 to 60 µg per spot on silica TLC plates and bioautography was performed according to standard methods (Section 3.3.1.5).

3.3.2.2 Antibiotic production in genus-specific production medium - *Micromonospora*

One loopful of spores was used to inoculate a 100 ml Erlenmeyer flask containing 10 ml modified Aretz (MA) medium (10 g starch; 10 g glucose; 10 g glycerol; 5 g peptone; 2 g yeast extract; 1 g NaCl; 3 g $CaCO_3$; 0.6 g L-valine; 0.5 g $MgSO_4.7H_20$; distilled water to 1 l; this medium was modified by the addition of $MgSO_4.7H_20$ and L-valine; the cornsteep liquor was excluded from the medium; the pH was not adjusted and ranged from pH 7.1 to pH 7.35) (Aretz *et al.*, 2000). Cultures were incubated at 30°C for 7 days with constant shaking. Cell mass was harvested by filtering cultures through 8 cm discs cut from coffee filters. The cell mass was placed in a sterile universal and 3 ml of methanol was added and the mixture was vortexed vigorously for 2 min. The methanol was removed with an automatic pipette and placed in a 2 ml Eppendorf tube. The solvent was allowed to evaporate and the residue was redissolved in 100 µl of methanol. The culture filtrate was split between two universals, and 3 ml of ethyl acetate was added to the one universal and 3 ml of toluene was added to the other universal. The mixtures were vortexed vigorously for 2 min and the phases allowed to separate for 30 min. The solvent layer was removed with an automatic pipette, placed in a 2 ml Eppendorf tube and allowed to evaporate in a fumehood. The residue was redissolved in 100 µl of the solvent used for extraction. Extracts were tested for

activity using the standard spot testing method followed by bioautography. Extracts were also tested by a disc diffusion assay.

For the disc diffusion assay, discs (0.5 cm diameter) were punched from Whatman no. 2 filter paper, placed in a glass petri dish, autoclaved and dried in a 60°C oven. Sterile discs were spotted with 20, 30 and 40 µl of extract (two discs at each concentration were prepared for every extract) and dried at 30°C in a sterile glass petri dish. One loopful of the test bacteria was used to inoculate a universal containing 10 ml Luria broth. The cultures were vortexed briefly and incubated at 37°C for 18-24 h with constant shaking. The OD_{600} was determined, the cultures were diluted to OD_{600}=0.5 with sterile Luria broth and 100 µl of the diluted culture was spread-plated onto Luria agar. Plates were incubated at 28°C for approximately 30 min to allow the surface of the agar to dry. Impregnated filter discs were aseptically transferred to the surface of the agar using a sterile needle and plates were incubated for 48 h at 37°C. All plates were prepared in duplicate and the results are an average of the two. The activity was determined as follows: activity (mm^2) = total area of inhibition (mm^2) - area of filter disc (mm^2).

3.3.3 PCR screening

PCR primers which target genes involved in the biosynthesis of aminoglycosides, ansamycins, glycopeptides and PKS-II were tested (Table 3.2). The Foxy and Roxy primers for PCR screening for the biosynthetic genes involved in the production of glycopeptide antibiotics target one of the monooxygenase genes (*oxyB)* and amplify a DNA fragment of 591 bp (Wood *et al.*, 2007).

The primers for PCR screening for the biosynthetic genes involved in the production of ansamycins target the 3-amino-5-hydroxy-benzoic acid (AHBA) synthase gene. The primers ANSA-F and ANSA-R amplify a DNA fragment of 641 bp (Wood *et al.*, 2007).

The primers for PCR screening for the biosynthetic genes involved in the production of Type-II polyketides target the tandem PKS gene pair, ketosynthase alpha (KS_α) and ketosynthase beta (KS_β). The forward primer, ARO-PKS-F, binds towards the 3' end of the KS_α gene and the reverse primer ARO-PKS-R, binds towards the 5' end of the KS_β gene. The ARO-PKS primers amplify a DNA fragment of 492 bp to 630 bp (Wood *et al.*, 2007).

The primers for PCR screening for the biosynthetic genes involved in the production of aminoglycosides target the 2-deoxy-*scyllo*-inosose (DOI) synthase involved in the synthesis of 2-deoxystreptamine antibiotics. The primers were designed from the *gntB* gene sequence from *M. echinospora* ATCC 15835 (involved in gentamicin biosynthesis) and the *tbmA* gene from '*Streptomyces tenebrarius*' ATCC 17920 (involved in the biosynthesis of tobramycin). These primers amplify a DNA fragment of 642 bp (V. Darji, pers. comm.).

Genomic DNA was used as a template for PCR screening. Actinomycetes were grown in 10 ml YEME broth for 18-72 h at 30°C with constant shaking. Genomic DNA was extracted using a modified version of the method of Wang *et al.* (1996) as outlined in Chapter 2, Section 2.3.2.1. PCR was carried out in 50 µl reaction volumes. Each reaction contained 2 mM $MgCl_2$, 0.1 U Super-Therm *Taq* polymerase (JMR Holdings, USA), 150 µM of each dNTP, 0.5 µM of each primer and 200-500 ng template DNA. PCR was performed using a Techne thermal cycler, model TC-512. The PCR programme used was: an initial denaturation at 96°C for 2 min, followed by 30

cycles of denaturation (96°C for 45 s), annealing for 30 s (Foxy/Roxy at 60°C; ANSA-F/ANSA-R at 56°C; ARO-PKS-F/ARO-PKS-R at 64°C; DINOS-F/DINOS-R at 56°C), and extension (72°C for 2 min), and a final extension at 72°C for 5 min. The PCR products were electrophoresed on 1.5% agarose gels containing 10 µg/ml ethidium bromide in 1 X TAE buffer (Sambrook *et al.*, 1989) at 100 V for 1 h and visualised on GelDoc XR System (BioRad). A *Pst*I digestion of λ DNA was included on all gels as a molecular size marker. A positive control was included for all reactions. *'Streptomyces polyantibioticus'* strain SPR was the positive control for the ARO-PKS-F/ARO-PKS-R primers, *Amycolatopsis lurida* NRRL 2430^T was the control for Foxy/Roxy primers, *Amycolatopsis mediterranei* NRRL B-3240^T was the control for the ANSA-F/ANSA-R primers, and *M. echinospora* NRRL 2985^T was the control for the DINOS-F/DINOS-R primers.

Table 3.2 PCR primers used for the PCR screening for antibiotic biosynthetic genes.

Primer name	Primer sequence	Primer length	Source
Foxy	5' - CTGGTCGGCAACCTGATGGAC - 3'	21-mer	Wood *et al.*, 2007
Roxy	5' - CAGGTACCGGATCAGCTCGTC - 3'	21-mer	
ANSA-F	5' - CCSGCSTTCACSTTCATCTC - 3'	20-mer	Wood *et al.*, 2007
ANSA-R	5' - AISYGGAICATIGCCATGTAG - 3'	21-mer	
ARO-PKS-F	5' - GGCAGCGGITTCGGCGGITTCCAG - 3'	24-mer	Wood *et al.*, 2007
ARO-PKS-R	5' - CGITGTTIACIGCGTAGAACCAGGCG - 3'	26-mer	
DINOS-F	5' - CTGMTSGCCGCSCTGCTSTTC - 3'	21-mer	V. Darji
DINOS-R	5' - GGTAGCCSCGCTTGTTGTCGAA - 3'	22-mer	

Key: I, inosine; S = G or C; Y = C or T; M = A or C.

3.3.4 The characterisation of the antimycobacterial compound produced by *Streptomyces speibonae* strain PK-Blue^T

3.3.4.1 Culture conditions

A spore suspension of PK-Blue was prepared from an HM agar plate and was used to inoculate a 100 ml Erlenmeyer flask containing 10 ml HM broth. The pre-culture was incubated at 30°C for 18-24 h with constant shaking. This was used to inoculate a 1 l Erlenmeyer flask containing 100 ml HM and incubated at 30°C for 48 h with constant shaking. The 100 ml culture was used to inoculate a 5 l Erlenmeyer flask containing 400 ml HM which was incubated at 30°C for 4 days with constant shaking. PK-Blue was only maintained on agar for four weeks and was not subcultured more than twice. For long term maintenance, 20% glycerol stock cultures were kept at -70°C.

3.3.4.2 Solvent extraction

A 500 ml HM culture of strain PK-Blue was used for solvent extraction (Figure 3.2) using a method adapted from Sensi *et al.* (1960). The culture was filtered through two coffee filters (size 102) and the pH of the culture filtrate was adjusted to pH 2 with 1 M HCl. The culture filtrate was transferred to a 1 l blue-top glass bottle and 150 ml (30% volume) of ethyl acetate was added. This mixture was shaken vigorously for 20 min to allow the antimicrobial compound to be extracted into the organic solvent. The mixture was left for 30 min to separate into

two layers. The upper organic phase was removed with a glass pipette and transferred to a 1 l blue-top glass bottle to which 500 ml (i.e. equal to the initial culture volume) sterile 10 mM Tris-HCl (pH 7) was added. The two liquids were mixed by vigorous shaking for 15 min to allow the antimicrobial compound to be back-extracted into the aqueous solution. The upper solvent phase was removed and discarded. The aqueous phase was poured into an 800 ml glass beaker and placed in the fumehood overnight to allow the excess solvent to evaporate. The aqueous solution was poured into a 1 l round-bottom flask, freeze-dried and concentrated 50 times by dissolving in sterile water. The concentrated sample was filter-sterilised using an MSI AcetatePlus Cameo syringe filter, pore size 0.22 µm (OSMONICS).

3.3.4.3 Anion exchange chromatography

An anion exchange column was prepared and packed with DE52 (Whatman) according to the manufacturer's instructions. The DE52 matrix was prepared in 100 mM Tris-HCl (pH 7) and packed into a 10 ml glass pipette. The packed column was washed with 30 ml (three column volumes) 10 mM Tris-HCl (pH 7). The 50 times concentrated sample was diluted to one times concentration in 30 ml 10 mM Tris-HCl (pH 7), the pH was adjusted to pH 7 and the sample was applied to the column. Bound compounds were eluted with a step gradient as follows: 20 mM NaCl 10 mM Tris-HCl (pH 7); 50 mM NaCl 10 mM Tris-HCl (pH 7); 100 mM NaCl 10 mM Tris-HCl (pH7); 250 mM NaCl 10 mM Tris-HCl (pH 7); and 500 mM NaCl 10 mM Tris-HCl (pH 7). Five millilitre (5 ml) fractions were collected manually and every second fraction was tested for antimicrobial activity by spot testing and bioautography against *M. aurum*. Active fractions were pooled and further purified by ethyl acetate extraction (Section 3.3.4.2) and freeze dried. The freeze-dried sample was concentrated 25 times. At all stages of the extraction process, the purity of the extract was determined by TLC. The developed chromatograms were treated with cerium (IV) ammonium sulphate (CAS) (63 g $CeNH_4SO_4$ was dissolved in 500 ml 1 M H_2SO_4 and made up to 1 l with deionised water) and heated slowly at 110°C. Organic compounds were detected by the development of brown spots. The presence of the active compound was also monitored by scanning the extracts (1 in 200 dilution in H_2O) from 200 to 400 nm with a Beckman DU-530 spectrometer.

3.3.4.4 NMR, X-ray crystallography and mass spectrometry

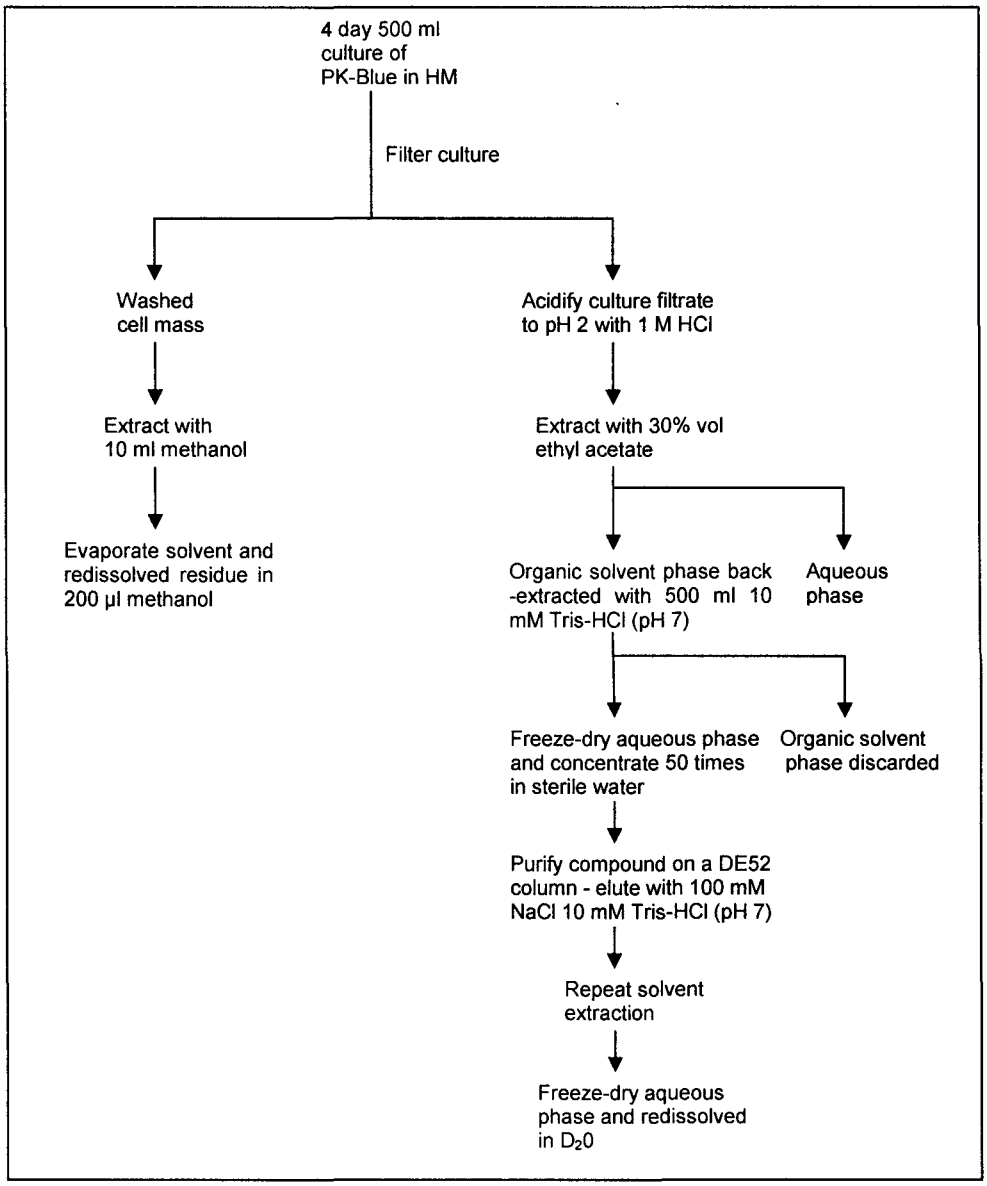
For nuclear magnetic resonance (NMR) analysis, the ethyl acetate extraction method was used and the 50 times concentrated sample was purified on a DE52 column as described above. The antimicrobial compound was further purified by repeating the solvent extraction, however, the compound was back-extracted into sterile deionised water (pH 7) instead of 10 mM Tris-HCl, and freeze-dried. Approximately 12 mg of the freeze-dried compound was dissolved in 200 µl D_20 (deuterated water) in an Eppendorf tube, frozen at -70°C for 2 h and freeze-dried for 6 h. The freeze-dried compound was redissolved in 200 µl D_20, frozen at -70°C for 2 h and freeze-dried again. This step was carried out four times. Approximately 5 mg of the deuterated compound was dissolved in 2 ml of D_20, to which an excess of $NaBH_4$ was added, and left at room temperature for 18 h. Acetic acid was added in a dropwise fashion until the extract was just acidic (pH was monitored by litmus paper). To remove the Na^+ ions, 1 ml of an acidic CM52 cation exchanger (Whatman), prepared according to the manufacturer's instructions, was added to the extract and left for 30 min. The extract was filtered through Whatman no. 2 filter paper to remove the resin. The extract was taken to dryness in a boiling water bath. The residue was redissolved in 5 ml methanol which was evaporated under a stream of nitrogen gas. This final step was repeated three times with the addition of 50 µl of acetic acid to acidify the compound each time it was

redissolved in methanol. After the final evaporation step, the residue was redissolved in D_20. NMR analysis was performed on a Varian *Unity* Inova 600 MHz NMR spectrometer and was performed as a service at the NMR Unit, University of Stellenbosch, South Africa.

For X-ray crystallography, approximately 10 mg of the 25 times concentrated sample was gradually dissolved in 400 µl of chloroform, isopropanol, methanol and toluene in separate glass vials. Vials were covered with Parafilm and a sterile needle was used to pierce several small holes in the Parafilm. The vials were placed at 4°C for 10 days to allow slow evaporation of the solvent and crystals to form. X-ray crystallography was performed on a Nonius KappaCCD diffractometer. X-ray crystallography and elemental analysis were performed as a service at the Department of Chemistry, University of Cape Town, South Africa.

For Electrospray mass spectrometry (ESMS), approximately 10 mg of the compound (crystallised in methanol) was dissolved in methanol. ESMS was performed on a Waters API G-TOF spectrometer (Ultima) at the Central Analytical Facility, University of Stellenbosch, South Africa.

Figure 3.2 Extraction scheme used to extract the antimicrobial compound, PK-B, produced by *S. speibonae* PK-Blue^T.

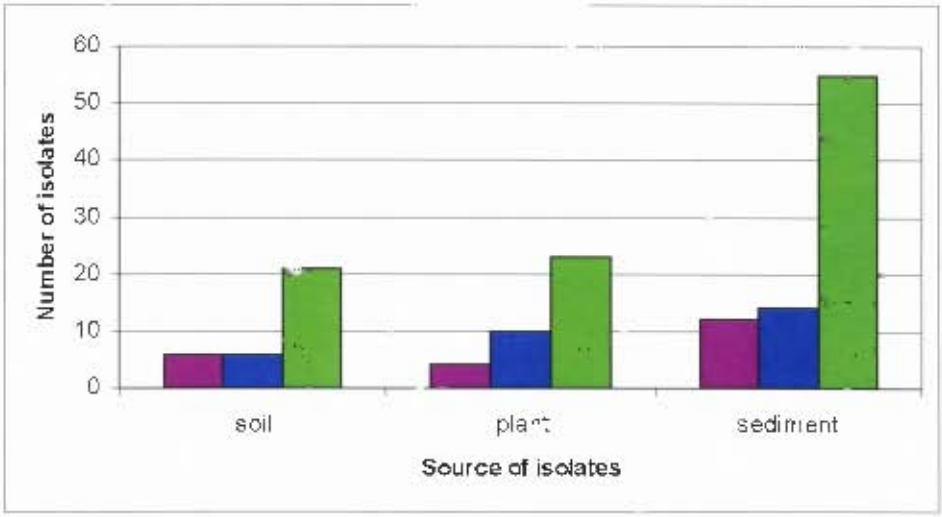


3.4 Results

3.4.1 Antibacterial testing of actinomycete isolates

Of the 30 isolates selected for full characterisation (Chapter 2), 24 had antimicrobial activity against *M. aurum* A+. Initially, 21 strains were isolated from the various soil samples, of which six strains were selected for full characterisation. All six of these isolates were found to have activity against *M. aurum* A+. Only four of the ten strains isolated from plants had activity (40%). However, as 23 strains were initially selected from plants, only 17% of the total number of plant isolates had activity (Figure 3.3). Likewise, 12 of the 14 strains isolated from sediment samples had antimicrobial activity (86%), but as 55 strains were initially selected the percentage of strains with activity is actually 22%.

Figure 3.3 Graph showing the distribution of actinomycete strains with antimicrobial activity against *M. aurum* A+.



Key: purple, the number of actinomycete isolates from each source that had antibacterial activity against *M. aurum* A+; blue, the total number of actinomycetes selected for full characterisation from each source; green, the total number of actinomycetes initially selected from each source.

3.4.1.1 Antibacterial testing by standard agar overlays

The results from the agar overlays against *E. coli*, *Ent. faecium*, *M. aurum* and *S. aureus* are presented in Table 3.3. Of the 30 isolates chosen for full characterisation, 18 (60%) had activity, on at least one medium, against *M. aurum*. All 13 *Streptomyces* strains exhibited antibiosis against *M. aurum*, while only three of the eight *Micromonospora* strains had antibacterial activity against *M. aurum*. AC41 (a *Gordonia* species) and M25 (a *Nocardia* species) were also active against this test bacterium. As M25 grew poorly on the three standard test media, antimicrobial activity was also determined on SM. On SM, the average activity zone against *M. aurum* was Z = 2958 mm^2. Ten isolates had activity against *S. aureus*, all of which were *Streptomyces* species. Only four strains had antibiosis against the Gram-negative test bacterium. All four of the isolates that had activity against *E. coli* were streptomycetes. Berg1C had relatively good activity against *E. coli*. YEME was found to be the best medium for antibiotic production and many of the isolates only had activity on this medium. Riet1C and Zand4Y were found to be the most active when grown on CZ and these two strains were subsequently grown in this medium for solvent extraction and testing by the modified Eli Lilly method.

Table 3.3 Antimicrobial testing of the 30 isolates against *M. aurum*, *S. aureus*, *E. coli* and *Ent. faecium*, using the standard agar overlay method

Actinomycete strain		Source	Medium	Production of aerial mycelium*	Activity against *M. aurum* (mm^2) 7 days	Activity against *M. aurum* (mm^2) 11 days	Activity against *S. aureus* (mm^2)	Activity against *Ent. faecium* (mm^2)	Activity against *E. coli* (mm^2)
AC41	*Gordonia*	Plant	CZ	-	/	/	/	/	/
			7H9	-	/	/	/	/	/
			YEME	-	244	255	/	/	/
Berg1C	*Streptomyces*	Sediment	CZ	+	/	/	/	/	1304
			7H9	+	/	/	/	/	/
			YEME	+	2008	2101	/	/	/
Berg2S	*Streptomyces*	Sediment	CZ	+	/	/	/	/	/
			7H9	+	/	/	/	/	/
			YEME	+	667	3458	1439	/	/
Berg4Y	*Streptomyces*	Sediment	CZ	+/s	/	/	/	/	/
			7H9	+/s	/	/	214	/	/
			YEME	+/s		2275	610	/	/
CGM31	*Micromonospora*	Sediment	CZ	-	/	73	/		
			7H9	-	/	/	/	/	/
			YEME	-	/	148	/	/	/
DG41	*Micromonospora*	Plant	CZ	-	/	/	/	/	/
			7H9	-	/	/	/	/	/
			YEME	-	/	/	/	/	/
Hel32	*Streptomyces*	Soil	CZ	-	46	283	98	69	/
			7H9	+	743	942	70	49	1457
			YEME	s	528	1709	141	/	189
HMC25	*Knbbella*	Soil	CZ	+	/	/	/	/	/
			7H9	+	/	/	/	/	/
			YEME	+	/	/	/	/	/
M25**	*Nocardia*	Soil	CZ	s	/	/	/	/	/
			7H9	-	/	/	/	/	/
			YEME	s	/	/	/	/	/
MuizA5S	*Micromonospora*	Sediment	CZ	-	/	/	/	/	/
			7H9	-	/	/	/	/	/
			YEME	-	/	/	/	/	/
Muiz4Y	*Streptomyces*	Sediment	CZ	+	962	895	1633	/	/
			7H9	+	933	113	/	/	/
			YEME	+	962	3594	214	251	132
NX03U2	*Streptomyces*	Sediment	CZ	+	/	/	137	/	/
			7H9	+	/	/	234	452	/
			YEME	+	/	2098	619	723	123
PBPE	*Micromonospora*	Plant	CZ	-	/	/	/	/	/
			7H9	-	/	/	/	/	/
			YEME	-	/	246	/	/	/

Actinomycete isolate		Source	Medium	Production of aerial mycelium*	Activity against *M. aurum* (mm^2) 7 days	Activity against *M. aurum* (mm^2) 11 days	Activity against *S. aureus* (mm^2)	Activity against *Ent. faecium* (mm^2)	Activity against *E. coli* (mm^2)
PHelU5	*Streptomyces*	Soil	CZ	+	/	/	/	/	/
			7H9	+	/	/	/	/	/
			YEME	+	1897	2395	/	/	/
PLU1	*Micromonospora*	Plant	CZ	-	/	/	/	/	/
			7H9	-	/	/	/	/	/
			YEME	-	/	/	/	/	/
Q41	*Kribbella*	Soil	CZ	+	/	/	/	/	/
			7H9	+	/	/	/	/	/
			YEME	+	/	/	/	/	/
Riet1C	*Streptomyces*	Sediment	CZ	+	/	2930	3267	/	/
			7H9	+	/	50	/	/	/
			YEME	+	/	413	318	/	/
ShaleUP	*Streptomyces*	Soil	CZ	+	/	/	/	/	/
			7H9	+	/	/	/	/	/
			YEME	+		1714	/	/	/
TVU1	*Micromonospora*	Plant	CZ	-	/	/	/	/	/
			7H9	-	/	/	/	/	/
			YEME	-	v	1885	/	/	/
VleiA3C	*Micromonospora*	Sediment	CZ	-	/	/	/	/	/
			7H9	-	/	/	/	/	/
			YEME	-	/	/	/	/	/
Zand2Y	*Streptomyces*	Sediment	CZ	-	25	153	/	/	/
			7H9	-	/	/	/	/	/
			YEME	s	149	1549	106	/	/
Zand4Y	*Streptomyces*	Sediment	CZ	+	/	4996	4430	/	/
			7H9	+	/	/	/	/	/
			YEME	+	1821	1970	657	/	/
Zand8Y	*Streptomyces*	Sediment	CZ	+	/	2185	50	509	/
			7H9	+	/	/	402	/	/
			YEME	+	/	2981	96	/	/
Zand9Y	*Streptomyces*	Sediment	CZ	-	236	2606	57	/	/
			7H9	+	/	/	66	/	/
			YEME	+	587	2985	85	/	/

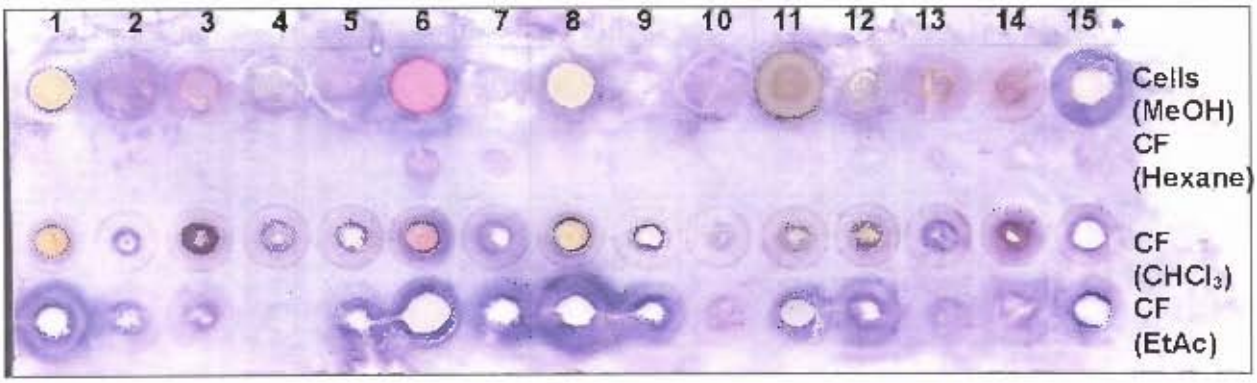
Activity is the average for a minimum of two separate experiments. The first column of results against *M. aurum* are from the stab inoculated actinomycetes that were incubated for 7 days at 30°C, while the second column shows the results for the stab inoculated actinomycetes that were incubated for 11 days at 30°C. For antibacterial testing against *E. coli*, *Ent. faecium* and *S. aureus*, the stab inoculated actinomycetes were incubated for 11 days at 30°C. The overlaid test bacteria were incubated at 37°C for 24 h for *E. coli* and *S. aureus*, while *Ent. faecium* and *M. aurum* plates were incubated for 48 h at 37°C. The six isolates that did not exhibit antimicrobial activity in any of the methods tested were excluded from the table. Excluded isolates: CA21, GIU1, GIU2, GIU3, NX0141 and RAU1. Symbols: +, aerial mycelium was produced; -, aerial mycelium was not produced; s, the production of aerial mycelium was sparse; v, production of aerial mycelium was variable; /, no antimicrobial activity; *, presence of aerial mycelium was recorded from 11 day stab inoculated actinomycetes; **, M25 grew poorly on all three test media, therefore antimicrobial activity was determined on SM, see text (Section 3.4.1.1).

A comparison of the antimicrobial activity against *M. aurum* after 7 days and 11 days incubation clearly shows that antimicrobial production generally improved when the cultures were incubated for 11 days. This may be due to the fact that some of the strains had not sporulated at 7 days. In the genus *Streptomyces*, the production of secondary metabolites normally coincides with the development of aerial hyphae when grown on solid medium, as a number of regulatory genes that are involved in the production of secondary metabolites are also involved in the production of spores and aerial hyphae (Bibb, 2005).

3.4.1.2 Solvent extraction, TLC and bioautography

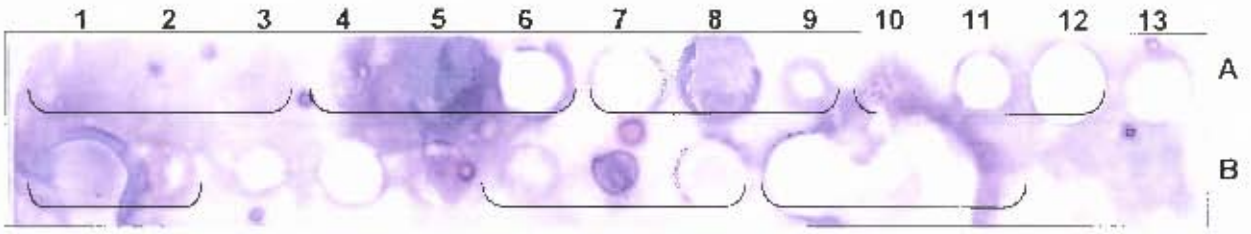
The results for the TLC against *M. aurum*, *M. bovis* and *M. smegmatis* are presented in Table 3.4. At least one extract from 19 isolates was found to be active against *M. aurum*. There was a correlation between the activity detected against *M. aurum* and *M. smegmatis*. *M. bovis* appeared to be less sensitive and generally clear activity was only detectible when 20 µl of the extracts were spotted. Obtaining clear results from spot testing and TLC of some crude extracts was problematic (Figure 3.4 and Figure 3.5). This was mainly due to overloading of the silica plate, especially when testing more polar compounds. The polar compounds spread when the test culture was dabbed on the plates. Generally, for spot testing, 30 µl of extract (spotted in three aliquots of 10 µl) was the most that could be applied, while for plates that were developed, 40 µl was the upper limit (although this was also dependent on the solvent the compound was dissolved in). Despite this problem, spot testing of extracts followed by bioautography has many advantages. This method is relatively quick, it allows one to test many extracts simultaneously, and it is more sensitive and less labour intensive than the disc diffusion assays (especially as the discs are easily contaminated).

Figure 3.4 Bioautography results for spot testing of extracts against *M. aurum* A+



Results for the initial testing against *M. aurum* (2.5 µl of each extract was spotted). Key: **1**, Hel32; **2**, M25; **3**, DG41; **4**, Zand4Y; **5**, Berg4Y; **6**, Berg2S; **7**, PHelU5; **8**, Berg1C; **9**, Muiz4Y; **10**, RAU1; **11**, ZandBY; **12**, CGM31; **13**, PLU1; **14**, VloiA3C; **15**, NXG3U2; CF, culture filtrate; MeOH, methanol, $CHCl_3$, chloroform; EtAc, ethyl acetate. As no antimicrobial activity was detected for the hexane CF extracts from any of the strains, this solvent was not routinely used for solvent extractions.

Figure 3.5 Bioautography results for spot testing of extracts against *M. bovis*.



Results from the initial testing against *M. bovis* (5 µl of each extract was spotted). Unless stated, the three extracts from each isolate were spotted in the order (left to right) methanol, chloroform, ethyl acetate. Key: **A1-3**, AC41; **A4-6**, Berg4Y; **A7-9**, Zand4Y; **A10-12**, NX03U2; **A13**, ShaleUP (methanol extract); **B1-2**, ShaleUP (chloroform, ethyl acetate); **B3**, Berg2S (chloroform extract); **B4**, Zand9Y (methanol extract); **B5**, Zand9Y (chloroform extract); **B6-8**, Muiz4Y; **B9-11**, Riet1C; **B12**, 10 µl methanol (control); **B13**, 10 µl chloroform (control).

Antibiotics were extracted from both the cells and the culture filtrate of eight actinomycete strains, of which six were *Streptomyces* strains. Generally, the culture filtrate extracts were more active than the cell extracts. The culture filtrate extracts of nine isolates inhibited the growth of *M. aurum*, while no activity was detected from their cell extracts. Although the streptomycetes appear to be the most active, they generally grew better than the other genera which may account for the increased antibiotic production. Both the methanol-cell extracts and the chloroform-CF extracts of AC41 (a *Gordonia* species) and Zand9Y (a *Streptomyces* species) were active against *M. aurum*, but no activity was detected from the ethyl acetate extracts. As ethyl acetate has an intermediate polarity, it is possible that both of these isolates produce two different antibiotics each. When the chloroform and methanol extracts of Zand9Y were separated by TLC, the chloroform extract had an active spot with an R_f =0.49, while the methanol extract had an active spot on the origin [when the TLC plates were developed in a solvent system of EtAc:MeOH (90:10; v/v)]. This finding also suggests that Zand9Y may produce two different antimicrobial compounds (Table 3.5). Two *Streptomycete* strains, Riet1C and ShaleUP, had similar activity profiles (Table 3.4). Only the cell extracts of both these strains had activity against *M. aurum*. However, the chloroform-CF extracts of both these strains inhibited the growth of *M. smegmatis*. This may suggest that both Riet1C and ShaleUP produce two different antimycobacterial compounds.

As only limited information was obtained on the antibacterial agents produced by each isolate, one can not identify the antibiotics present. However, if the data from all the tests is combined it is possible to make a preliminary identification of some compounds. Three *Micromonospora* strains, CGM31, DG41 and PBPE produced pH sensitive, orange to yellow coloured methanol extracts which had an absorption peak at 259 nm (Table 3.5). The extracts from these isolates were orange to yellow at neutral pH and changed to a red-brown colour with the addition of 50 mM NaOH. A number of anthracycline antibiotics were found to have these characteristics, including four deoxy-glycoside antibiotics (analogues of daunorubicin) produced by *Micromonospora* sp. strain ATCC 31366. These compounds are reported to have antibacterial and antitumor activity (Cassinelli *et al.*, 1980). Anthracycline antibiotics are synthesized by a Type-II PKS and a DNA fragment of the expected size was obtained by PCR for CGM31 and DG41 when screened with the ARO-PKS primers that target the PKS-II tandem gene pair (Table 3.8). Therefore, it is possible that these three *Micromonospora* strains produce an anthracycline-type antibiotic.

Table 3.4 Bioautography results for spot testing of solvent extracts against *M. aurum*, *M. bovis* and *M. smegmatis*.

Actinomycete isolate and genus to which it belongs		Source	*M. aurum*			*M. bovis*			*M. smegmatis*		
			Cells MeOH	CF EtAc	CF $CHCl_3$	Cells MeOH	CF EtAc	CF $CHCl_3$	Cells MeOH	CF EtAc	CF $CHCl_3$
AC41	*Gordonia*	plant	+	-	+	-	-	-	-	-	-
Berg1C	*Streptomyces*	sediment	+	+	+	+	+	+	+	+	+
Berg2S	*Streptomyces*	sediment	+	+	+	-	-	+	+	+	+
Berg4Y	*Streptomyces*	sediment	-	+	+	-	+	-	-	IND	-
CGM31	*Micromonospora*	sediment	-	+w	+w	/	/	/	/	/	/
DG41	*Micromonospora*	plant	-	+w	+w	-	-	-	-	+w	+w
Hel32	*Streptomyces*	soil	+	+	+	+	+	+	+	+	+
HMC25	*Kribbella*	soil	-	-	-	/	/	/	/	/	/
M25	*Nocardia*	soil	-	+w	+w	-	-	-	-	-	-
MuizA5S	*Micromonospora*	sediment	-	-	-	/	/	/	/	/	/
Muiz4Y	*Streptomyces*	sediment	+w	+	+	-	+	+	-	+	+
NX03U2	*Streptomyces*	sediment	+	+	+	-	+	+	+w	+	+
PBPE	*Micromonospora*	plant	/	/	/	/	/	/	/	/	/
PHelU5	*Streptomyces*	soil	-	+	+	IND	IND	IND	IND	IND	IND
PLU1	*Micromonospora*	sediment	-	-	-	/	/	/	/	/	/
Q41	*Kribbella*	soil	-	-	-	/	/	/	/	/	/
Riet1C	*Streptomyces*	sediment	-/+	-	-	+	-	+	-	-	+
ShaleUP	*Streptomyces*	soil	-/+	-	-	+	-	-	-	-	+
TVU1	*Micromonospora*	plant	-	-	+	/	/	/	/	/	/
VleiA3C	*Micromonospora*	sediment	-	+w	-	/	/	/	/	/	/
Zand2Y	*Streptomyces*	sediment	-	-	+	-	-	-	-	-	-
Zand4Y	*Streptomyces*	sediment	-	+w	-	+	+	-	+w	IND	IND
Zand8Y	*Streptomyces*	sediment	+	+w	+	+	IND	+	+	-	+
Zand9Y	*Streptomyces*	sediment	+	-	+	+	-	-	+	-	+

Key: IND, indistinct, the presence of activity could not be confidently assigned; /, the activity of the *Kribbella* strains and selected *Micromonospora* strains was not tested using the standard solvent extraction method; +, antimicrobial activity was detected; +w, antimicrobial activity was weak; -, no antimicrobial activity was detected; CF, culture filtrate; MeOH, methanol; $CHCl_3$, chloroform; EtAc, ethyl acetate.

Similarly, the methanol extracts from the cells of TVU1 and VleiA3C were a red-orange colour and both had common adsorption peaks at 256 and 271 nm (Table 3.5). A literature search identified a number of angucycline antibiotics with the same physical characteristics (Rohr & Thiericke, 1992). Angucycline antibiotics are also synthesized by Type-II PKSs and both these isolates were positive when screened with the ARO-PKS primers.

Table 3.5 Physical properties of the methanol-cell extracts and chloroform-culture filtrate extracts for the actinomycete isolates.

Isolate	Colour of MeOH extract from cells	Scan 200 to 700 nm	Colour of $CHCl_3$ extract from CF	Properties of the compounds*	pH of culture filtrate
AC41	None	240, 260, 284	None	$CHCl_3$ CF R_f=0.49**	4.39
Berg1C	None	IND	None	/	NC
Berg2S	Red-orange	231, 246, 274	Pinkish-red	$CHCl_3$ CF R_f=0.71	7.97
Berg4Y	None	283	None	/	7.08
CGM31	Yellow-brown	229, **259**	Pink	pH sensitive	NC
DG41	Pale pink/yellow	225, 251, **259**	Bright pink	pH sensitive	NC
Hel32	Yellow-brown	IND	Yellow-brown	MeOH cells R_f=0.91	NC
HMC25	Yellow-brown	IND	Yellow-brown	/	NC
M25	Yellow	253	Very pale yellow	/	8.01
Muiz4Y	Pale yellow	221, 250	None	/	8.15
NX03U2	Pale purple-brown	219, 253	Very pale pink	MeOH cells and $CHCl_3$ CF R_f=0.41	7.05
PBPE	Orange	221, 251, **259**	None	pH sensitive	NC
PHelU5	Pale yellow	224, 258	Very pale yellow	/	NC
Q41	Yellow brown	IND	Yellow brown	/	NC
Riet1C	Black-green	243, 263, 279, 308	None	/	7.72
ShaleUP	Pale yellow	223, 253, 324	None	$CHCl_3$ CF R_f=0.49	8.58
TVU1	Orange	244, **256**, **271**, 315	None	$CHCl_3$ CF R_f=0.49	NC
VleiA3C	Red-orange	207, **256**, **271**	Pink	pH sensitive	NC
Zand2Y	None	253	None	/	8.78
Zand4Y	Brown-green	238, 271	Very pale yellow	pH sensitive	7.55
Zand8Y	Dark brown	IND	Pale brown	MeOH cells R_f=0.93	8.77
Zand9Y	Pale pinkish-brown	222, 245, **257**, 269	None	$CHCl_3$ CF R_f=0.49 and MeOH cells, active spot on origin	5.03

The pH of the culture filtrates are averages of two readings. The methanol extracts were diluted 1 in 500 or 1 in 1000 and scanned from 200 to 700 nm. Symbols: IND, indeterminate (it was not possible to determine the absorption peaks for some crude extracts); NC, no change (original pH of YEME was pH 7.3 and slight changes in pH from pH 7.2 to pH 7.4 were recorded as no change); pH sensitive, refers to the colour of the extract changing with the addition of 50 mM NaOH and/or 50 mM HCl; *, TLC plates were developed in a solvent system of EtAc:MeOH; 90:10 (v/v); **, chloroform CF extract had an active spot with an R_f=0.49; /, no significant characteristic. The methanol extracts were cell extracts, while the chloroform extracts were extracted from the CF. The absorbance values in boldface are those discussed in Section 3.4.1.2.

3.4.1.3 Modified Eli Lilly Method

The results for the modified Eli Lilly method against *M. aurum* are presented in Table 3.6. Generally, there was a good correlation between the results from the modified Eli Lilly method and solvent extraction. The solvent extracts from five strains, DG41, M25, VleiA3C, Zand2Y and Zand8Y, had weak to moderate activity against *M. aurum* (Figure 3.4), however, activity was not detected for these strains using the modified Eli Lilly method (Table 3.6). This result may be due to the fact that the antimicrobial compounds were only produced in very

small quantities and therefore were not detected by this method. The growth of two strains, M25 and Zand2Y was also poor on agar plates, which may have resulted in the lack of detectible activity. The other possibility is that the antimicrobial compounds were not produced when grown on agar, as some actinomycetes have been reported to only produce antibiotics in liquid culture, where there is better aeration and availability of nutrients (Iwai & Ōmura, 1982).

Table 3.6 Antimicrobial activity of the actinomycete isolates against *M. aurum* using the modified Eli Lilly method.

Isolate		Source	Methanol	Ethyl acetate	Chloroform	Toluene	Hexane
AC41	*Gordonia*	plant	+	-	+	-	-
Berg1C	*Streptomyces*	sediment	-	+	+	-	+
Berg2S	*Streptomyces*	sediment	+	-	-	-	-
Berg4Y	*Streptomyces*	sediment	+	-	-	-	-
CGM31	*Micromonospora*	sediment	+	-	+	-	-
DG41	*Micromonospora*	plant	-	-	-	-	-
Hel32	*Streptomyces*	soil	+	-	+	+	+
HMC25*	*Kribbella*	soil	+	+	+	-	-
M25	*Nocardia*	soil	-	-	-	-	-
MuizA5S	*Micromonospora*	sediment	-	-	-	-	-
Muiz4Y	*Streptomyces*	sediment	-	+	+	+	+
NX03U2	*Streptomyces*	sediment	-	-	+	-	-
PBPE	*Micromonospora*	plant	+w	-	-	-	-
PHelU5	*Streptomyces*	soil	-	-	+	-	-
PLU1	*Micromonospora*	plant	-	-	-	-	-
Q41*	*Kribbella*	soil	+	+	+	-	-
Riet1C	*Streptomyces*	sediment	-	-	+	-	-
ShaleUP	*Streptomyces*	soil	-	-	+	-	-
TVU1	*Micromonospora*	plant	-	-	+	-	-
VleiA3C	*Micromonospora*	sediment	-	-	-	-	-
Zand2Y	*Streptomyces*	sediment	-	-	-	-	-
Zand4Y	*Streptomyces*	sediment	-	-	-	+	-
Zand8Y	*Streptomyces*	sediment	-	-	-	-	-
Zand9Y	*Streptomyces*	sediment	-	-	-	+	-

Unless indicated otherwise in the results section, isolates were grown on YEME for 11 days at 30°C. The solvents are arranged in order of decreasing polarity: methanol, 5.1; ethyl acetate, 4.4; chloroform, 4.1; toluene, 2.4; hexane, 0.0 (http://www.phenomenex.com/phen/Doc/z366.pdf); * the two kribbellae strains were grown on production medium for 5 days at 30°C.

3.4.2 The effects of alternative growth media on antibiotic production

3.4.2.1 Production of antibiotics by *Kribbella* strains HMC25^T and Q41^T

The production of antibiotics by both HMC25 and Q41 improved when cultured in production medium. When these two strains were initially tested against the 14 test bacteria (excluding *M. tuberculosis*) listed in Table 3.1, both only had activity against the *Micrococcus* sp. For Q41, the average activity against the *Micrococcus* sp. was Z =2073 mm^2 and 3958 mm^2 on CZ and YEME, respectively. While the average activity for HMC25 against the *Micrococcus* sp. was Z =126 mm^2 and 528 mm^2 on CZ and YEME, respectively. When grown in YEME, the solvent extracts from both kribbellae did not have any detectable antimicrobial activity against *M. aurum* (Table 3.4). However, the solvent extractions of cultures grown in production medium had moderate activity against *M. aurum*, *M. bovis*, *M. smegmatis* and *S. aureus*. It was found that for Q41 the highest antibiotic activity was observed in the methanol cell extract from a production-medium culture grown at 37°C for 5 days, while the methanol cell extract from a production-medium culture of HMC25 incubated at 30°C for 5 days had the best activity. When TLC was performed on these extracts and developed in a solvent system of methanol:chloroform (95:5; v/v), the extracts from both HMC25 and Q41 exhibited two activity spots against *M. aurum* (data not shown). One active spot had an R_f =0.59 and the second active spot remained at the origin. When these extracts were tested against *M. bovis* and *M. smegmatis*, in the same solvent system, two activity spots were detected for HMC25 and Q41. The one active spot was at the origin, while a second active spot was detected with an R_f=0.72, suggesting that both kribbellae produce two different antimicrobial compounds.

3.4.2.2 Antibiotic production in genus-specific production medium - *Micromonospora*

Generally, the *Micromonospora* isolates grew very well in MA. The only exception was RAU1, which did not grow in this medium. Table 3.7 shows the results for the ethyl acetate and toluene CF extracts for the other seven *Micromonospora* strains against *M. aurum*. These extracts were also spot tested against *M. bovis* and *M. smegmatis*, and the activity was the same as those reported for *M. aurum* (data not shown). It can be seen that all seven isolates had activity against *M. aurum*, including PLU1 and MuizA5S. These two strains had no detectable antimicrobial activity when tested by overlays, standard solvent extraction and the modified Eli Lilly method. No activity was detected for the MeOH cell extracts from any of the *Micromonospora* isolates cultured in MA. It was found that the yield of crude extract (for *Micromonospora* isolates cultured in MA) ranged from 900 µg to 14.3 mg per 1 ml of solvent extract. The majority of these extracts were brightly coloured. Scans from 200 to 600 nm of these crude extracts were complex, so it was not possible to determine the absorption peaks of the dominant compounds.

Table 3.7 Activity against *M. aurum* for seven *Micromonospora* isolates cultured in modified Aretz medium (MA).

Strain	Toluene		Ethyl acetate	
	Solvent extract spot test	Disc diffusion (mm^2)	Solvent extract spot test	Disc diffusion (mm^2)
CGM31	+	79	+	-
DG41	-	133	-	-
MuizA5S	+	79	+	138
PBPE	+	201	-	24
PLU1	-	-	-	113
TVU1	-	93	+	154
VleiA3C	-	346	+	-

The disc diffusion assay results are the average activity recorded from two experiments and the activity was calculated as described in Section 3.3.2.2. Symbols; +, antibiosis was observed against *M. aurum*; -, no detectible activity against *M. aurum*. The activity from the spot testing of these extracts against *M. bovis* and *M. smegmatis* was the same as that reported for *M. aurum*.

3.4.3 PCR screening

The results of the PCR screening with primers that target the gene clusters involved in the biosynthesis of glycopeptides (*oxyB* primers) and PKS-II (ARO-PKS primers) are presented in Table 3.8. All 30 isolates were negative when screened with the primers that target the AHBA synthase gene (involved in ansamycin biosynthesis). Likewise, all 30 isolates were negative when screened for the DOI synthase gene (involved in the biosynthesis of 2-deoxystreptamine-containing aminoglycosides). As *Micromonospora* strains MuizA5S, PLU1, RAU1 and TVU1 are phylogenetically most closely related to *M. echinospora*, which produces the aminoglycoside gentamicin, it was surprising that none of these four isolates were positive when screened for the presence of the DOI synthase gene (Wagman & Weinstein, 1980).

It was found that 75% of the isolates which had antimicrobial activity (18 of the 24 active isolates) were positive for the presence of the Type-II PKS gene pair. However, as the ARO-PKS primers may amplify a DNA fragment from a spore-pigment PKS, one can not assume that this implies that all 18 isolates have the potential to produce aromatic polyketide antibiotics (Wood *et al.*, 2007). In fact, only two streptomycetes, Berg2S and PHelU5, were found to be negative when screened with these primers. Only three (12.5%) of the active isolates were positive when screened for the presence of the glycopeptide monooxygenase B gene (*oxyB*). The positive strains included two *Micromonospora* species and a *Nocardia* species. The one member of the family *Micromonosporaceae* that is a known glycopeptide producer is *Actinoplanes teichomyceticus* AB8327^T, which produces teicoplanin (Somma *et al.*, 1984). Although a literature search identified a number of glycopeptide antibiotics produced by *Nocardia* species including, actinoidin A2 by *Nocardia* sp. strain F-AAJ-193 (Dingerdissen *et al.*, 1987), most articles refer to strains of *Nocardia orientalis* which produce vancomycin [*N. orientalis* has subsequently been transferred to the genus *Amycolatopsis*, as *Amycolatopsis orientalis* (Lechevalier *et al.*, 1986)]. Of the six isolates that had no detectible antimicrobial production (CA217, GIU1,

GIU2, GIU3, NX0141 and RAU1), only RAU1 was found to be positive when tested with the ARO-PKS primers (data not shown). Both the *Kribbella* isolates, Q41 and HMC25, were negative for all four primer pairs. It is interesting to note that when the nine other *Kribbella* type strains were screened with these primers, a DNA fragment of the correct size (640 bp) was amplified with the ANSA primers for *K. alba* (data not shown), although this stain has no reported antimicrobial activity (Li *et al.*, 2006).

Table 3.8 Results for the PCR screening for the monooxygenase B gene (*oxyB*) and the Type-II polyketide synthase gene pair.

Isolate	Source	ARO-PKS	*oxyB*	Isolate	ARO-PKS	*oxyB*	Source
AC41	plant	-	-	PBPE	-	-	plant
Berg1C	sediment	+	-	PHelU5	-	-	soil
Berg2S	sediment	-	-	PLU1	+	+	plant
Berg4Y	sediment	+	-	Q41	-	-	soil
CGM31	sediment	+	+	Riet1C	+	-	sediment
DG41	plant	+	-	ShaleUP	+	-	soil
Hel32	soil	+	-	TVU1	+	-	plant
HMC25	oil	-	-	VleiA3C	+	-	sediment
M25	oil	+	+	Zand2Y	+	-	sediment
MuizA5S	sediment	-	-	Zand4Y	+	-	sediment
Muiz4Y	sediment	+	-	Zand8Y	+	-	sediment
NX03U2	sediment	+	-	Zand9Y	+	-	sediment

Key: +, DNA fragment of the expected size amplified; -, no fragment was amplified.

3.4.4 The characterisation of the antimycobacterial compound produced by *Streptomyces speibonae* strain PK-Blue^T

Streptomyces speibonae strain PK-Blue^T was isolated by J. M. Pule and T. Kwetane (Meyers *et al.*, 2003), and was found to produce an antimicrobial compound that is effective against *M. aurum* A+. This work forms part of a study to characterise this antimicrobial compound (PK-B) which was started by Lerato Legoete in 2002. The solvent extraction method was adapted from the one used by Ms Legoete (pers. comm.) and all results presented here are the author's work.

The antimicrobial activity of PK-Blue was determined on 7H9 agar against the 15 test bacteria listed in Table 3.1. Weak activity was detected against *Citrobacter braaki* strain 90 (area of the zone of inhibition (Z) =16 mm^2); *Klebsiella pneumoniae* strain 66 (Z=47 mm^2); *Pseudomonas aeruginosa* ATCC 27853 (Z=35 mm^2), *Proteus mirabilis* strain 87 (Z=63 mm^2) and *Serratia* strain 83 (Z=56 mm^2). However, slightly stronger activity was detected against *M. aurum* A+, *M. bovis* BCG and *M. smegmatis* LR 222 where the activity was Z =182 mm^2, 168 mm^2 and 176 mm^2, respectively. However, no activity was detected against *M. tuberculosis*. Both the methanol cell extract and the CF solvent extract had activity against *M. aurum*, *M. bovis* and *M. smegmatis*. The

methanol cell extract had two active spots against these three test bacteria, with an R_f=0.89 and R_f=0.75, when the TLC plates were developed in a MeOH:EtAc (80:20; v/v) solvent system (data not shown). However, as the antibiosis was weak for both compounds, it was decided to focus on the predominant active compound, PK-B, which was extracted from the CF (Figure 3.2).

Initially, it was thought that the ability of the active compound to be extracted into an organic solvent at low pH and then subsequently back-extracted into an aqueous solution when the pH was increased (Figure 3.2), was due to a protonation/deprotonation reaction, most likely due to a carboxylic acid group. However, ^{13}C NMR data did not support the presence of a carboxylic acid group to account for the acidic nature of the compound. Elemental analysis of PK-B found C, 28.10; H, 4.70; N, 1.57. A partial elemental formula for this compound was thus determined to be $C_{21}H_{43}N0_x$ (x, the amount of oxygen present can not be measured and is assumed to account for the remaining ~65%). It is possible that PK-B contains additional elements that were not detected by elemental analysis (the compound was not assayed for sulphur or phosphorous). The molecular weight (MW) obtained from ESMS was *m/z* (mass-to-charge ratio) 365.07 (inaccurate weight), with two additional peaks detected at 122.07 and 243.13.

An extensive literature search for acid-like compounds with a similar partial elemental formula identified a number of tetramic acids including cryptocin, an antimycotic tetramic acid isolated from an endophytic fungus, *Cryptosporiopsis cf. quercina* (Li *et al.*, 2000). PK-B was found to share a number of features with cryptocin, including an UV absorption peak in methanol at λ 204 nm, similar elemental formula (empirical formula of cryptocin is $C_{21}H_{31}N_1O_4$) and a similar mass peak (the former having an $(M + H)^+$ peak at 362) (Li *et al.*, 2000). Selected physico-chemical properties of compound PK-B are shown in Table 3.9.

Table 3.9 Physico-chemical properties of antimycobacterial compound PK-B.

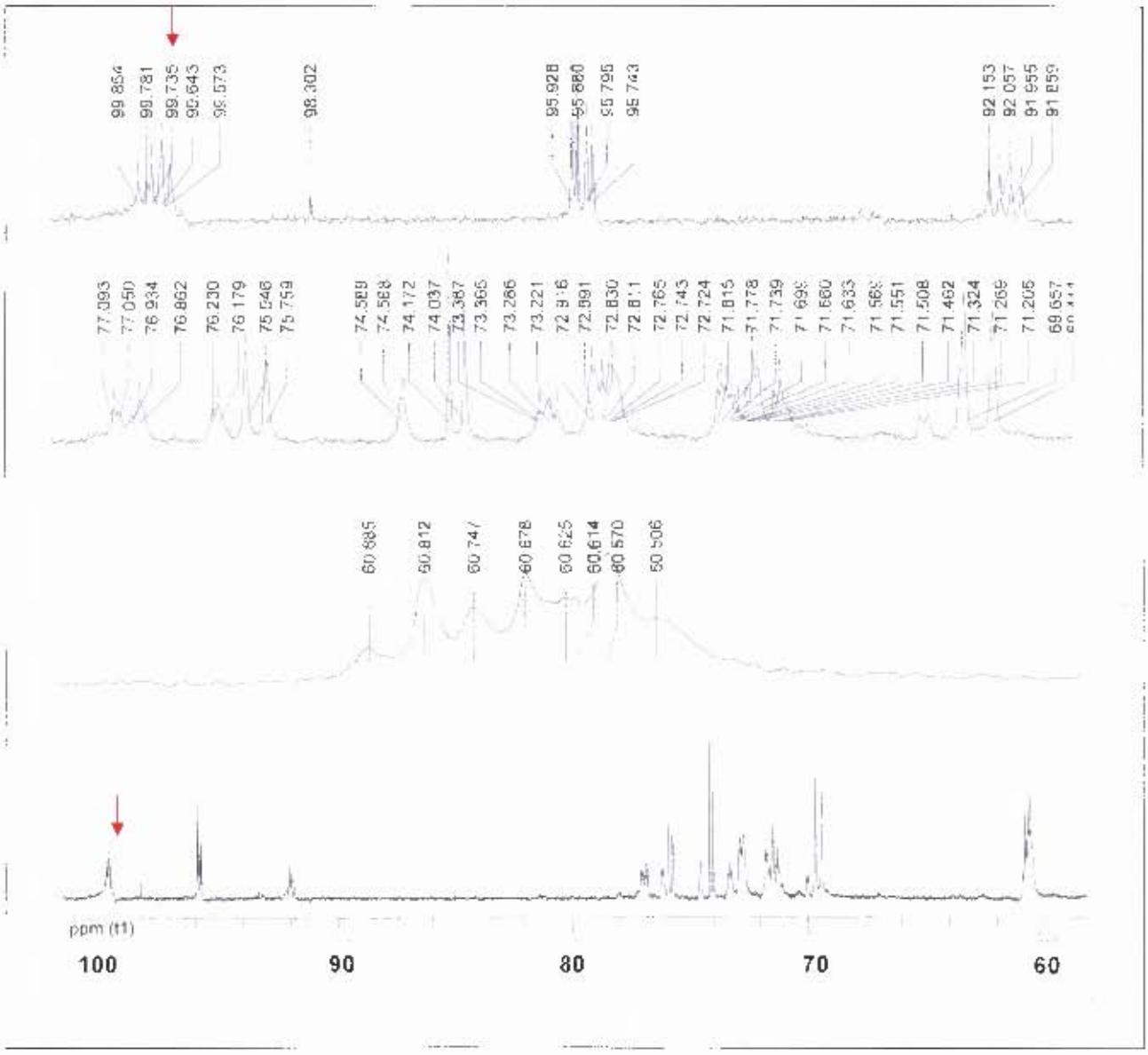
Appearance	Yellow-brown crystals
Partial elemental analysis	C, 28.10; H, 4.70; N, 1.57
Melting point (°C)	> 300
Molecular weight (*m/z*)	365.07
UV λ_{max} nm (in MeOH)	204, 226 and 287
Solubility	Soluble in water and methanol, slightly soluble in isopropanol
R_f in MeOH:EtAc (80:20; v/v)	0.82
Ninhydrin test	Positive*
Fehlings reagent	Positive

All data was determined on a 25-times concentrated sample that had been purified on a DE52 column.
*, ninhydrin test was positive for the presence of an imino (=NH) group.

Attempts to determine the structure of PK-B by NMR were unsuccessful. Many of the peaks in the ^{13}C NMR spectrum were broadened or doubled, which complicated the determination of the structure of the compound. The doubling of peaks may either be the result of the presence of two structurally related compounds or due to

the presence of two energetically similar forms of the same compound. Li *et al.* reported that the structural elucidation of cryptocin was impeded by the doubling of ^{13}C NMR spectra due to the presence of an equilibrium between two energetically similar forms of the compound (Li *et al.*, 2000). A similar finding was reported by Graupner *et al.* (1997). These authors described the compound dihydromaltophilin, a fungicidal tetramic acid produced by a *Streptomyces* species (Graupner *et al.*, 1997). In a review article on naturally occurring tetramic acids, it was stated that the NMR spectra of this family of antibiotics can be complicated by the presence of several tautomeric forms of the tetramic acid ring (Royles, 1995). In an attempt to simplify the NMR spectra, PK-B was treated with sodium borohydride to reduce any functional groups that may be present. However, this was unsuccessful as similar spectra (^{1}H NMR and ^{13}C NMR) were obtained (^{1}H NMR data is not shown; ^{13}C spectrum is shown in Figure 3.6).

Figure 3.6 ^{13}C NMR spectrum obtained from PK-B in D_2O.



The spectrum was obtained from a sample of PK-B that had been treated with $NaBH_4$ to reduce the functional groups, however, the spectrum was similar to that obtained without $NaHBH_4$ treatment (the peaks are still doubled). The red arrows mark the position of the C-atom at δ 99.7 ppm that is characteristic for a tetramic acid moiety.

From these findings it is possible that PK-B may be a tetramic acid. Additional results support this theory. Firstly, PK-B has three absorption peaks when dissolved in methanol, at 204, 226 and 287 nm. The presence of a peak at 287 nm is a characteristic of the tetramic acid moiety (Segeth *et al.*, 2003), while an absorption peak at 204 nm is a common feature of a number of tetramic acids. Secondly, the ^{13}C spectrum has a peak at δ 99.7 ppm (Figure 3.6), which is the characteristic resonance for a. carbon in a tetramic acid moiety of many 3-acyltetramic acids (Michael *et al.*, 2002). Thirdly, the acidic nature of PK-B may be the due to the acidic proton that forms part of the tetramic acid ring (the proton attached to the nitrogen atom). Lastly, many tetramic acids, including tirandalydigin and cryptocin, have been found to crystallise as a sodium salt, with sodium coordinating with each of the oxygen atoms in the molecule (Brill *et al.*, 1988; Li *et al.*, 2000). As the elemental analysis performed on PK-B can only accurately determine the amounts of C, H and N present, it is possible that the compound crystallised with sodium ions which could not be detected. This would account for the partial elemental formula determined for PK-B ($C_{21}H_{43}N0_x$).

As the structure could not be determined by NMR, X-ray crystallography was attempted. Due to the insolubility of PK-B in many solvents, only the sample that had been crystallised in methanol was suitable for X-ray analysis. However, meaningful results could not be obtained as the unit cell value for the crystals was too low (Dr Hong Su, pers. comm.). The one possible explanation for this finding was that the compound had fragmented. The elemental composition of these crystals was found to be C, 30.41; H, 7.80; N, 7.88, from which a partial formula of $C_5H_{14}NO_x$ can be inferred. The spectra obtained from ESMS had two minor peaks at *m/z* 122.07 and 243.13. The sum of these molecular weights is 365.20, which is approximately the mass of the major peak. Therefore, it is possible that the compound had fragmented.

3.5 Discussion

The aim of this part of the study was to identify antibiotics produced by actinomycetes that are effective against *M. tuberculosis*. Ideally, when identifying novel antimicrobials that are effective against a specific pathogen it is best to use the intended target organism in the initial screening program (Peláez, 2006). However, due to its slow growth rate and its pathogenic nature, it is not practical to use *M. tuberculosis* for the routine screening of compounds. *M. aurum* A+ is a non-pathogenic, fast growing mycobacterium that is reported to have a similar antibiotic susceptibility profile to *M. tuberculosis* (Chung *et al.*, 1995). Therefore *M. aurum* A+ was used for all preliminary screening and antibiotic characterisation. From the results of this study, it was found that *M. aurum* A+ and *M. smegmatis* LR 222 have similar antibiotic susceptibilities, while *M. bovis* BCG (Tokyo) was less sensitive to the tested compounds. Although the cell extracts from the two kribbellae grown in production medium had activity against these three mycobacteria, no antimicrobial activity was detected against *M. tuberculosis*. Likewise, the compound PK-B had activity against *M. aurum*, *M. bovis* and *M. smegmatis*, but no antimicrobial activity was detected against *M. tuberculosis*.

Of the 30 isolates chosen for full characterisation, antimycobacterial activity against *M. aurum* was detectible in 24 isolates (80%). It is estimated that 74% of the natural products derived from actinomycetes exhibit some form of antimicrobial activity (Bérdy, 2005). Of the six isolates that had no detectible activity, four were *Kineococcus* species. The only secondary metabolites that kineococci have been reported to produce are carotenoid pigments (Phillips *et al.*, 2002). All four *Kineococcus* isolates produced an orange pigment that was soluble in methanol. Although these methanol extracts did not have antimicrobial activity, their absorption peaks were similar to those reported for carotenoid pigments (Phillips *et al.*, 2002) (data not shown). The only other isolates that did not exhibit antimicrobial activity against *M. aurum* were *Micromonospora* species RAU1 and *Gordonia* species NX0141. However, the former failed to grow in MA, in which the other *Micromonospora* strains had enhanced antibiotic production.

The standard agar overlay method found that 57% of the isolates (17 of 30) had activity against *M. aurum* on one or more media. However, the antimycobacterial activity was limited to two genera. All 13 *Streptomyces* strains had activity, as did three of the *Micromonospora* strains (equates to 38% of the micromonosporae). These results are in line with the findings of Peláez (2006) who reported that the incidence of antimicrobial activity in standard agar diffusion assays ranges between 30 and 80% of the isolates, depending on the taxa examined.

The finding that many of the isolates only had activity on YEME agar, a rich medium, is surprising as many secondary metabolites are only produced in response to starvation (Iwai & Ōmura, 1982; Tormo *et al.*, 2003). However, as the cell density in YEME was significantly higher than in the other test media, YEME cultures may have already exhausted the C- and N-supply by 11 days incubation. Media which are deficient in either of these metabolites have been shown to trigger the production of antibiotics, including cephalosporin and vancomycin (Lawrence, 1999). It has also been reported that the production of secondary metabolites can be triggered when cultures enter stationary phase (Bibb, 2005). Tormo *et al.* (2003) reported that growth in either nutrient deficient media or media with alternative carbon sources (other than glucose) can improve metabolite production.

Catabolite repression by glucose has been found to inhibit the production of antibiotics in a number of *Streptomyces* species, including actinomycin production by *Streptomyces antibioticus*, neomycin production by *Streptomyces fradiae* and streptomycin biosynthesis by *Streptomyces griseus* (Iwai & Ōmura, 1982). Therefore, when deciding which media to include in a screening program, at least one medium with an alternative carbon source should be included.

From the results for the overlay experiments it can be seen that only 20% of the isolates had activity against *E. coli* and no isolates had activity that was specific to this Gram-negative test bacterium. Bérdy (2005) reported that only 1.5% of antimicrobial compounds are specific to Gram-negative microorganisms, which may account for the lack of antibiotics effective against Gram-negative pathogens that are under development (Bush *et al.,* 2004). Thirteen of the 18 (72%) active isolates only had activity against the Gram-positive test bacteria. It is estimated that 30% of microbial natural products are specific to Gram-positives (Bérdy, 2005).

Most isolates that exhibited antimycobacterial activity in the initial overlay experiments, also had activity when tested by solvent extraction. As actinomycetes are slow-growing microorganisms, they do not lend themselves to HTS (Lawrence, 1999). The results presented here show that the screening of actinomycetes is still worthwhile. Small scale extraction of 10 ml cultures yielded sufficient compound to perform a number of assays. As these small cultures are convenient to grow and extract, they can be adapted to batch screening schemes. Fifteen to 20 cultures could readily be processed in a day by one person. The majority of the extracts remained stable for several months when stored at -20°C, as has been reported by other authors (Horan, 1999). As all isolates were screened for antibiotic production within one month of isolation, compounds extracted from strains isolated at different times could be directly compared. There was generally a good correlation between the findings from the modified Eli Lilly method and solvent extractions, and the former may be used as a preliminary screen to determine which solvents to use for extraction from larger liquid cultures.

The production of antibiotics by many of the rare actinomycete genera may be restricted under standard culture techniques. It has been reported that by altering the constituents of the culture media, previously unknown antibiotics may be discovered (Iwai & Ōmura, 1982). In this study, it was found that when the two *Kribbella* strains were cultured in medium developed for the production of antibiotics by *Nocardioides* species, antibiotic production was significantly increased. Both HMC25 and Q41 did not inhibit *M. aurum*, *M. bovis* and *M. smegmatis* when tested by standard overlays or solvent extraction. However, when cultured in production medium, the methanol cell extracts from both these isolates inhibited the growth of these three test bacteria (Section 3.4.2.1). The effect of growth temperature on antibiotic production was also evident as, although the growth of Q41 was reduced when cultured at 37°C, antibiotic production was increased. It has been reported that the production of secondary metabolites is usually limited to a narrow temperature range of 5 to 10°C and that the optimal growth temperature need not be the best temperature for antibiotic production (Iwai & Ōmura, 1982).

Likewise, enhanced antibiotic production was observed when the seven isolates belonging to the genus *Micromonospora* were cultured in MA medium. Two strains, MuizA5S and PLU1, did not have detectible activity against *M. aurum* when tested by overlays, the modified Eli Lilly method and solvent extraction. However, when

cultured in MA, antimycobacterial activity was detected by disc diffusion and solvent extraction. This medium was modified by the addition of $MgSO_4$ and L-valine. $MgSO_4$ is reported to increase the production of antibiotics in the genus *Micromonospora*, including the production of fortimicin by *Micromonospora olivasterospora* (Yamamoto *et al.,* 1977), while L-valine is reported to be required for the production of the depsipeptide antibiotics, korkormicins, by *Micromonospora* sp. strain C39500 (Lam *et al.,* 1995).

None of the isolates were positive when screened with the ANSA and DINOS primers. However, it must be considered that a negative result may in fact be a false negative and may be the result of the primers failing to bind to the target gene (Wood *et al.*, 2007). The one limitation of PCR screening is that, given the genetic diversity that is likely to be present in the biosynthetic genes, the true potential of strains to produce antibiotics may be underestimated. In a recent study by Ayuso and co-workers, a number of PCR primers were designed that targeted the ketosynthase gene of PKS Type-I and Type-II pathways. Despite attempts to optimise PCR conditions only 50% of the 12 avermectin domains present in *S. avermitilis* could be detected (Ayuso *et al.*, 2005). When these primers were tested on a number of actinomycete genera, they found that only in the genus *Streptomyces* was there a correlation between antimicrobial activity and the occurrence of biosynthetic genes. From the results presented in this study, it is clear that a screening program based solely on PCR screening is likely to miss a number of strains that produce bioactive compounds. For this method to be viable, it must be combined with culture-based techniques.

Structural elucidation of the compound PK-B was complicated by a number of factors. However, from the results presented here, it is possible that PK-B is a tetramic acid and this information should be used to guide future attempts to determine the structure of this compound. Possible methods that could be used to overcome the problems incurred include determining the NMR spectra with the compound dissolved in pyridine, which can be used to obtain spectra which are not as broad as those obtained in D_2O. This method was used by Sata *et al.* (1999b) to overcome the broadening of spectra obtained in CD_3OD when determining the structure of the tetramic acids rubrosides A-H. Generally, tetramic acids only contain C, H, N and O. However, rubrosides A-H (Sata *et al.*, 1999b) and pachydermin (Lang *et al.*, 2006) contain chlorine (Figure 3.1). As the partial elemental formula determined for PK-B suggests that this compound may contain other elements, elemental analysis should be repeated testing for the presence of sulphur, phosphorous and chlorine. PK-B has antimicrobial activity against *M. aurum* A+, *M. bovis* BCG and *M. smegmatis* LR 222, however, no antimicrobial activity was detected against *M. tuberculosis*. As PK-B is known to have antimycobacterial activity, future studies of this compound should attempt to improve its potency and to extend its antimycobacterial spectrum to include *M. tuberculosis*. Recent studies has reported the characterisation of the biosynthetic gene clusters involved in the production of the tetramic acids streptolydigin (Yu *et al.*, 2006) and α-lipomycin (Bihlmaier *et al.*, 2006). Therefore, combinatorial biosynthesis could be attempted, based on the premise that PK-B is a tetramic acid. This will require that the biosynthetic gene cluster be cloned, sequenced and characterised.

As expected, *Streptomyces* was found to be the most prolific antibiotic producing genus. In fact, all 13 *Streptomyces* isolates had antimicrobial activity against *M. aurum* A+. Nearly 30% of the strains initially isolated from soil were found to produce antimicrobial compounds effective against *M. aurum* A+. Twelve of the 14 strains isolated from sediment had activity against *M. aurum* A+. Only four plant isolates had activity against

M. aurum A+, of which three strains were *Micromonospora* species and the fourth was a *Gordonia* species. This study has shown that non-streptomycete antibiotic producing actinomycetes can be isolated from plants. However, given the low numbers of strains isolated from plants, for a natural product screen of indigenous plants to be successful, a large number of plant species should be used. Many natural product screens focus on the rarer, non-streptomycetes. However, as it is estimated that the genus *Streptomyces* could potentially produce 150 000 antimicrobials, of which only 3% have been reported, and given the ease with which these strains can be isolated from terrestrial environments, they should not be overlooked as a source of novel antibiotics (Watve *et al.*, 2001; Lam, 2007).

3.6 References

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CHAPTER 4

The application of the *gyrB* gene to resolve species relationships within the genus *Kribbella*

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4.1 Summary

Given the advances in molecular biology, many microbial taxonomists feel that a sequencing based method should be developed that can replace DDH for species delineation. Multilocus sequence analysis (MLSA) is an extension of MLST, a method that has been used extensively in epidemiological studies. MLSA could be adapted to environmental and taxonomic studies. MLSA would require the identification of a core set of genes that could successfully resolve the intrageneric relationships within the genus under investigation. The potential of the *gyrB* gene to be used for phylogenetic studies has been investigated within a number of actinomycete genera, including *Gordonia*, *Micromonospora* and the whorl-forming *Streptomyces*. This study aimed to determine whether the *gyrB* gene can discriminate between members of the genus *Kribbella*. Previous studies have found that a *gyrB*-based genetic distance of 0.014 correlates to a DNA relatedness of 70% and those strains with a genetic distance of greater than 0.014 are likely to be distinct species. In this study, the *gyrB*-based genetic distances between *Kribbella* type strains were found to range from 0.0525 to 0.1081, supporting their classification as distinct species. Phylogenetic analysis based on the *gyrB* gene had improved resolution compared to that based on the 16S rRNA gene sequence. The *gyrB* based trees were also more robust as shown by increased bootstrap values. The *gyrB* gene and 16S rRNA gene sequences were concatenated and this chimeric sequence was used for phylogenetic analysis. The topography of this tree was most similar to that of the *gyrB* tree. Based on this study, the *gyrB* gene can be used to classify *Kribbella* isolates to the species level.

CHAPTER 4

The application of the *gyrB* gene to resolve species relationships within the genus *Kribbella*

4.2 Introduction

The current species definition defines a bacterial species as including "strains with approximately 70% or greater DNA-DNA relatedness and with 5°C or less ΔT_m" and that "phenotypic characteristics should agree with this definition" (Wayne *et al.*, 1987). Although the selection of a 70% threshold was empirically based, this definition was only meant to be a guideline. However, some taxonomists have rigidly enforced these artificial boundaries when classifying novel species and the classification of some unique genospecies has not been supported by phenetic characterisation (Rosselló-Mora, 2006).

The 16S rRNA gene can distinguish between species up to a level of 98.5% sequence similarity, while DDH is superior for closely related species (Stackebrandt & Ebers, 2006). The 16S rRNA gene lacks the sensitivity to resolve close species relationships, especially between recently evolved species, due to its slow evolution rate. Similarly, at a level of >99% sequence similarity, sequencing errors are more significant than for distantly related strains (Fox *et al.*, 1992; Keswani & Whitman, 2001).

Despite the supremacy of DDH in bacterial taxonomy, there are a number of disadvantages to this method. Firstly, data generated can not be used to create a comparative database. Secondly, a number of parameters can affect the results, including genome size and DNA purity, with reciprocal values differing by up to 15% (Rosselló-Mora, 2006; Stackebrandt & Ebers, 2006). Thirdly, DDH is time consuming and laborious. Lastly, as this method requires a high level of technical expertise, it is usually only performed by specialised laboratories (Gevers *et al.*, 2005).

The ad hoc committee for the re-evaluation of the species definition in bacteriology proposed that new molecular based methods should be developed to replace DDH (Stackebrandt *et al.*, 2002). Molecular techniques which could be used in bacterial taxonomy include DNA profiling methods, such as AFLP and PCR-RFLP, DNA arrays and the sequencing of housekeeping genes. Ideally, any new typing system should allow for the construction of a database, be reliable and practical. Most importantly, the results obtained from this method should agree with current classifications schemes (Stackebrandt *et al.*, 2002; Rosselló-Mora, 2006).

Housekeeping genes, which code for proteins involved in central metabolism, evolve faster than rRNA genes and can be used to distinguish between closely related organisms (Maiden, 2006). Zeigler proposed that selected protein coding genes could be used to predict whole genome relatedness. The criteria used by Zeigler when selecting candidate genes were: the genes should be widely distributed among bacterial genomes, they must be present in single copy, the ideal length would be from 900 to 2250 bp to allow for rapid sequencing, but also have sufficient length to be informative, and the gene sequences must be useful in predicting whole

genome relatedness. Thirty two protein coding genes fulfilled these criteria, of which the *recN* gene was found to be the best predictor of whole genome relatedness. Zeigler found that the sequence of a single protein-coding gene may be sufficient to distinguish between species (Zeigler, 2003). However, as more whole genome sequences have been published, it has become apparent that many of the 32 genes originally identified by Zeigler no longer fulfil the selection criteria as they are present.in <95% of bacterial genomes or are not single copy (Coenye *et al.,* 2005).

Studies have found that a number of protein coding genes can infer phylogenetic relationships within different bacterial genera. These include the *rpoD* gene within the genus *Pseudomonas* (Yamamoto *et al.,* 2000), the HSP60 gene (which codes for a chaperonin protein) within the genus *Staphylococcus* (Goh *et al.*, 1996) and the recombination/repair gene *recA* within the genus *Mycobacterium* (Blackwood *et al.*, 2000). As phylogenetic analysis based on a single gene can be affected by horizontal gene transfer (HGT) and variable mutation rates (Coenye *et al.,* 2005), a number of typing schemes compare multiple loci, thereby overcoming the effects of HGT.

Multilocus sequence typing (MSLT) has been used extensively in epidemiology and there are published MLST schemes for a number of human pathogens, including *Neisseria* species (over 7550 isolates included in the database), *S. aureus* (1530), *Haemophilus influenzae* (563) and *E. coli* (764) (Maiden, 2006). MLST can readily distinguish between strains, even within clonal populations (Maiden *et al.*, 1998). The main advantage of MSLT is that it allows for the creation of databases that can be accessed over the internet, allowing isolates to be compared to published strains without having to obtain and culture the strains (Maiden, 2006).

Multilocus sequence analysis (MLSA) is an extension of MLST. Unlike MLST, in MLSA the sequences obtained are concatenated and used to construct a phylogenetic tree (Gevers *et al.,* 2005). By comparing a number of loci, the robustness and the discriminatory power of the phylogenetic analysis is increased. Phylogenies obtained from MLSA have been found to correlate with DDH and whole genome sequences (Gevers *et al.,* 2005; Konstantinidis *et al.*, 2006). The immediate bottleneck in the widespread application of this method will be gene selection and primer design. Santos & Ochman proposed a method that could be used to design universal primers to amplify a number of protein coding genes. They identified 39 housekeeping genes that fulfilled the criteria of having two highly conserved regions separated by at least 100 amino acids and designed degenerate primers for ten of these genes. These primers were tested within ten taxonomically diverse genera, however no high G+C Gram-positive species were included in the analysis. The average success rate of the primers was only 60% and the primers that targeted the *gyrB*, *lepA* and *recA* genes were the most successful (Santos & Ochman, 2004).

As more bacterial genomes have been published, it has become apparent that it is unlikely that a core set of genes will be found that can be used for all taxa. Instead, a core set of genes that are family or genus-specific should be identified for inclusion within MLSA schemes (Gevers *et al.*, 2005). Candidate genes should include those that have already been found to discriminate between isolates at the species level. Within the Class *Actinobacteria* genes that have been investigated include the *gyrB*, *rpoB*, *recA* and *ppk* genes within the genera

Microbacterium and *Aureobacterium* (Richert *et al.*, 2007), while the nitrogenase iron protein gene *nifH* has been used to classify *Frankia* strains (Gtari *et al.*, 2007).

The *gyrB* gene codes for the β-subunit of DNA gyrase, a type II DNA topoisomerase, which introduces negative supercoils into closed circular DNA molecules (Harayama & Kasai, 2006). The *gyrB* gene has been used for the phylogenetic analysis within the non-actinobacterial genera *Pseudomonas* (Yamamoto & Harayama, 1995), *Acinetobacter* (Yamamoto *et al.*, 1999) and *Aquaspirillum* (Watanabe *et al.*, 1999). One of the reasons why the *gyrB* gene was selected for phylogenetic studies is that, as HGT occurs infrequently in informational genes that are involved in transcription and translation, it is assumed to not undergo HGT (Harayama & Kasai, 2006). There are over 2790 bacterial *gyrB* sequences available, of which 409 are for members of the Class *Actinobacteria* (http://www.mbio.co.jp/icb; accessed July 2007).

The ability of the *gyrB* gene to infer inter- and intraspecies relationships has also been investigated within a number of actinobacterial genera, including *Gordonia* (Shen *et al.*, 2006a), *Microbacterium* (Richert *et al.*, 2005), the slow-growing *Mycobacterium* (Kasai *et al.*, 2000a) and the whorl-forming *Streptomyces* (Hatano *et al.*, 2003). Kasai *et al.* used the *gyrB* within the genus *Micromonospora* to reclassify a number of species as strains and elevate some subspecies to species. Kimura's 2-parameter model was used to construct a distance matrix and they found that a *gyrB*-based genetic distance of 0.014 correlated to 70% DNA homology (Kasai *et al.*, 2000b). Subsequent studies by Hatano and co-workers found that a *gyrB* genetic distance of 0.014 corresponded to a *gyrB* sequence similarity of 98.5% (Hatano *et al.*, 2003).

The 16S rRNA gene sequence similarities between *Kribbella* species range from 97.5 to 99.1%. Although isolates can be differentiated based on phenetic characteristics, DDH is usually required to distinguish them as unique species. The aim of this study was to evaluate the ability of the *recN* and *gyrB* genes to resolve species relationships within the genus *Kribbella*. Genes that can distinguish between species could ultimately be included within a MLSA scheme. This classification system could be used in taxonomic studies and for species delineation within the genus *Kribbella*.

4.3 Materials and Methods

4.3.1 Bacterial strains, cultivation and DNA extraction

All strains were grown in YEME at 30°C for 3 to 5 days with constant shaking, except for *K. antibiotica* which was grown at room temperature (Table 4.1).

Table 4.1 Actinomycete stains used in this study.

Strain	Strain number	16S rRNA gene accession number	Source
Kribbella alba	DSM 15500^T	AY082062	DSMZ, Germany
Kribbella antibiotica	DSM 15501^T	AY082063	DSMZ, Germany
Kribbella flavida	CIP 107494^T	AY253863	*Collection de l'Institut Pasteur*, France
'Kribbella hippodromi'	DSM 19227^T	EF472955	Lab isolate
Kribbella jejuensis	CIP 108509^T	AY253866	*Collection de l'Institut Pasteur*, France
Kribbella karoonensis	Q41^T	AY995146	This study
Kribbella koreensis	CIP 108301^T	AY253865	*Collection de l'Institut Pasteur*, France
Kribbella lupini	DSM 16683^T	AJ811962	M. Trujillo
Kribbella sandramycini	DSM 15626^T	AY253864	DSMZ, Germany
Kribbella swartbergensis	HMC25^T	AY995147	This study
Kribbella solani	CIP 108508^T	AY253862	*Collection de l'Institut Pasteur*, France
Kribbella solani	Strain YB2	EF623891	Lab isolate
Kribbella yunnanensis	DSM 15499^T	AY082061	DSMZ, Germany
Nocardia brasiliensis	ATCC 19296^T	NA	I. Shankland*
Streptomyces janthinus	NRRL B-3365^T	NA	D. Labeda**

Key: *, Diagnostic Bacteriology Laboratory (C18) at Groote Schuur Hospital, Cape Town, South Africa; **, Actinobacterial Culture Collection, USDA Agricultural Research Service; NA, not applicable.

For genomic DNA extraction, strains were grown in 10 ml YEME broth for 18-72 h at 30°C with constant shaking. Except for HMC25, Q41 and *K. antibiotica*, genomic DNA was extracted from all actinomycete strains using a modified version of the method of Wang *et al.* (1996) as outlined in Chapter 2, Section 2.3.2.1. A large scale genomic DNA extraction method (Mandel & Marmur, 1968) was used to extract genomic DNA from HMC25, Q41 and *K. antibiotica*. A loopful of spores was used to inoculate a 100 ml Erlenmeyer flask containing 10 ml YEME which was incubated for 24-48 h with constant shaking. This 10 ml culture was used to inoculate a 1 l Erlenmeyer flask containing 100 ml YEME and the culture was incubated for 5 to 7 days with constant shaking. HMC25 and Q41 were incubated at 30°C, while *K. antibiotica* was incubated at room temperature (~22°C).

4.3.2 PCR amplification of the *recN* gene

Primers MS-*recN*-F1 and MS-*recN*-R5 (Table 4.2) are based on the *recN* sequences from eight members of the Class *Actinobacteria*: *Mycobacterium avium* subsp. *paratuberculosis* strain K-10 (accession number NC_002944), *Mycobacterium bovis* AF2122/97 (BX248339), *Mycobacterium leprae* strain TN (AL583921),

Mycobacterium tuberculosis H37Rv^T (BX842577), *Mycobacterium tuberculosis* CDC 1551 (NC_002755), a partial sequence from *Nocardia brasiliensis* ATCC 19296^T (P. Meyers), *Streptomyces avermitilis* MA-4680^T (AP005046) and *Streptomyces coelicolor* A3(2) (AL939110).

Primer MMSN-*recN*-F2 is based on 9 *recN* sequences: *Micromonospora aurantiaca* (A. Cook); *M. avium* subsp. *paratuberulosis*, *M. leprae*, *M. tuberculosis*, *N. farcinica*, *N. brasiliensis*, *S. avermitilis*, *S. coelicolor* and *Streptomyces janthinus* NRRL B-3365^T (primer designed by G. Everest).

Primer *recN*-R1455 was designed using the *recN* sequence from *N. brasiliensis*, *M. aurantiaca* and 17 streptomycete sequences (primer designed in this study).

Table 4.2 PCR primers used to amplify the *recN* gene.

Primer	Sequence	Length (bp)	Annealing temp (°C)	Source
MS-*recN*-F1	5' - GGYRCIGGCAAGACSATGGTGG - 3'	22	57	P. Meyers
MS-*recN*-R5	5' - ACYGCIGCCYIGCCGCCGAC - 3'	20	59	P. Meyers
MMSN-*recN*-F2	5' - TGCACGGGCARRICGAYCAG - 3'	20	53	G. Everest
recN-R1455	5' - GACICCSGCGTCGACCTCGTCG - 3'	22	61	This study

Redundancy code: I = inosine; R = A or G; S = C or G; Y = C or T.

PCR was carried out in 50 µl reaction volumes. Each reaction contained 2 mM or 4 mM $MgCl_2$ (Table 4.3), 0.1 U Super-Therm *Taq* polymerase (JMR Holdings, USA), 150 µM of each dNTP, 0.5 µM of each primer and 500-1000 ng template DNA. PCR was performed using a Techne thermal cycler, model TC-512. The PCR programme used was an initial denaturation at 96°C for 2 min, followed by 30 cycles of denaturation (96°C for 45 s), annealing for 30 s (annealing temperatures and PCR conditions are given in Table 4.3), and extension (72°C for 2 min), and a final extension at 72°C for 5 min. The PCR products were electrophoresed on 1% agarose gels containing 10 µg/ml ethidium bromide in 1 X TAE buffer (Sambrook *et al.*, 1989) at 100 V for 1 h and visualised on a GelDoc XR System (BioRad). A *Pst*I digestion of λ DNA was included on all gels as a molecular size marker.

Table 4.3 PCR conditions and expected product size for the three combinations of *recN* primers.

Primer combination	Expected size of product (bp)	PCR conditions	Annealing temperature (°C)	Positive control
MMSN-*recN*-F2/MS-*recN*-R5	1100	2 mM $MgCl_2$ and 10% glycerol	60	*Streptomyces janthinus* NRRL B-3365^T
MS-*recN*-F1/*recN*-R1455	1382	2 or 4 mM $MgCl_2$ and 5% glycerol	56	*Streptomyces janthinus* NRRL B-3365^T
MMSN-*recN*-F2/*recN*-R1455	1075	2 or 4 mM $MgCl_2$ and 5% glycerol	62	*Streptomyces janthinus* NRRL B-3365^T

4.3.3 PCR amplification of the *gyrB* gene

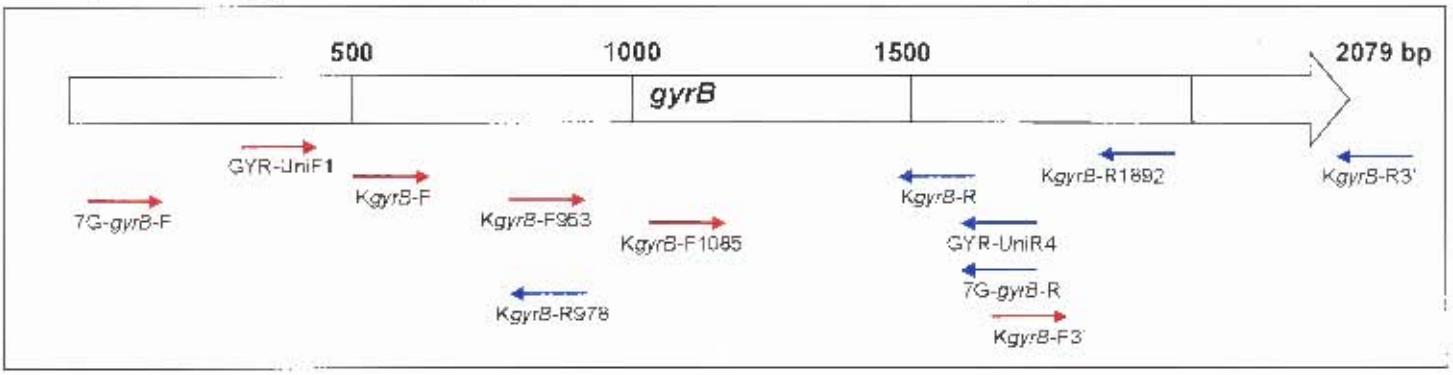
Twelve PCR primers were used to amplify and sequence the *gyrB* gene for thirteen *Kribbella* strains, which included all type strains and two lab isolates (Table 4.1). The primer sequences and relative binding positions are given in Table 4.4 and Figure 4.1. Numbering is based on the full length *Streptomyces avermitilis* MA-4680 *gyrB* sequence (accession number AP005038) (Figure 4.2). Unless stated, all primers were designed in this study.

Table 4.4 PCR primers used to amplify the *gyrB* gene.

Primer	Primer sequence	Primer length	Annealing position***	Annealing temperature (°c)
K*gyrB*-F	5' - WCCCGGACGAGGAYCTGGCCGAG - 3'	23	544-567	62
K*gyrB*-F953	5' - CSGTGCACACBTTCGCGAACG - 3'	21	953-973	59
K*gyrB*-R978	5' - GATCGCGTTCGCGAAVGTGTGCAC - 3'	24	978-955	59
K*gyrB*-F1085	5' - GGCTCACCGGTGACGACRTCCG - 3'	22	1085-1106	61
K*gyrB*-R	5'- CGATCCGGGCCTTCTCGACGTTCAG - 3'	25	1534-1510	62
K*gyrB*-F3'	5' - CGCSCAGCCGCCGCTGTACAAG - 3'	22	1743-1764	62
K*gyrB*-R1892	5' - CCSAGRCCCTTGWAGCGCTGG - 3'	21	1892-1872	59
K*gyrB*-R3'	5' - CGBACSTCSGGCAGCGCGCG - 3'	20	191-172 §	63
7G-*gyrB*-F **	5' - GTICGYAWVCGICCSGGHATGTAC - 3'	24	157-180	55
7G-*gyrB*-R **	5' - CCGTCVACRTCRGCR CSTCSGCCATS - 3'	24	1665-1642	59
GYR-UniF1*	5' - CAIGCIGGIGGYAARTTYG - 3'	19	394-412	46
GYR-UniR4*	5' - CCSTCIACITCIGCRTCSGYCAT - 3'	23	1664-1642	52

Unless stated, the primers were designed as part of this study. *, primers were modified from Yamamoto & Harayama 1995;**, primers designed by P. Meyers (pers. comm.). ***, numbering is based on the full-length *Streptomyces avermitilis* MA-4680 *gyrB* gene; §, birds in the 3' region flanking the *gyrB* gene (Figure 4.2). Redundancy code: B = C, G or T, H = A, C or T; I = Inosine, R = A or G; S = C or G; V = A, C or G, W = A or T; Y = C or T.

Figure 4.1 Binding position of the PCR primers used to obtain a partial fragment of the *gyrB* gene.



The block arrow represents the full length (2079 bp) of the *S. avermitilis* MA-4680 *gyrB* gene. The red arrows represent forward primers and blue arrows present reverse primers.

Primers GYR-UniF1 and GYR-UniR4 are based on the *Pseudomonas* primers UP-1 and UP-2r, respectively (Yamamoto & Harayama, 1995) (P. Meyers, pers. comm.). Primers K*gyrB*-F and K*gyrB*-R were designed based on the partial *gyrB* sequences (amplified with the GYR-UniF1 and GYR-UniR4 primers) from *Kribbella alba* DSM 15500^T, *Kribbella antibiotica* DSM 15501^T and *Kribbella solani* CIP 108508^T.

Primers K*gyrB*-F953, K*gyrB*-F1085, K*gyrB*-F3', K*gyrB*-R978 and K*gyrB*-R1892 were based on the full length *gyrB* gene sequences from *Mycobacterium tuberculosis* H37Rv^T (accession number BX842572), *Nocardia farcinica* IFM 10152 (NC_006361), *Streptomyces coelicolor* A3(2) (AL939125) and *Streptomyces avermitilis* MA-4680^T (AP005038), as well as the partial *gyrB* sequence from HMC25, *K. sandramycini* and *K. antibiotica* obtained in this study. Primer K*gyrB*-R3' was based on the 300 bp that flanks the 3' end of the *gyrB* gene from *N. farcinica*, *S. avermitilis* and *S. coelicolor*.

Primers 7G-*gyrB*-F and 7G-*gyrB*-R were based on 13 *gyrB* sequences from seven actinobacterial genera: *Bifidobacterium longum* AE014685, *Corynebacterium diphtheriae* BX248354, *Corynebacterium efficiens* AP005214, *Corynebacterium glutamicum* AP005274, *Frankia* sp. strain CcI3 CP000249, *Mycobacterium avium* subsp. *paratuberculosis* AE017227, *Mycobacterium bovis* BX248334, *Mycobacterium leprae* AL583917, *Mycobacterium tuberculosis* H37Rv BX842572, *Nocardia farcinica* AP006618, *Streptomyces avermitilis* AP005038, *Streptomyces coelicolor* AL939118 and *Thermobifida fusca* NC_007333 (Primers designed by P. Meyers).

Genomic DNA was extracted as described in Section 4.3.1. PCR was carried out in 50 µl reaction volumes. Each reaction contained 2 mM or 4 mM $MgCl_2$ (Table 4.5), 0.1 U Super-Therm *Taq* polymerase (JMR Holdings, USA), 150 µM of each dNTP, 0.5 µM of each primer and 500-1000 ng template DNA. PCR was performed using a Techne thermal cycler, model TC-512. PCR with the 7G-gyrB-F/7G-*gyrB*-R primers was carried out in 25 µl reaction volumes. The PCR programme used was an initial denaturation at 96°C for 2 min, followed by 30 cycles of denaturation (96°C for 45 s), annealing for 30 s (annealing temperatures are given in Table 4.5), and extension (72°C for 2 min), and a final extension at 72°C for 5 min. The PCR products were electrophoresed on 1% agarose gels containing 10 µg/ml ethidium bromide in 1 X TAE buffer (Sambrook *et al.*, 1989) at 100 V for 1 h and visualised on a GelDoc XR System (BioRad). A *Pst*I digestion of λ DNA was included on all gels as a molecular size marker.

4.3.4 Phylogenetic analysis of the *gyrB* gene

The amplified DNA was purified with either a Cleanmix kit (TA050CLN; Talent, Italy) or MSB® Spin PCRapace kit (Invitek). The purity and quantity of the purified PCR product was determined with a Nanodrop™ spectrophotometer. DNA (55 to 60 ng) was prepared for sequencing using a Big Dye terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and analysed on an Applied Biosystems 3130 Genetic Analyser. Electrophoresis was performed by GeneCare Genetics (Pty) Ltd, South Africa or Macrogen Inc., Seoul, Korea. Chromatograms were edited with Chromas version 2.01 (Technelysium Pty Ltd, Australia) and sequences were assembled in DNAMAN version 4.13; Lynnon BioSoft. Aligned sequences were edited manually. Local alignments were obtained by performing a standard nucleotide-nucleotide BLAST search (*blastn*) (Altschul *et al.*, 1997) of the GenBank database. For phylogenetic analysis, sequences were aligned using CLUSTAL_X,

version 1.81 (Thompson *et al.*, 1997). Phylogenetic analyses were conducted using MEGA version 3.1 (Kumar *et al.*, 2004) and neighbour-joining (Saitou & Nei, 1987); minimum evolution (Rzhetsky & Nei, 1993) and maximum parsimony (Fitch, 1971) trees were constructed. The 16S rRNA gene sequences were downloaded from GenBank (accession numbers listed in Table 4.1) and a phylogenetic tree was constructed as described above. The edited 16S rRNA and *gyrB* gene sequences were concatenated in-frame (the 16S rRNA gene sequence followed by *gyrB* gene sequence) and phylogenetic analysis was performed using the standard method. Edited *gyrB* nucleotide sequences were translated into amino acid sequences in DNAMAN and were aligned using CLUSTAL_X, version 1.81. Phylogenetic analysis of the protein sequences was performed as for nucleotide sequences.

The *gyrB* genetic distances were determined by aligning the sequences in DNAMAN (multiple sequence alignment) and the number of base substitutions was determined manually. The *gyrB* genetic distance was calculated using the equation from Kimura's 2-parameter model: $K = -(\frac{1}{2}) \ln \{(1-2P-Q)\sqrt{(1-2Q)}\}$, where K = evolutionary distance per site; P = fraction of nucleotides showing transitions; Q = fraction of nucleotides showing transversions (Kimura, 1980). All graphs were plotted in Microsoft® Office Excel 2003.

Table 4.5 PCR conditions used for the different *gyrB* PCR primer combinations.

Primer combination	Expected size of product* (bp)	PCR conditions	Annealing temperature (°C)
KgyrB-F/KgyrB-R	958	2 mM $MgCl_2$	60-62
K*gyrB*-F953/K*gyrB*-R3	537	2 mM $MgCl_2$	60
GYR-UniF1/GYR-UniR4	1230	2 mM $MgCl_2$	48-52
K*gyrB*-F1085/K*gyrB*-R	452	2 mM $MgCl_2$	61
K*gyrB*-F/K*gyrB*-R978	400	2 mM $MgCl_2$	57-59
K*gyrB*-F1085/K*gyrB*-R1892	807	2 mM $MgCl_2$	60
K*gyrB*-F953/ K*gyrB*-R1892	939	2 mM $MgCl_2$	60
K*gyrB*-F3'/K*gyrB*-R3'	**520	2 mM $MgCl_2$, 5% glycerol	62
K*gyrB*-F953/K*gyrB*-R3'	**1278	2 mM $MgCl_2$, 2.5% glycerol or DMSO***	61
K*gyrB*-F1085/K*gyrB*-R3'	**1145	4 mM $MgCl_2$, 2% glycerol or DMSO***	61-63
7G-*gyrB*-F/7G-*gyrB*-R	1462	4 mM $MgCl_2$, 5% glycerol	60

*, expected product size based on the full length *Streptomyces avermitilis gyrB* gene; **, due to the variability in length of the 3' flanking region, the minimum expected size is given; ***, reducing the concentration of the K*gyrB*-R3' primer from 0.5 µM to 0.25 µM improved the amplification.

Figure 4.2 Sequence of the full length *Streptomyces avermitilis* MA-4680[1] *gyrB* gene sequence, showing the PCR primer binding sites.

Streptomyces avermitilis gyrB accession number AP005038
SEQ length: 2079 bp
Composition 415 A; 715 C; 655 G; 294 T; 0 OTHER
Percentage: 20% A; 34% C; 32% G; 14% T; 0%OTHER

Molecular Weight (kDa): ssDNA: 641.40 dsDNA: 1282.0
ORIGIN

```
1     GTGGCCGATT CCGGCAACCC CAACGAGAAC ATCCCGTCCA CCGACGCCGG CAACGGCGAG
61    GCCAACACCT CGATCGGCGA GGTCGCAGCC TTCGAACGGC GAGTCACAGC CTCGTACGAC
121   GCCAGCGCCA TCACCGTCCT CGAGGGTCTG GACGCGGTCC GCAAGCGACC CGGTATGTAC   7G-gyrB-F
181   ATCGGCTCGA CCGGTCAGCG CGGACTTCAC CACCTTGTGT ACCAGGTCGT CGACAACTCC
241   GTCGACGAGG CGCTGGCCGG CCACGCCGAC ACGATCGACA TCACGATCCT CGCCGACGGT
301   GGCGTGCGCG TCATCGACAA CGGCCGTGGC ATCCCCGTGG GCATCGTCGC CTCCGAGGGC
361   AAGCCGGCCC TCGAGGTCGT GCTGACCGTC CTGCACGCGG GCGGCAAGTT CGGCGGCGGC   GYR-UniF1
421   GGCTACGCGG TCTCCGGCGG TCTGCACGGC GTCGGCGTGT CCGTCGTGAA CGCCCTGTCC
481   AGCAAGGTCT CCGTCGAGGT CAAGACCGAC GGCTTCCGCT GGACGCAGCA ATACAAGATG
541   GGCGTCCCGA CAGCCCCGCT CCCCAGAAC GAGGCTACGG ACGAGACCGG CACCTCGTTC   KgyrB-F
601   ACCTTCTGGG CCGACGCGGA CATCTTCGAG ACCACCGAAT ACTCCTTCGA GACGCTCTCA
661   CGGCGCTCCC AGGAGATGGC GTTCCTCAAC AAGGGTCTGA CGATCAAACT CACTGATGAG
721   CGCGAGTCGG CGAAGGCCAC CGCCGGCGCG GACGAGGCCG GTGCGGACGA GAAGGACGAG
781   GTCAAGACCG TCACGTACCA CTACGAGGGC GGCATCGTCG ACTTCGTGAA GTACCTCAAC
841   TCCCGCAAGG GAGACCCGGT GCACCCCACC GTCATCGACC TCGAGGCCGA GGACAGGGAC
901   AAGAGCCTGT CCCTCAAGGT CGCGATCGAG TGGAACAGCG GCTACACCGA GGGCGTGTAC   KgyrB-F953 (underlined)
961   TCCTTCGCCA ACATCATCCA CACCCACGAG GGCGGCACGC ACGAGGAGGG CTTCCGCGCG   KgyrB-R978(boldface)
1021  GCGCTGACCT CGTTGATCAA CAAGTACGCG CGCGACAAGA AGCTGCTGCG CGAGAAGGAC
1081  GACAACCTCA CGGGCGACGA CATCCGCGAG GGTCTGACCG CGATCATCTC GGTCAAGCTG   KgyrB-F1085
1141  AGCGAGCCGC AGTTCGAGGG CCAGACAAAG ACCAAGCTGG GCAACACCGA GGCGAAGACC
1201  CTCGTCCAGA AGGCGCTCTA CGAGCACCTC AACGACTTGC TGGACCGCAA CCCGAACGAG
1261  GCCGCGGACA TCATCCGCAA GTCGATCCAG GCGGCCACCG CGCGCGTGTC GGCCGCAAG
1321  GCGCGTGACC TCACCCGCCG CAAGGGCTTC CTTGAGTCGG CGTCCCTGCC GGGCAAGCTC
1381  TCCGACTGCC AGTCGAACGA CCCCACCAAG TGCGAGATCT TCATCGTCGA GGGTGACTCC
1441  GCCGGCGGTT CGGCCAAGTC CGGCCGCAAC CCGCACTTCC AGGCGATCCT CCCGATCCGA
1501  GGAAAGATCC TCAACGTCGA GAAGGCGCGG ATCGACAAGA TCCTGCAGAA CCAGGAGATC   KgyrB R
1561  CAGGGCTGA TCTCCGCCTT CGGTACCGGA ATCCACGACG ACTTCGACAT CGAGAAGCTC
1621  CGCTATCACA AGATCATCCT GATGGCGGAC GCCGACGTCG ACGGCCAGCA CATCAACACC   GYR UniR4(underlined)/
1681  CTGCTGCTGA CCTTCCTGTT CCGCTTCATG CGGCCGCTGG TCGAGGCCGG GCACGTGTTC   7G-gyrB-R (boldface)
1741  CTCTCCCGCC CGCCGCTCTA CAAGCTCAAG TGGGGCCGGT ACGACCTCGA GTACGCGTAC   KgyrB-F3'
1801  TCGGACCGCG AGCGCGACGC CCTGATCGAG CTCGGCCGCC AGGCGGGCAA GCGCGTCAGG
1861  GAAGACTCGA TCCAGCGCTT CAAGGGTCTC GGTGAGATGA ACGCCGAGGA ACTGGCATC   KgyrB-R1892
1921  ACGACGATGG ACCACGAGCA CGGCGTCCTC GGCCAGGTCA CCCTCGATGA CGCCGCCCAG
1981  GCCGACGACC TGTTCTCGGT CCTGATGGGC GAGGACGTCG AGGCTCGCCG CGCGTTCATC
2041  CAGCGCAACG CCAAGGACG. CCGCTTCCTC GACA.CTGA
```

Streptomyces avermitilis gyrB 3' flanking region accession number AP005038
SEQ length: 300 bp;
Composition 56 A; 100 C; 94 G; 50 T; 0 OTHER
Percentage: 18.7% A; 33.3% C; 31.3% G; 16.7% T; 0.0%OTHER
Molecular Weight (kDa): ssDNA: 92.55 dsDNA: 184.99
ORIGIN

```
1     GTCGGTCTCA GCTGACCGCA TCAGGAAGGA TCTTCACCAG CAATGACCGA CGAGAACACT
61    CCGAGCACTC CTGAAGAAGA GGGCTAGATC GCCCTGCGTG TCGAGCCCGT CGGGCTCGAG
121   ACCGAGATGC AGCGCTCGTA CCTCGACTAC GCGATGTCCG TCATCGTCGC GCGCGCGCTG   KgyrB-R3'
181   CCCGACGTAC GGGACGATCT CAAGCCCGTC CACCCGCGTG TGCTGTACGC CATGTACGAC
241   GGCGGCTACC GGCCCGAGAA GGGCTTCTAC AAGTGCGCCC GTGTCGTCGG CGACGTCATC
```

The 1232 bp *gyrB* fragment used for analysis is highlighted in blue.

4.4 Results

4.4.1 Assessing the potential of the *recN* gene within the genus *Kribbella*

Most combinations of PCR primers failed to amplify the *recN* gene from any *Kribbella* strains. A DNA fragment of the expected size (1100 bp) was amplified with the MMSN-*recN*-F2/MS-*recN*-R5 primers for HMC25, *K. sandramycini* and *K. flavida*, however, the band was found to be a doublet with the lower band formed by the reverse primer MS-*recN*-R5, only. Attempts were made to separate the bands and excise the correct amplicon from the gel, however, the sequence obtained from this template DNA was of poor quality as contaminating DNA from the second band resulted in high background levels. A BLAST search of a 210 bp sequence obtained from HMC25 was found to be 53% homologous to a *Streptomyces* sp. putative repair gene. A single band was obtained for *K. solani* with this primer combination. However a BLAST search found that the 462 bp sequence was most similar to a uracil phosphoribosyltransferase gene from *Deinococcus geothermalis* DSM 11300^T (CP000359) (70% homology) and *Arthrobacter aurescens* ATCC BAA-1386 (CP000474) (62% homology). *In silico* analysis found that both primers would bind to the *D. geothermalis* DSM 11300^T gene or the flanking regions. Twelve basepairs (12 bp) of the MMSN-*recN*-F2 primer, including the last 4 bp at the 3' end of the primer, would bind to this target DNA (data not shown). The *recN* study was thus abandoned in favour of the *gyrB* gene.

4.4.2 Sequence analysis of the *gyrB* gene

The GYR-Uni-F1/GYR-Uni-R4 primers, adapted from the *Pseudomonas gyrB*-primers, only amplified a DNA fragment from *K. alba* DSM 15500^T, *K. antibiotica* DSM 15501^T and *K. solani* CIP 108508^T (Table 4.6). From these three partial *gyrB* sequences, regions that were highly conserved, within the genus *Kribbella*, were identified and *Kribbella*-specific primers were designed. Although a fragment was amplified for ten of the 13 kribbellae with the K*gyrB*-R3' primer, the amplification was weak and the sequence of the 3' end of the *gyrB* gene was only obtained for four *Kribbella* species (Table 4.6).

Nucleotide substitutions in the third codon position (the wobble position) are generally silent and do not affect the protein sequence. A comparison of the nucleotide and translated protein sequences of the *Kribbella gyrB* genes found that most of the nucleotide substitutions occurred in synonymous positions, with substitutions in the third codon positions ranging from 18% to 52%. Phylogenetic studies of the *gyrB* gene in the genera *Microbacterium* (Richert *et al.*, 2005) and *Pseudomonas* (Yamamoto & Harayama, 1995) also reported that most nucleotide substitutions occurred in synonymous positions. DNA gyrase is required for DNA replication and transcription. Therefore, due to functional constraints, the evolution rate of the GyrB protein is slow.

The 5' region of the *gyrB* gene was found to be variable, which may account for primer combinations containing GYR-UniF1 or 7G-*gyrB*-F failing to amplify the *gyrB* gene of some species. Therefore a common length of over 1600 bp was only obtained from eight *Kribbella* species (Table 4.6). From a multiple sequence alignment it was found that the maximum length of common *gyrB* sequence was 1232 bp and, unless stated, all phylogenetic analysis was based on these truncated sequences.

Table 4.6 The primer combinations that successfully amplified the *gyrB* gene and the total length of the *gyrB* sequence obtained for all the *Kribbella* strains.

Kribbella strain	GYR-uni F1/ GYR-uni R4	K*gyrB*-F/ K*gyrB*-R	K*gyrB*-R/ K*gyrB*-F1085	K*gyrB*-F/ K*gyrB*-R978	K*gyrB*-F953/ K*gyrB*-R1892	7G-*gyrB*-F/ 7G-*gyrB*-R	K*gyrB*-R3'*	Consensus length (bp)
K. alba	+	+	+	/	+	+	/	1624
K. antibiotica	+	+	+	+	+	/	/	1235
K. flavida	/	+	/	+	+	+	/	1606
'K. hippodromi'	/	+	+	+	+	/	+	1672
K. jejuensis	/	+	+	/	+	/	+	1293
K. karoonensis	/	+	+	+	+	+	+	1890
K. koreensis	/	+	+	+	+	/	/	1233
K. lupini	/	+	+	+	+	+	/	1601
K. sandramycini	/	+	+	+	+	+	/	1614
K. swartbergensis	/	+	+	+	+	/	/	1279
K. solani (type strain)	+	+	/	/	+	/	+	1669
K. solani YB2	/	+	+	+	+	/	/	1264
K. yunnanensis	/	+	+	/	+	+	/	1606

*, the K*gyrB*-R3' fragments were amplified with either the K*gyrB*-F1085 or K*gyrB*-F953 primers.

4.4.3 Phylogenetic analysis and determination of the *gyrB*-based genetic distances

The *gyrB* genetic distances between the *Kribbella* type strains ranged from 0.0525 (*Kribbella jejuensis* CIP 108509^T versus *Kribbella solani* CIP 108508^T) to 0.1495 (*Kribbella alba* DSM15500^T versus *Kribbella antibiotica* DSM 15501^T) (Table 4.7). Kasai *et al.* (2000b) reported that in the genus *Micromonospora* a *gyrB*-based genetic distance of 0.014 correlates to 70% DNA relatedness. It was subsequently found that a genetic distance of 0.014 correlates to 98.5% *gyrB* sequence similarity (Hatano *et al.*, 2003). If these threshold values are applied to the genus *Kribbella*, all type strains would be recognised as distinct species (Figure 4.3).

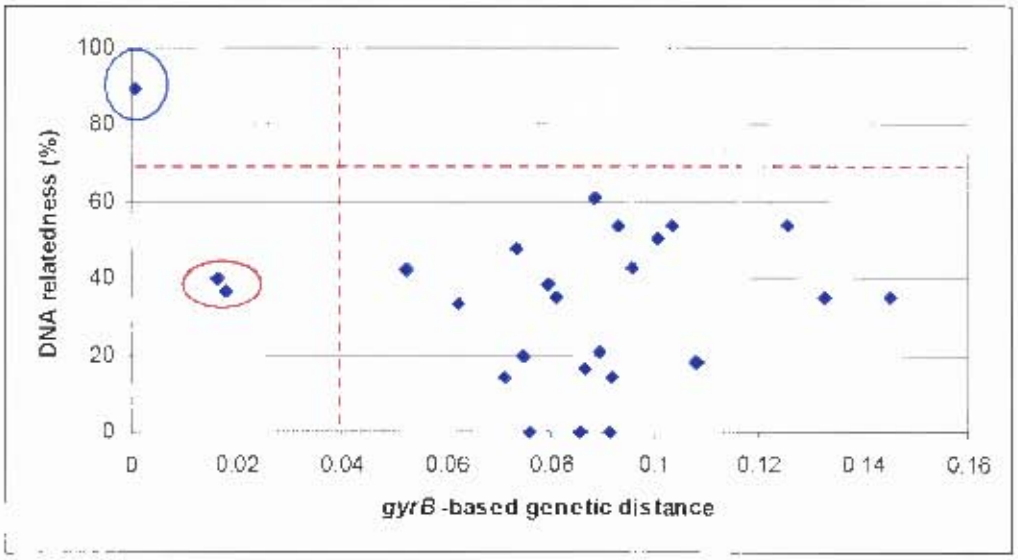
A *gyrB*-based genetic distance of less than 0.04 could be used as a cut-off point to determine whether DDH is required. There are only three data points that are under this threshold value (Table 4.7 and Figure 4.3). The *gyrB*-based genetic distance between the two strains of *Kribbella solani* is 0 (as the *gyrB* sequence similarity is 100%), which indicates that they are strains of the same species (Figure 4.3, data point encircled in blue). This was confirmed by DDH which showed that the DNA relatedness between these strains is 89.3% ±3.1%. The *gyrB* sequence similarity between *'K. hippodromi'* S1-4^T and *K. solani* CIP 108508^T is 98.22% and the *gyrB*-based genetic distance is 0.0164 (Figure 4.3, data point encircled in red, the second data point is the comparison of *'K. hippodromi'* versus *K. solani* YB2). Based on the *gyrB*-based genetic distances reported in other genera, the DNA relatedness between these two strains would be expected to be less than 70%. This was confirmed by DDH which showed that the DNA relatedness between *'K. hippodromi'* and *K. solani* CIP 108505^T is 40.4% ±3.8%. Although the 16S rDNA sequence similarity between these two strains is 99.64%, based on phenetic characterisation (G. Everest pers. comm.), *gyrB* gene sequence analysis and DNA relatedness, *'K. hippodromi'* S1-4^T should be recognised as a distinct species.

Table 4.7 Comparison of the *gyrB*-based genetic distances and *gyrB* sequence similarities for all *Kribbella* strains for which there is published DNA relatedness data.

***Kribbella* strains compared**	***gyrB*-based genetic distance (1232 bp)**	***gyrB* genetic distance over 390 bp***	***gyrB* 1232 nt sequence similarity (%)**	**GyrB AA sequence similarity (%)**	**DNA relatedness (%)**	**Source of DDH data**	**16S rRNA gene sequence similarity (%)**
K. alba vs *K. antibiotica*	0.1495	0.1256	89.48	91.80	35.4	Li *et al.*, 2006	98.21
K. alba vs *K. flavida*	0.0814	0.0936	92.32	94.80	35.4	Li *et al.*, 2006	99.11
K. alba vs *K. koreensis*	0.1034	0.0864	90.42	92.63	53.8	Li *et al.*, 2006	98.83
K. alba vs *K. sandramycini*	0.1081	0.1131	89.91	91.65	18.4	Li *et al.*, 2006	98.55
K. alba vs *K. yunnanensis*	0.0932	0.0747	91.14	93.38	53.8	Li *et al.*, 2006	97.81
K. antibiotica vs *K. flavida*	0.1080	0.0784	92.99	93.36	18	Li *et al.*, 2004	98.44
K. antibiotica vs *K. koreensis*	0.1256	0.0921	91.43	92.19	53.8	Li *et al.*, 2004	98.65
K. antibiotica vs *K. sandramycini*	0.1329	0.0980	91.42	91.41	35.4	Li *et al.*, 2004	98.58
K. antibiotica vs *K. yunnanensis*	0.0920	0.0832	92.59	93.75	14.4	Li *et al.*, 2006	97.86
K. flavida vs *K. jejuensis*	0.0738	0.0755	93.53	95.68	47.8	Song *et al.*, 2004	97.51
K. flavida vs *K. koreensis*	0.0751	0.0641	92.93	92.57	20	Sohn *et al.*, 2003	98.81
K. flavida vs *K. sandramycini*	0.0960	0.1086	90.86	90.80	43	Park *et al.*, 1999	98.94
K. flavida vs *K. solani* (type strain)	0.1006	0.0728	91.16	92.33	50.4	Song *et al.*, 2004	98.61
K. flavida vs *K. yunnanensis*	0.0626	0.0385	94.01	95.30	33.3	Li *et al.*, 2006	98.11
'K. hippodromi' vs *K. solani* (type strain)	0.0164	0.0160	98.22	100	40.4	P. Meyers	99.64
'K. hippodromi' vs *K. solani* YB2	0.0164	0.0160	98.22	100	36.6	P. Meyers	100
K. jejuensis vs *K. koreensis*	0.0896	0.1055	91.57	95.68	20.9	Song *et al.*, 2004	97.75
K. jejuensis vs *K. sandramycini*	0.0762	0.0873	92.76	94.60	0	Song *et al.*, 2004	97.88
K. jejuensis vs *K. solani* (type strain)	0.0525	0.0640	94.77	98.93	42.5	Song *et al.*, 2004	98.15
K. koreensis vs *K. sandramycini*	0.0914	0.1040	91.54	93.63	0	Sohn *et al.*, 2003	98.67
K. koreensis vs *K. solani* (type strain)	0.0868	0.0864	91.8	96.08	16.4	Song *et al.*, 2004	98.08
K. koreensis vs *K. yunnanensis*	0.0715	0.0462	93.39	94.83	14.4	Li *et al.*, 2006	97.84
K. lupini vs *K. sandramycini*	0.0796	0.0980	90.72	90.89	38.5	Trujillo *et al.*, 2006	98.69
K. sandramycini vs *K. solani* (type strain)	0.0859	0.0891	91.71	93.87	0	Song *et al.*, 2004	98.54
K. sandramycini vs *K. yunnanensis*	0.0886	0.0980	90.57	92.12	61.2	Li *et al.*, 2006	98.65
K. solani (type strain) vs *K. solani* YB2	0	0	100	100	89.3	P. Meyers	100

The DNA-relatedness was determined by DDH. Key: nt, nucleotide; AA, amino acid (nucleotide sequences were translated in DNAMAN); *, *gyrB*-based genetic distance determined for the 390 bp fragment (1010 to 1400 bp, *S. avermitilis* numbering).

Figure 4.3 Graph of *gyrB*-based genetic distances versus DNA relatedness.



The two data points in the red oval are the comparisons between '*K. hippodromi*' versus the *K. solani* CIP 108508^{T} and '*K. hippodromi*' versus *K. solani* YB2, respectively. The data point in the blue oval is the comparison between *K. solani* CIP 108508^{T} and *K. solani* YB2. The horizontal dashed line indicates the 70% DNA relatedness threshold, while the vertical dashed line indicates the proposed *gyrB* genetic distance threshold at which DDH is warranted.

A comparison of the sequence similarities for the *gyrB* gene nucleotide sequences to the translated GyrB protein sequences, found that on average the protein sequence similarity was 1.65 % higher than the corresponding nucleotide sequence similarity. The only exceptions were *Kribbella flavida* CIP 107494^{T} versus *Kribbella koreensis* CIP 108301^{T}, *K. flavida* versus *K. sandramycini* DSM15626^{T}, and *K. antibiotica* DSM15501^{T} versus *K. sandramycini* where the protein sequence similarities were slightly lower than the nucleotide sequence similarities. A possible explanation for this finding is that *K. flavida* and *K. sandramycini* had the fewest nucleotide substitutions in synonymous positions, 18% and 21%, respectively. The relationship between the 16S rRNA gene sequence similarity and *gyrB* gene sequence similarity, and DNA relatedness is not linear with the R^2 values of 0.1782 and 0.0524, respectively (Figure 4.4).

Phylogenetic analysis based on the *gyrB* gene was found to be more robust (more bootstrap values greater than 40%) and better resolved (longer branch lengths) than those based on the 16S rRNA gene (Figure 4.5 and 4.6). The trees that were constructed from the concatenated sequence of the 16S rRNA gene and the *gyrB* gene had similar topographies to the *gyrB* tree (Figure 4.7).

Figure 4.4 A comparison of the sequence similarity of the *gyrB* and 16S rRNA gene, and DNA relatedness.

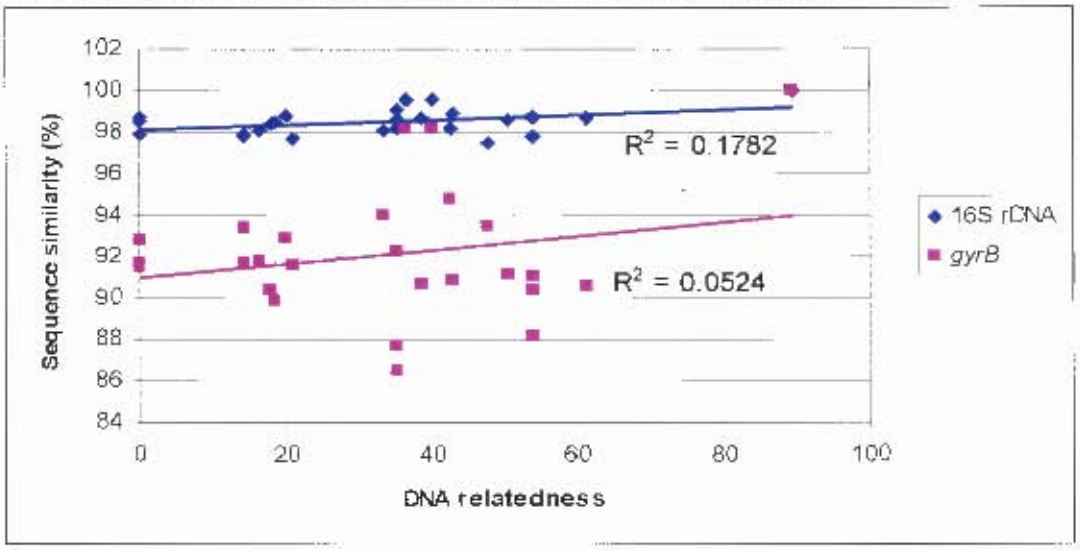
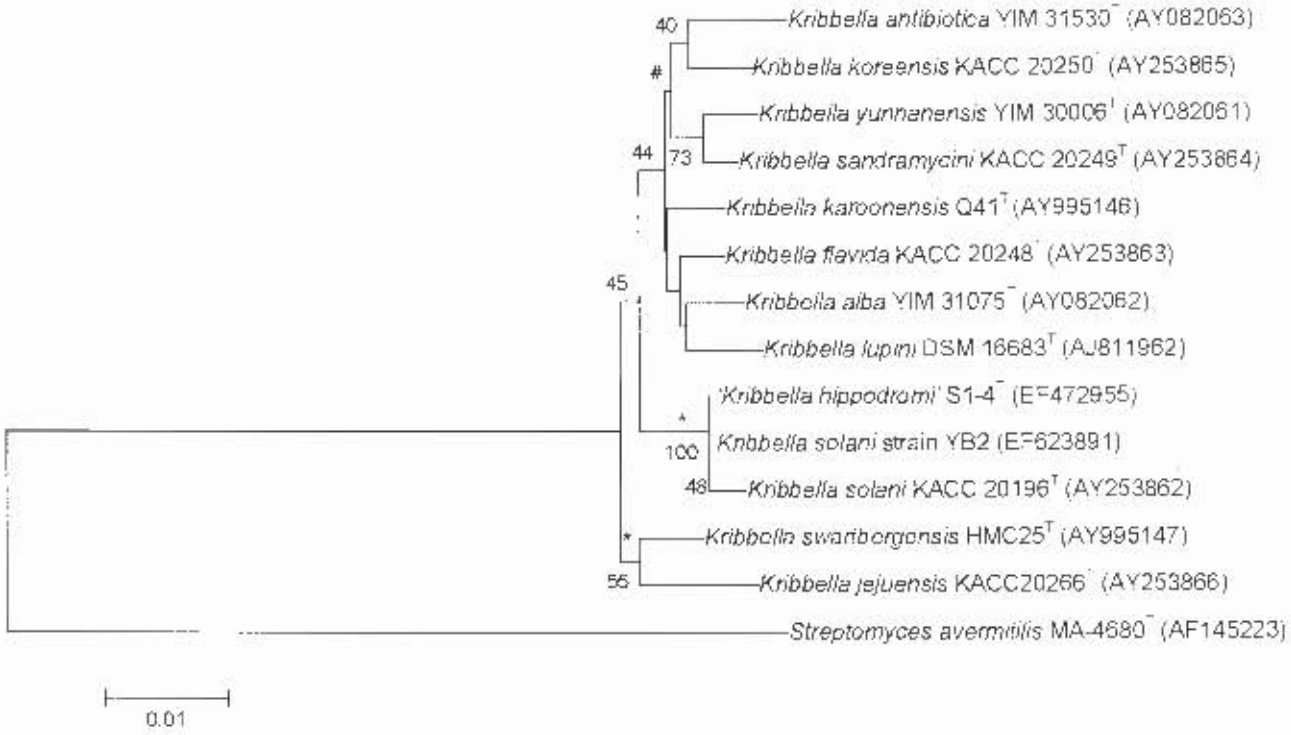
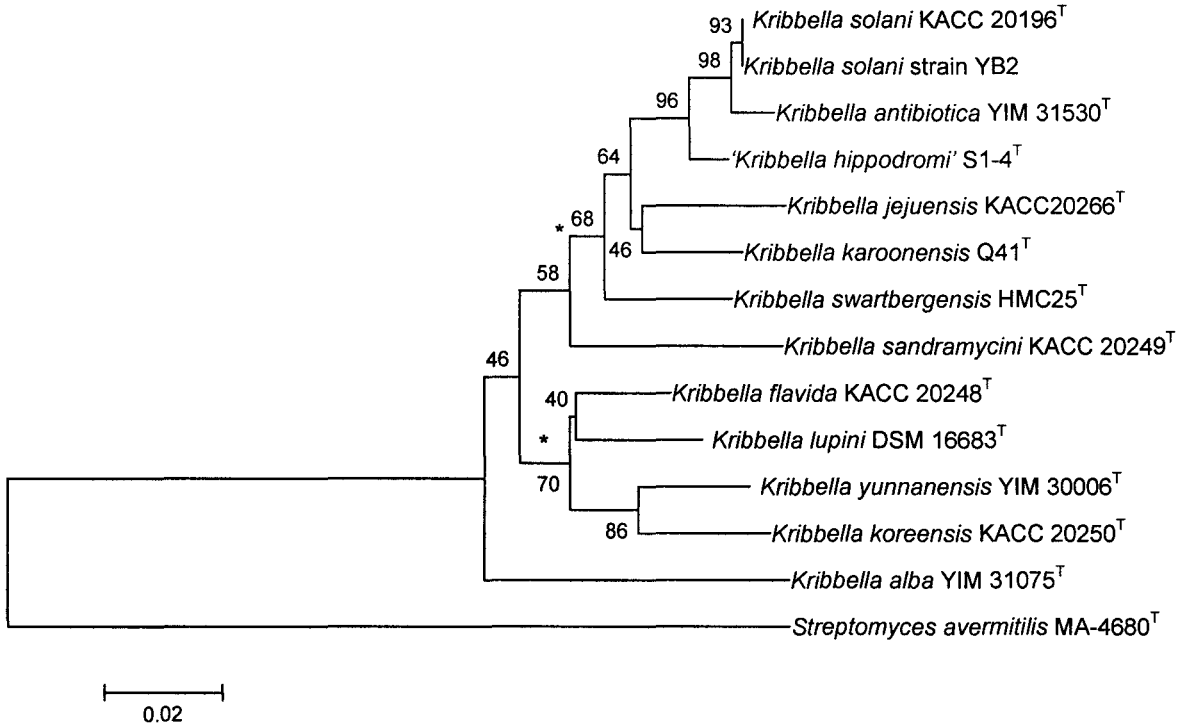


Figure 4.5 Unrooted 16S rRNA gene phylogenetic tree of the genus *Kribbella*. The tree was based on 1396 bp of common sequence and was obtained using the neighbour-joining method. Bootstrap values are based on 1000 resampled datasets and only values greater than 40% are shown. The bar represents 0.01 nt substitutions per nt position. *Streptomyces avermitilis* MA-4680^T was set as the outgroup.



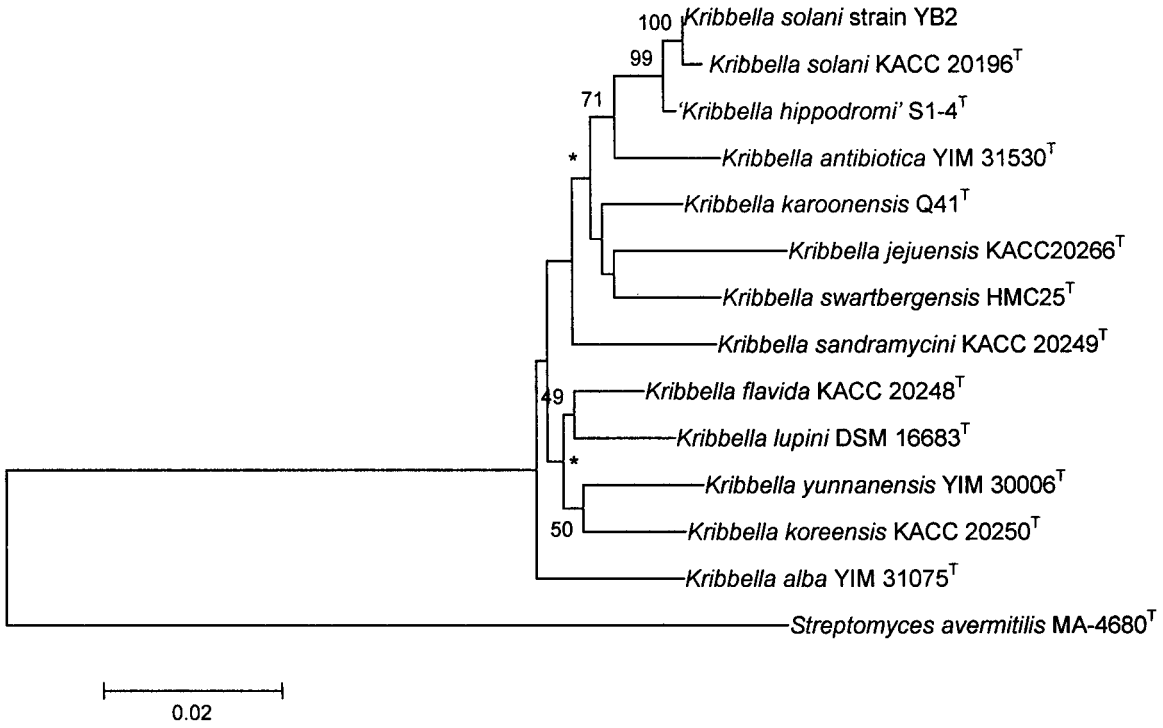
Asterisks (*) denote clusters that were conserved using the neighbour-joining, minimum evolution and maximum parsimony methods to construct the phylogenetic trees. A hash denotes the cluster that was conserved using the neighbour-joining and minimum evolution methods to construct the phylogenetic trees.

Figure 4.6 Unrooted *gyrB* gene phylogenetic tree of the genus *Kribbella*. The tree is based on 1232 bp of common sequence and was obtained using the neighbour-joining method. Bootstrap values are based on 1000 resampled datasets and only values greater than 40% are shown. The bar represents 0.02 nt substitutions per nt position. *Streptomyces avermitilis* was set as the outgroup.



Asterisks (*) denote clusters that were conserved using the neighbour-joining, minimum evolution and maximum parsimony methods to construct the phylogenetic trees. The accession number of *Streptomyces avermitilis gyrB* gene sequence is AP005038. All *Kribbella gyrB* sequences were determined in this study.

Figure 4.7 Unrooted phylogenetic tree of the genus *Kribbella* constructed from the concatenated 16S rRNA and *gyrB* gene sequences. The tree is based on 2696 bp of common sequence and was obtained using the neighbour-joining method. Bootstrap values are based on 1000 resampled datasets and only values greater than 40% are shown. The bar represents 0.02 nt substitutions per nt position. *Streptomyces avermitilis* was set as the outgroup.



Asterisks (*) denote clusters that were conserved using the neighbour-joining, minimum evolution and maximum parsimony methods to construct the phylogenetic trees. The 16S rRNA gene sequence accession numbers are listed in Table 4.1. The accession numbers of the *Streptomyces avermitilis gyrB* and 16S rRNA genes sequence are AP005038 and AF145223, respectively. All *Kribbella gyrB* sequences were determined in this study.

4.5 Discussion

The bacterial *recN* gene encodes a recombination and repair protein, and is required for homologous recombination and the SOS response (Skaar *et al.*, 2002). Genes involved in homologous recombination are one of the driving forces behind bacterial adaptive responses and, ultimately, bacterial speciation (Radman *et al.*, 2000). Unlike informational genes that are involved directly in DNA replication, it is possible that the *recN* gene has a faster evolution rate. As all the primers tested in this study were based on *recN* sequences from *Mycobacterium, Nocardia* and *Streptomyces* species, it is likely that the primers failed to bind to the *Kribbella recN* gene as these genes have significantly different sequences from *Mycobacterium*, *Nocardia* and *Streptomyces* (at least in the primer-binding regions). The *recN* gene appears to be highly variable, even within a single genus. In an extension of his 2003 study, Zeigler amplified and sequenced the *recN* gene from 68 strains of the genus *Geobacillus*. However, in this study a total of 41 forward primers and 34 reverse primers were required to obtain the *recN* gene sequence from these strains (Zeigler, 2005). As Zeigler's initial study focused on 44 genome sequences from 16 genera, due to the bias in available genome sequences, the application of the *recN* gene in bacterial taxonomy still needs to be tested in more diverse taxa (Zeigler, 2003; Rosselló-Mora, 2006).

The widespread application of the *gyrB* gene in bacterial taxonomy may be limited due to a number of factors. Firstly, there are few highly conserved regions within the *gyrB* gene that can be used to design primers, therefore, it may be difficult to design primers for diverse genera from the limited number of sequences available. Secondly, *gyrB* primers may also amplify the *parE* gene, as this gene has high sequence similarity to *gyrB* in these conserved regions. The *parE* gene is a paralogue of *gyrB* and codes for the β-protein of topoisomerase IV (Harayama & Kasai, 2006). Thirdly, an additional *gyrB* gene has been identified in some antibiotic biosynthetic gene clusters, such as that for novobiocin, which confers antibiotic resistance to the novobiocin-producing strain (Steffensky *et al.*, 2000). The sequence of this second *gyrB* gene differs from the wild type *gyrB* gene sequence. In order to circumvent this problem in some actinobacterial genera, the *gyrB* gene would need to be cloned and several colonies sequenced in order to obtain the sequence from both *gyrB* genes (Harayama & Kasai, 2006).

The GYR-UniF1 and GYR-UniR4 primers which were based on the *Pseudomonas* primers UP-1 and UP-2r, respectively (Yamamoto & Harayama, 1995), failed to amplify the required product from most of the *Kribbella* species. However, a partial *gyrB* sequence from three *Kribbella* species was sufficient to design the initial PCR primers, K*gyrB*-F and K*gyrB*-R. Richert *et al.* (2005) reported that the *Pseudomonas* (universal) primers UP-1 and UP-2r were unsuccessful when tested in the genus *Microbacterium*. In the study on the genus *Micromonospora* by Kasai *et al.* (2000b), the 1.2 kb *gyrB* fragment amplified by the UP-1 and UP-2r primers was used as a template for a second PCR using additional primers. It is probable that within the Class *Actinobacteria*, family or genus-specific *gyrB* primers will need to be designed. This task will be aided by the growing number of sequences available in public databases (Harayama & Kasai, 2006).

In this study, it was found that the 5' region of the *gyrB* gene within the genus *Kribbella* was more variable than 3' end of the gene, where there are large conserved areas that can be used to design PCR primers. Richert *et al.* reported that the 5' terminus of the *gyrB* gene within the genus *Microbacterium* is also highly variable. When

the variable region was excluded from their analysis, they found that the *gyrB* sequence similarities between strains increased from 69.2%-85-6% to 76.7%-88.6%, respectively (Richert *et al.*, 2005). This reported variability at the 5' terminus should be taken into account when designing primers to amplify the *gyrB* gene of a particular genus based on the *gyrB* sequences of closely related genera.

From a multiple sequence alignment of all the *Kribbella gyrB* gene sequences, a highly variable region was identified. This 390 bp variable region, spanning from 1010 bp to 1400 bp, is flanked by well conserved regions, and would be amplified by the primer combination K*gyrB*-F953/K*gyrB*-R. A 537 bp fragment is amplified by this primer combination and the full length of the fragment can be sequenced with just these two primers. The *gyrB*-based genetic distances based on this 390 bp region were calculated for all pairwise strain combinations and were found to correlate well with the *gyrB*-based distances determined for the conserved 1232 bp fragment (Table 4.7). Therefore, this region could potentially be included in a MLSA scheme for the genus *Kribbella*.

Although the phylogenetic analyses of the 16S rRNA gene of the genus *Kribbella* provides good resolution of the *Kribbella* type strains, the tree is not robust, with only three bootstrap values greater than 50% (Figure 4.5). The phylogenetic tree of the *gyrB* gene was found to have improved resolution and was more robust (Figure 4.6). The *gyrB* gene sequence analysis was able to resolve the *K. solani* - '*K. hippodromi*' cluster. The *gyrB* gene sequence of *K. antibiotica* was found to be most closely related to that of *K. solani* and '*K. hippodromi*'. The topographies of the 16S rRNA gene trees and the *gyrB* trees differ. A comparison of the *gyrB* gene sequences for all *Kribbella* species, found that the *gyrB* gene sequences of *K. alba*, *K. antibiotica* and *K. sandramycini* differed substantially from that of their closest phylogenetic neighbours (based on 16S rRNA gene analysis), which could possibly account for the different branching orders (data not shown). Within the genus *Micromonospora*, the topography of the *gyrB* tree (Kasai *et al.*, 2000b) differs from the 16S rRNA gene tree (Kroppenstedt *et al.*, 2005). Likewise, in the *Gordonia gyrB* tree the clustering of closely related strains seems to be maintained, while the clustering of more distantly related species differs (Shen *et al.*, 2006b). In this study, it was found that the *gyrB* sequence similarity for the *Kribbella* type strains ranged from 86.55% to 94.77%, which is equivalent to 166 to 64 nucleotide substitutions. For closely related strains, minor differences in the gene sequences used for phylogenetic analysis may alter the topography of the trees. This effect would be less apparent for more divergent species, such as *Gordonia* where the *gyrB* sequence similarity is reported to range from 79.3% to 97.2% (Shen *et al.*, 2006a). The phylogenetic trees constructed with the concatenated 16S rRNA and *gyrB* gene sequences were less robust and not as well resolved as the tree based on the *gyrB* sequence only (Figure 4.7). However, the topography of concatenated tree was most similar to the *gyrB* tree. The branching order of the concatenated trees was not affected by the order in which the genes were concatenated (data not shown).

This is no linear correlation between the pairwise *gyrB* gene comparisons and DNA relatedness (Figure 4.4). This finding has been reported in other actinobacterial genera (Richert *et al.*, 2005). The *gyrB*-based genetic distance determined using Kimura's two parameter model was found to be useful for species delineation within the genus *Kribbella*. From Figure 4.3, a *gyrB* genetic distance of less than 0.04 could be defined as the level at which DDH experiments should be performed, as above this threshold it is unlikely that the DNA relatedness would be above 70%. For strains with a genetic distance between 0.014 and 0.04, DDH should be performed

between strains that have similar phenetic characteristics. Although the 16S rRNA gene sequence similarity between *K. solani* CIP 108508^T and '*K. hippodromi*' is 99.64%, these strains differ phenetically. The *gyrB* genetic distance between these strains is 0.0164, therefore using the 0.014 threshold, they would be considered distinct species. This finding was substantiated by DDH, which found that the DNA relatedness between these strains is 40.4% ±3.8%.

This study found that there is a good correlation between the phylogenetic analyses based on *gyrB*-based genetic distance and DNA relatedness data. From results presented here, the *gyrB* gene is superior to the 16S rRNA gene sequence for resolving species relationships within the genus *Kribbella*. The increased resolution allowed for the differentiation of closely related species. The *gyrB*-based genetic distance based on a 390 bp fragment was found to be a good predictor of species relationships. When classifying *Kribbella* strains, the *gyrB* genetic distance based on this partial sequence could be determined prior to extensive phenetic characterisation. Strains that were found to have a genetic distance of greater than 0.04 could then be subjected to full polyphasic characterisation.

Actinomycete taxonomists should evaluate the potential of different genes for inclusion within family or genus-specific typing schemes. Within the genus *Kribbella*, phylogenies based on the *gyrB* gene are robust and well resolved. This study found that the *gyrB* gene sequence is sufficient to resolve species relationships within the genus *Kribbella*. However, as the genus gets bigger with the description of novel *Kribbella* species, MLSA may be considered and this gene could be included in such a scheme. Although it is unlikely that sequencing based methods will replace DDH in the near future, such sequence-based methods can be used as a guide to determine when DDH is necessary.

4.6 References

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CHAPTER 5

General Discussion

Actinomycete taxonomy is constantly evolving and technological advances have been matched by adaptations to classification schemes. Early morphology based taxonomy was superseded by chemotaxonomy, which was later surpassed by genotypic methods. Although genomics-based methods have dominated bacterial taxonomy for the last decade, it has become apparent that a classification scheme based on a single methodology is flawed. Even though polyphasic taxonomy is laborious and time consuming, it is still the favoured taxonomic method as it integrates genotypic, phenetic and phylogenetic data, thereby giving the best reflection of a strain's biology.

It is estimated that the genus *Streptomyces* has the potential to produce over 150 000 antimicrobials, of which only 3% have been discovered (Watve *et al.*, 2001). Clearly, actinomycetes are still a source of novel bioactive compounds and recent screening programs have isolated novel actinomycete species and genera, which produce unknown metabolites (Lam, 2006). There are currently a number of anti-infective and anticancer agents undergoing clinical development that are derived from actinomycetes (Lam, 2007). Advances in microbial genetics have resulted in the introduction of new methods including heterologous expression, whole-genome scanning and metagenomics (Van Lanen & Shen, 2006). Diazepinomicin and coelichelin are two examples of the increasing number of actinomycete derived natural products that have been isolated by a genome mining approach (Lam, 2007). However, developments in microbiology have shown that many of the so called unculturable strains are in fact culturable under standard laboratory conditions. These strains can therefore be cultured and directly tested for antibiotic production, without the need for lengthy and costly cloning procedures. Even though many recent natural product screening programs are focused on exotic sources of microorganisms, such as deep sea trenches and Antarctic glaciers, traditional terrestrial sources (including fauna and flora) and near-shore sediment should not be overlooked as a source of novel antibiotic-producing actinomycetes.

The main aim of this project was to isolate actinomycetes from indigenous plants, sediment and soil samples, and to screen isolates for the production of antimicrobial compounds. Thirty isolates were selected for full characterisation, employing a polyphasic approach.

Ten actinomycete strains were isolated from the leaves of different indigenous plant species. These isolates belonged to the genera *Gordonia*, *Kineococcus* and *Micromonospora*. Although the number of actinomycetes isolated from leaves was significantly lower than the numbers isolated from soil and sediment samples, it is worthwhile to isolate from leaves as, from the findings of this study, they are clearly a potential source of novel actinomycetes. There are over 9000 plant species in the fynbos biome (Manning, 2003) and the systematic screening of this previously unexplored source is likely to result in the isolation of novel actinomycetes. As very few actinomycetes were isolated from the plant species with hairy, leathery leaves (a characteristic feature of fynbos), future studies may consider excluding those plant species with very hairy, hard leaves. Interestingly, three *Kineococcus* strains were isolated from the leaves of a *Geranium* species, while a *Micromonospora*

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species was isolated from a *Pelargonium* species. Both these plant genera belong to the Family *Geraniaceae*. There are over 250 South African *Pelargonium* species, as well as other indigenous *Geranium* species (http://www.ecocam.com/species/Great_Green_Ten/South_Africa.html), therefore, other plant species belonging to this family could be screened for the presence of endophytic actinomycetes.

The eight *Micromonospora* strains that were characterised in this study were isolated from either sediment or leaves. Phylogenetic and phenetic characterisation identified four strains as potentially novel species. Phylogenetic analysis found that two strains from different sources, TVU1 and MuizA5S, were closely related, however, based on phenetic characterisation they could be distinguished from each other and their closest phylogenetic neighbours. DDH studies confirmed that these strains are distinct genomospecies. Therefore, TVU1 and MuizA5S represent the type strains of novel species, for which the names '*Micromonospora tulbaghiae*' sp. nov. and '*Micromonospora psammotica*' sp. nov., respectively, are proposed. Although PLU1 and RAU1 were isolated from the leaves of different plant species, phylogenetically they can not be resolved as distinct species. Based on physiological and phylogenetic characterisation, both these strains can be distinguished from their closest neighbours, *M. echinospora* and *M. rosaria*. It is possible that PLU1 and RAU1 represent strains of a novel *Micromonospora* species which has formed a beneficial relationship with plants.

Three *Kineococcus* strains were isolated from the leaves of *G. incanum* and a fourth strain was isolated from the leaves of *C. album*. Based on phylogenetic analysis of the 16S rRNA gene, all four isolates are most closely related to *Kineococcus* species (Figure 2.4). Interestingly, the three type strains of the genus *Kineococcus* do not form a monophyletic cluster, and *K. marinus* was found to be most closely related to *Quadrisphaera granulorum* DSM 44889^T. Although the phenetic characteristics of the four strains isolated in this study are similar, the morphological features of CA21 differ from those of GIU1, GIU2 and GIU3. Based on published findings (Yokota *et al.*, 1993; Phillips *et al.*, 2002), CA21 appears to resemble a *Kineococcus* species (both on agar plates and under a SEM), while GIU1, GIU2 and GIU3 appear to have the morphological characteristics of *Kineosporia* species. Although none of the validly published *Kineococcus* species was isolated from plants, four of the five type strains of the genus *Kineosporia* were isolated from plants. It is possible that members of the Family "*Kineosporiaceae*" have adapted to this environmental niche.

Thirteen *Streptomyces* strains, isolated from sediment and soil samples, were selected for characterisation. Based on the results reported here, additional characterisation should be performed on five strains to determine whether they are novel species. Berg2S and Berg4Y were isolated from a sediment sample and belong to the grey series. Phenetically, both these strains can be distinguished from their closest phylogenetic neighbours, however, DDH will be required to determine whether these strains are distinct genomospecies. Although many of the *Streptomyces* strains that were classified as belonging to either the green or blue series could not be distinguished from *S. cyaneus*, two isolates, Riet1C (blue series) and ShaleUP (green series), appear to be novel. Phylogenetically, both these strains appear to be distinct species and phenetic characterisation allowed them to be differentiated from their closest phylogenetic neighbours. Likewise, strain NX03U2 was isolated from a sediment sample and belongs to the red series. Based on phenetic and phylogenetic characterisation, this strain can be distinguished from its closest phylogenetic neighbours, *S. novaecaesareae* and *S. aureocirculatus*. As there are only limited phenetic data available for these type strains, in order to distinguish NX03U2 as a

unique species, additional phenetic testing should be conducted directly comparing this strain to the type strains of *S. novaecaesareae* and *S. aureocirculatus*. Preliminary antibiotic testing found that both Berg2S and NX03U2 had relatively strong antimicrobial activity against *M. aurum*, *M. bovis* and *M. smegmatis*, and future studies on these strains could include additional characterisation of the antibiotics they produce.

Of the 30 isolates selected for full characterisation, 80% were found to inhibit the growth of *M. aurum* A+. The majority of the antibiotic producing strains were isolated from either soil or sediment. Only four plant isolates had antimycobacterial activity. However, two factors may account for the finding that plant isolates generally do not produce antibiotics. Firstly, no streptomycetes isolated from leaves were selected for full characterisation. All *Streptomyces* strains isolated from plants grew very weakly and generally only grew on plant extract medium. As it is not feasible to grow actinomycetes on this medium for small scale fermentations, where the presence of residual plant metabolites may be mistaken for microbially derived antibiotics, they were excluded from the study. Secondly, four of the ten plant isolates are *Kineococcus* species, a genus not known to produce antibiotics. As endophytic actinomycetes have been shown to be the source of novel antibiotics (Castillo *et al.*, 2002; 2003), indigenous South African plants may be an unexplored source of antibiotic producing actinomycetes.

Many programs which screen actinomycetes for antibiotic production seem to be biased towards the isolation of streptomycetes. In this study, it was found that antibiotic production was improved in the genera *Micromonospora* and *Kribbella* when strains were cultured in specific production media. Both the *Kribbella* species isolated in this study, HMC25 and Q41, had enhanced antibiotic production when cultured in *Nocardioides* antibiotic production medium and produced antimycobacterial compounds that were effective against *M. aurum* A+, *M. bovis* and *M. smegmatis*. Of the other nine *Kribbella* type strains, only *K. sandramycini* and *K. antibiotica* are reported to produce antibiotics. *K. sandramycini* produces sandramycin, which has moderate activity against Gram-positive bacteria (Matson & Bush, 1989). Therefore, in an extension of this study it may be worthwhile culturing all *Kribbella* strains in the *Nocardioides* production medium to test for antibiotic production. Of the eight *Micromonospora* strains characterised, only RAU1 had no detectible antimicrobial activity against *M. aurum* A+. The seven active *Micromonospora* isolates all had enhanced activity when cultured in medium adapted to improve the production of antibiotics by members of this genus. As micromonosporae are one of the dominant species isolated from plants and sediment (Kawamoto, 1989; Coombs & Franco, 2003), this medium could be included in the initial antibiotic screening of actinomycetes isolated from these sources.

M. aurum A+ was selected as a substitute for *M. tuberculosis* for routine antibiotic screening as this species is reported to have a similar antibiotic susceptibility profile to *M. tuberculosis* (Chung *et al.*, 1995). However, in this study it was found that *M. aurum* and *M. smegmatis* had similar antibiotic susceptibilities, while *M. bovis* was more resistant to the antibiotics tested. Compounds which inhibited the growth of *M. aurum*, *M. bovis* and *M. smegmatis*, failed to inhibit the growth of *M. tuberculosis*. In future studies it may be prudent to determine whether *M. bovis*, which is a member of the *M. tuberculosis* complex, or possibly another non-pathogenic, slow growing mycobacterium species, has a susceptibility profile which is more similar to that of *M. tuberculosis*.

Although *M. bovis* needs to be grown for at least 5 days, compared to *M. aurum*, bioautography against *M. bovis* would only require an additional incubation day so it could be used for routine antibiotic screening. However, as studies have shown that there are three large regions (each over 9 kb in length) missing from the genome of *M. bovis* BCG Connaught (Mahairas *et al.*, 1996), it is probable that the susceptibility profiles of *M. tuberculosis* and *M. bovis* are different.

Structural determination of the antimycobacterial compound PK-B was problematic. However, from the findings presented here, it is possible that *S. speibonae* PK-Blue^T produces a tetramic acid. Although PK-B was found to be inactive against *M. tuberculosis*, it is active against *M. aurum*, *M. bovis* and *M. smegmatis*. From the findings of this study, additional work on this compound is warranted. This may include performing ^{13}C NMR with the compound dissolved in pyridine, accurate mass determination and elemental analysis to determine the structure of this compound. Recent studies have characterised the biosynthetic gene cluster involved in the production of tetramic acids (Bihlmaier *et al.*, 2006) and these findings could be used as a guide for future combinatorial biosynthesis studies involving PK-B. Historically, tetramic acids were considered to be predominantly fungal metabolites or derived from marine invertebrates, however, it has become evident that many actinomycetes also produce these compounds (Royles, 1995). Although the aim of future combinatorial biosynthetic studies would be to improve the potency and antibacterial spectrum of PK-B, genes identified in these studies could also be used to design PCR primers for the PCR screening of isolates for the presence of genes involved in tetramic acid production.

Many natural product screening programs have shifted focus from terrestrial sources to unexplored sources such as marine habitats. With the exploration of these unique environmental niches, the true richness of microbial diversity has become apparent (Colwell, 1997). However, these finding have not been matched with advances in taxonomy and current methods lack the resolution to identify organisms to the species or even strain level. (Gevers *et al.*, 2005). It is essential that the overspeciation of antibiotic-producing genera that marked the mass isolations of the 1960s and 1970s is not repeated (Anderson & Wellington, 2001). To fully exploit this increased microbial diversity, improved taxonomic methods are required.

Many of the methods currently used in actinomycete taxonomy are labour intensive and time consuming. Therefore, there is a need to develop new methods that can be used for the rapid identification of isolates. An ideal method would be able to identify strains to the species level and be readily available to most laboratories. The potential of selected protein coding genes to predict phylogenetic relationships has been investigated in a number of bacterial genera. Many taxonomists feel that a sequencing based method could replace DDH.

One aim of this study was to determine whether the *gyrB* gene could be used to resolve closely-related species within the genus *Kribbella*. The advantages of performing a taxonomic study on this genus were that it is a small genus so all the type strains could be included in the analysis and there is extensive DNA relatedness data available. It was discovered that the *gyrB* gene could distinguish between closely related species within the genus *Kribbella* and that *gyrB* phylogenetic trees had improved resolution and were more robust than 16S rRNA trees. Kasai *et al.* (2000) reported that the *gyrB* gene could distinguish between members of the genus *Micromonospora*. When the *Pseudomonas* primers used by Kasai *et al.* were tested on the eight

Micromonospora strains isolated in this study, it was found that they only amplified the *gyrB* gene from two *Micromonospora* isolates, TVU1 and MuizA5S. Preliminary work has shown that the K*gyrB*-F/K*gyrB*-R and K*gyrB*-F1085/K*gyrB*-R primer combinations amplify a band of the expected size for four *Micromonospora* isolates, CGM31, PBPE, MuizA5S and TVU1. Therefore, as an extension to this study on the application of the *gyrB* gene in actinomycete taxonomy, the primers designed in this study could be tested on the eight *Micromonospora* isolates. It would be interesting to see whether phylogenies constructed with *gyrB* gene sequences could resolve the TVU1-MuizA5S and PLU1-RAU1 clusters. Another possibility would be to test these primers on the two *Gordonia* isolates, AC41 and NX0141. Phylogenetic analysis of the 16S rRNA gene found that both these strains were most closely related to the human pathogens *G. sputi* and *G. bronchialis*, respectively. As neither AC41 nor NX0141 could clearly be differentiated from their closest phylogenetic neighbours, it was decided not to perform full phenetic characterisation on these isolates. A recent study by Shen *et al.* (2006) found that the *gyrB* gene could be used for the phylogenetic analysis of the genus *Gordonia*, and, as in the genera *Kribbella* and *Micromonospora*, these phylogenies have improved resolution allowing for the separation of closely related species. Preliminary work has shown that the primer combination K*gyrB*-F/K*gyrB*-R978 amplified a fragment from NX0141 and AC41. Although this fragment was slightly larger than the expected size, a multiple sequence alignment of published *Gordonia gyrB* sequences and the *Kribbella gyrB* sequences obtained in this study found that *Gordonia* species have three insertions (each approximately 30 bp is length) in their *gyrB* genes. Therefore, the primers designed in this study could potentially be used to amplify the *gyrB* gene from the two *Gordonia* isolates and phylogenetic analysis could be performed to see whether they can be distinguished as distinct species.

There are over 400 *gyrB* gene sequences available in public databases from diverse actinobacterial genera, which can be used to design PCR primers for taxonomic studies. Although numerous genes have been identified as predictors of whole genome relatedness, including *recN*, *dnaX* (Zeigler, 2003) and *lepA* (Santos & Ochman, 2004), the application of some of these genes within actinomycete taxonomy may be restricted to those genes that can be successfully amplified with primers designed from the limited number of sequences currently available.

As housekeeping genes evolve at different rates and some are more prone to horizontal gene transfer, it is not prudent to base a classification scheme on a single gene. Gevers *et al.* (2005) proposed the implementation of MLSA for bacterial taxonomy. Genes that have already been found to form stable, well resolved phylogenies are excellent candidates for inclusion within MLSA schemes. It must still be determined how many housekeeping genes should be included in such a classification scheme. Many authors have stated that at least eight loci should be sequenced. However, a recent study by Konstantinidis *et al.* found that the phylogenetic relationships based on the gene sequences of most core genes were in fact stable and analyses based on three genes were robust for most well resolved species. However, when determining intraspecies relationships, the selection of genes was critical, as the tree topographies obtained from some genes did not correlate with the whole genome phylogenies based on the ANI (Konstantinidis *et al.*, 2006). Maiden (2006) also reported that if the constructed phylogeny has poor resolution, it is better to rather include a few diverse genes, than it is to include more genes. Ultimately, the number of loci selected should be based on the robustness of the phylogenetic clusters. For well resolved species, three loci should be sufficient, while for recently diverged species or for intraspecies analysis

six to eight loci should be included (Stackebrandt *et al.*, 2002). However, in the genus *Kribbella* (based on its current composition), *gyrB* is very good at resolving species relationships, although MLSA may need to be considered as the genus gains new members.

The main disadvantage of MLSA is that it only compares a small region of the genome. Konstantinidis & Tiedje (2005) proposed the concept of ANI, which compares the entire genome of sequenced microorganisms and this method has been identified by many authors as a potential replacement for DDH (Rosselló-Mora, 2006). However, the determination of ANI will remain limited to bacteria for which whole-genome sequences are available, such as medically relevant and industrially important strains. As DNA sequencing facilities are available to most laboratories and sequences can be obtained cheaply and rapidly, MLSA typing schemes are an ideal replacement for DDH. MLSA could be used in taxonomic studies including those of environmental isolates (Gevers *et al.*, 2005; Maiden, 2006). Studies have shown that the phylogenies obtained from MLSA correlate with classifications based on DNA relatedness (Devulder *et al.*, 2005). However, before it can be used in taxonomy, MLSA must be validated by performing studies in large genera for which there is extensive DNA relatedness data available (Rosselló-Mora, 2006).

Despite the potential of MLSA and ANI, other developments in taxonomy must not be overlooked. Numerous genomic based methods have been developed, including PCR-based fingerprinting methods such as AFLP, RIS-PCR and ribotyping. These methods can be used for the identification and classification of actinomycete species. Ribotyping has been automated, generating highly reproducible riboprints and is used for the identification of clinical isolates. New chemotaxonomic methods have been developed, including PyMS and FT-IR. Both these methods can be used for whole cell analysis and can be used for strain dereplication (Sanglier *et al.*, 1992; Zhao *et al.*, 2004). However, due to the cost of the necessary equipment and the need for standardised conditions, it is unlikely that these methods will be widely used in actinomycete taxonomy, especially for the classification of environmental isolates.

Many of the problems currently faced in bacterial taxonomy have been created by the rigid application of the original guidelines proposed by Wayne *et al.* (1987). It is essential that any new thresholds for species delineation identified by new methods are not dogmatically enforced. From the recent advances in bacterial taxonomy, it is apparent that the idea of a universal prokaryotic species concept is a utopian ideal and a threshold identified in one taxon may possibly not apply to other taxa, especially for highly clonal populations. Classification schemes based on these new methods should be seen as a guide and species identification should still agree with phenotypic and genomic data (Rosselló-Mora, 2006).

In the next few years, microbial taxonomists are likely to witness a shift away from the traditional phenetic based classification methods towards genomic based methods, which may include MLSA and ANI. In actinomycete taxonomy, MLSA schemes should be developed to classify species belonging to large genera, such as *Streptomyces*.

Actinomycetes have been isolated from most habitats and many appear to have adapted to survive in unique environmental niches including plants and animals (such as arthropods and marine invertebrates). The diversity

of actinomycetes is seemingly endless and is fortuitously matched by the range of natural products they produce. The classification schemes currently used in actinomycete taxonomy are based on phenotypic and genomic data, and the relationships inferred by these methods appear to reflect natural species relationships. However, improved taxonomic methods will soon be required if taxonomists are to recognise the full potential of actinomycete biodiversity.

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