SCREENING ENVIRONMENTAL ACTINOBACTERIA FOR ANTIMYCOBACTERIAL ANTIBIOTICS AND CHARACTERISATION OF *Kribbella stellenboschensis* sp. nov.

by

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"It's gone. It's done." – Frodo Baggins

(Lord of the Rings: The Return of the King)

List of Abbreviations

7H9	-	Middlebrook 7H9 (agar/broth)
A	-	adenine (DNA base)
AHBA	-	amino-5-hydroxy-benzoic acid
AM	-	aerial mycelium
ANI	-	average nucleotide identity
atpD	-	ATP synthase F1, beta subunit
ACT	-	artemisinin based combination therapy
BCE	-	before the common era
BMSA	-	basal mineral salts agar
bp	-	base pairs (DNA)
C	-	cytosine (DNA base)
CAS	-	Cerium (IV) ammonium sulphate
cm	-	centimeters
CSI	-	conserved signature indel
CZ	-	Czapek solution (agar/broth)
DNA	-	deoxy-ribonucleic acid
DDH	-	DNA-DNA hybridisation
dNTP	-	deoxyribonucleotide triphosphate
DPG	-	diphosphatidylglycerol
DSMZ	-	Deutsche Sammlung von Mikroorganismen und Zellkulturen
et al.	-	et alia, “and others”
EtBr	-	ethidium bromide
EtOAc	-	ethyl acetate
F	-	Forward
g	-	grams
G	-	guanine (DNA base)

oxyB	-	the glycopeptide monooxygenase B
gyrB	-	DNA gyrase subunit B gene
HGT	-	horizontal gene transfer
HIV	-	human immunodeficiency virus
HPLC	-	high performance liquid chromatography
HR-LCMS	-	high resolution liquid chromatography mass spectrometry
I	-	inosine (nucleoside)
IC_{50}	-	half maximal inhibitory concentration
i.e.	-	*id est*, “it is” or “that is (to say)”
ISP	-	International *Streptomyces* project
kb	-	kilobase pairs (DNA)
keto synthase	-	keto synthase
L	-	litres
LPSN	-	list of prokaryote names withstanding in nomenclature
M	-	molar
MDR	-	multi-drug resistant
MeOH	-	methanol
min	-	minutes
mL	-	milliliters
MLSA	-	multilocus sequence analysis
mm	-	millimeters
mm^2	-	square millimeters
mM	-	millimolar
mol%	-	mole percent
MTT	-	thiazolyl blue tetrazolium bromide
n/a	-	not available
N/A	-	not applicable
NCBI	-	National Center for Biotechnology Information

ng	-	nanograms
NMR	-	nuclear magnetic resonance spectroscopy
NRP	-	non-ribosomal peptide
NRPS	-	non-ribosomal peptide synthetase
nt	-	nucleotides (DNA)
OD	-	optical density
PC	-	phosphatidylcholine
PCR	-	polymerase chain reaction
pH	-	potential Hydrogen
PI	-	phosphatidylinositol
pLDH	-	parasite lactate dehydrogenase
R	-	reverse
RBR	-	relative binding ratio
recA	-	recombinase A gene
R_f	-	retention factor
RNA	-	ribonucleic acid
rpm	-	revolutions per minute
rpoB	-	RNA polymerase beta subunit
rRNA	-	ribosomal RNA
s	-	seconds
S	-	Svedberg
sp. nov.	-	*species nova*, “new species”
ssDNA	-	single stranded DNA
subsp.	-	subspecies
T	-	thymine (DNA base)
TB	-	tuberculosis
TE	-	Tris-EDTA
TLC	-	thin layer chromatography

T_m	-	melting temperature
TMPD	-	N, N, N′, N′-tetramethyl-p-phenylenediamine
tRNA	-	transfer RNA
trpB	-	tryptophan synthetase, beta subunit
U	-	units
vs	-	versus
v/v	-	volume for volume
WCSP	-	whole cell sugar pattern
WHO	-	world health organisation
w/v	-	weight for volume
XDR	-	extensively drug-resistant
ZOI	-	zone of inhibition
µg	-	micrograms
µL	-	microlitre
µM	-	micromolar
λ	-	phage Lambda
&	-	and
°C	-	degrees Celsius
>	-	“greater than” or “more than”
≥	-	“greater than or equal to”
<	-	“less than”

ABSTRACT

Soil was collected from a compost heap in a Mowbray suburban garden and a compost heap in a Plumstead suburban garden. The soil and 'worm tea' of a vermiculture farm from the same Mowbray suburban garden were also sampled. Using four different types of media (7H9, CZ, ISP2 and GOT) 135 isolates were putatively identified as actinobacteria based on colony morphology. These isolates were screened for antimycobacterial activity against the test bacterium *Mycobacterium aurum* A+. A *Kribbella* strain, isolated and identified by an intern in the lab, and a *Micromonospora* strain, isolated and identified during the authors Honours project, were also screened for antimycobacterial activity. Sixty-four (64) actinobacterial isolates displayed moderate antibiotic activity or higher (ZOI >1001 mm^2) based on the standard overlay method. *Kribbella* strain SK5 displayed very strong antimycobacterial activity (3309 mm^2).

Forty (40) of the actinobacterial strains that exhibited moderate/strong/very strong antimycobacterial activity and/or had interesting morphological features were selected for genus identification via a standard nucleotide-nucleotide *blastn* analysis of their 16S rRNA gene sequences. Thirty-one (31) strains were identified as *Streptomyces* species, six strains were identified as *Micromonospora* species, one strain was identified as a *Nocardia* species, one strain was identified as a *Kitasatospora* species, and one strain was identified as a member of the genus *Tsukamurella*. These isolates were subjected to phylogenetic analysis using the partial 16S rRNA gene sequences. Based on analysis of the 16S rRNA gene sequences, *Streptomyces* strain PR10 was found to be the most interesting of the *Streptomyces* isolates and should be pursued as a novel species (99.7% sequence similarity to the top *blastn* hit and less than 98.8% sequence similarity from the third *blastn* hit onwards). Further analysis of the gyrase subunit B (*gyrB*) gene sequence of the *Kitasatospora* isolate (strain PR3) revealed that the isolate is more closely related to members of the genus *Streptomyces*. Further evidence to support the assignment of strain PR3 to the genus *Streptomyces* (rather than *Kitasatospora*) is that it has two *Streptomyces*-specific *gyrB* gene indels signatures. *Tsukamurella* strain G4 was noted for characterisation as a novel species.

The potential for seven isolates to produce ansamycin, glycopeptide, non-ribosomal peptide, and/or Type-II polyketide antibiotics was determined by detection of antibiotic biosynthetic gene clusters using PCR. Strain M27 demonstrated the potential to produce all the aforementioned antibiotics. Strain Y10 demonstrated the potential to produce a non-ribosomal peptide antibiotic. Strains PR10, PR28, PR47 and UK1 demonstrated the potential to produce Type-II polyketide and non-ribosomal peptide antibiotics. The PCR products were sequenced and analysed via *blastn* to compare them to the known antibiotic biosynthetic gene sequences in the GenBank database. The non-ribosomal peptide synthetase (NRPS) A domain sequences were analysed using the NRPSpredictor2 software to identify the A domain substrate specificity.

Solvent extraction was done on the broth cultures of *Streptomyces* strains PR3, UK1 and Y30 and *Kribbella* strain SK5 to isolate the antimycobacterial compounds. It was found that the cell mass extract of the three

Streptomyces isolates had active compounds against *M. aurum* A+. The culture broth extract of the *Kribbella* isolate was found to have an active compound against *M. aurum* A+ and *Staphylococcus aureus* ATCC 25923. One-dimensional and two-dimensional TLC of the culture broth extract from strain SK5 revealed that a single compound was active against *M. aurum* A+ and *S. aureus* ATCC 25923. Nocardamine was purified from the culture broth extract of strain SK5 by Mr Kojo Acquah (PhD student, Department of Chemistry, University of Cape Town). In a side-by-side spot bioautography analysis of the purified nocardamine and the strain SK5 culture broth extract, it was found that the active compound in the culture broth extract was not nocardamine, because nocardamine only had activity against *M. aurum* A+ while the culture broth extract had activity against *M. aurum* A+ and *S. aureus* ATCC 25923.

Using the polyphasic taxonomic approach, *Kribbella* strain SK5 was tentatively characterised as a novel species, for which the name *Kribbella stellenboschensis* sp. nov. is proposed. The closest phylogenetic relatives were identified as the type strains of *Kribbella aluminosa*, *Kribbella karoonensis*, *Kribbella pittospori*, *Kribbella shriazensis*, '*Kribbella sindirgiensis*' and '*Kribbella soli*'. Genetic distances of 0.030 and 0.016 were calculated for '*K. soli*' and '*Kribbella sindirgiensis*', respectively, for the concatenated gene sequence of five housekeeping genes (*gyrB, rpoB, recA, relA*, and *atpD*). Thus, DNA-DNA hybridisation (DDH) will need to be carried out to confirm that strain SK5 is a separate species. Phenotypic differences were observed between strain SK5 and all the type strains of the most closely related species. Chemotaxonomically, strain SK5 possessed the key characters definitive of the genus *Kribbella*: i) MK-9(H_4) as the major menaquinone; ii) LL-diaminopimelic acid as the diagnostic diamino acid; iii) anteiso-$C_{15:0}$ and iso-$C_{16:0}$ as the major fatty acids (>10%); and iv) phosphatidylcholine in the polar lipid profile.

CHAPTER 1

INTRODUCTION

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1.1 Actinobacteria

Actinobacteria (phylum *Actinobacteria*) are Gram positive bacteria that contain a high G + C content in their DNA. *Actinobacteria* are one of the largest taxonomic units within the major lineages of the domain *Bacteria* (Figure 1). They are also amongst the richest sources of natural products with clinical applications (Barka et al., 2016; Goodfellow and Fiedler, 2010; Prescott et al., 2008; Buchanan, 1917). In 1997, Stackebrandt et al. established a hierarchical classification system for the actinobacteria using the 16S rRNA gene. As of 2017, the phylum *Actinobacteria* is comprised of six classes, 23 orders, and 53 families based on the outline stipulated on the Bergey's Manual Trust website (https://www.bergeys.org/outlines.html). The class *Actinobacteria* is comprised of nine orders, 11 suborders, and 45 families. The order *Actinomycetales* is the largest of the nine orders within the class *Actinobacteria* (http://www.bacterio.net/-classifphyla.html).

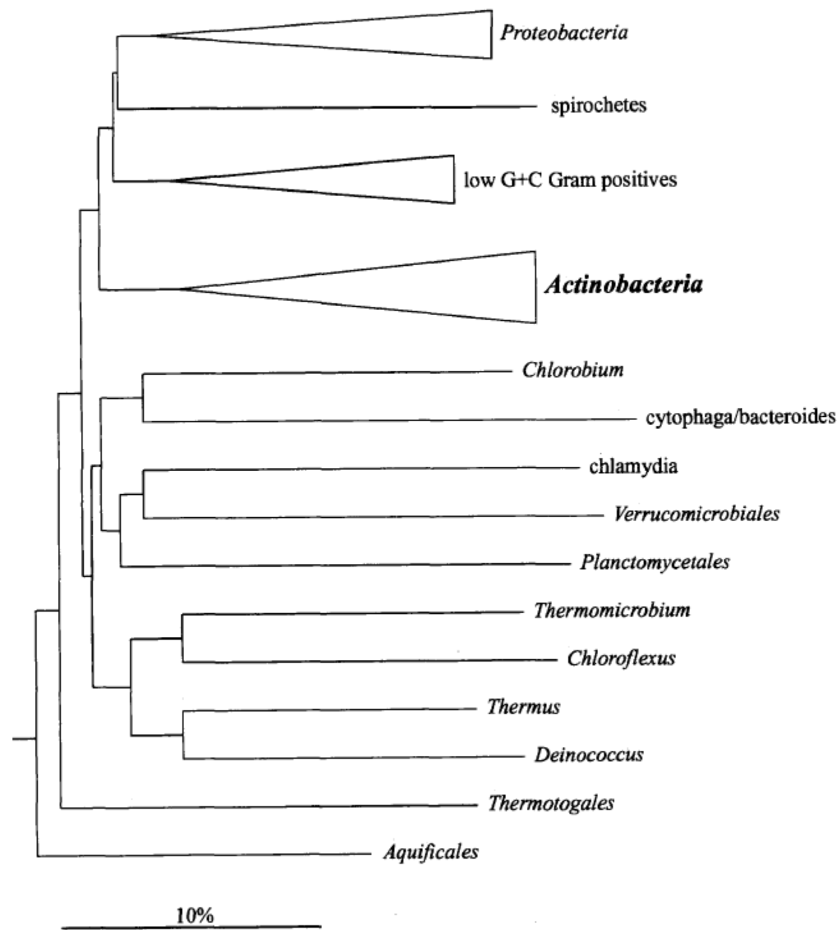

Figure 1: Phylogenetic position of the phylum *Actinobacteria* in the domain *Bacteria* based on 16S rRNA gene sequences. The scale bare indicates 10 nucleotide substitutions per 100 nucleotides. Figure taken from Stackebrandt et al. (1997).

The 16S rRNA gene and alternative ‘housekeeping’ genes have been used to establish the phylogeny of *Actinobacteria* and individual groups within the class separately (Verma et al., 2013). Actinobacteria display numerous morphologies: highly differentiated branched hyphae, fragmenting hyphae, rod shaped, and/or rod-coccoid forms (Ventura et al., 2007). The filamentous actinobacteria are commonly referred to as actinomycetes whose name is a derivative of the Greek words for ray (*aktis* or *aktin*) and fungi (*mukēs*), because of their resemblance to filamentous fungi. This is why they were originally believed to be a transitional form between bacteria and fungi (Barka et al., 2016).

Actinobacteria are mostly saprophytic, soil-dwelling microorganisms with the ability to utilise a broad range of nutritional resources. They also play important roles in decomposition and the cycling of nutrients. They are best known for the turnover of complex biopolymers. Despite the long focus on the soil-dwelling variants of actinobacteria, they have also been found to be prolific in other environments with variable temperatures, pH, and salinity, such as plants, animals, and the aquatic environment (Barka et al., 2016; Mincer et al., 2002). Being generally mesophilic, actinobacteria grow optimally between 25 and 30°C. However, thermophilic actinobacteria have been isolated from environments that experience temperatures up to 60°C (Edwards, 1993).

One of the most important and well-known features of actinobacteria is their ability to produce secondary metabolites that have a suite of different applications, particularly in the pharmaceutical industry. Actinobacteria are the source of a plethora of antibiotics discovered to date. Seventy (70) to 80% of all antibiotics were predominantly isolated from *Streptomyces* species in the early days of antibiotic research (Bérdy, 2005). The isolation of streptomycin from *Streptomyces griseus* in 1944 lead to the exploration, and exploitation, of the *Streptomyces* genus for the discovery of antibiotics (Barka et al., 2016; Watve et al., 2001). Today, the *Actinomycetales* are still the largest group from which bioactive metabolites have been isolated, with more than 10 000 active compounds having been discovered (Bérdy, 2005). To keep up in the ‘arms race’ against antibiotic resistance, researchers are looking towards unexplored habitats that are possibly teeming with biologically diverse organisms with chemically distinct natural compounds. Additionally, the importance for the discovery of ‘rare actinomycetes’ has been recognised, because their discovery increases the chances of finding structurally variable bioactive molecules (Bérdy, 2005).

However, to continue isolating from soil environments is not a futile task. Novel species of actinobacteria that demonstrate antibiotic activity are still continuously being isolated from soil environments (Genilloud, 2017; Také et al., 2017). One can even turn towards soil types that have not been well studied, such as vermiculture farms, which have previously been demonstrated as a rich source of the rarer actinobacteria (Yami et al. 2003). The isolation and characterisation of novel species is vital in the search for new antibiotics. Consequently, the procedures to describe a bacterium as a novel species have become increasingly sophisticated.

1.2 Bacterial taxonomy

1.2.1 A brief history

Since Aristotle first conceived the idea of a species approximately 2400 years ago, scientists have laboured over trying to establish a set of rules that would group organisms effectively (Richter & Rósselló-Móra, 2009). A reliable system of classification is a vital tool that is needed for the effective study of life. Taxonomy is a field consisting of three integral parts, namely classification, nomenclature and identification (Prescott et al., 2008). Natural classification is one of the oldest classification systems, which groups members based on shared characteristics that reflect the biological nature of the organisms. The taxonomic study of prokaryotic life is one of the youngest and more dynamic classification systems (Rósselló-Móra & Amann, 2001). Unlike in zoological classification, the evolutionary relationships between prokaryotes is not apparent from their morphological traits, as there are few observable differences between simple bacterial cells (Rósselló-Móra & Amann, 2001). Metabolic, physiological and morphological characteristics were the first features used to classify bacteria (Staley, 2006). However, these characteristics on their own are not sufficient to establish the evolutionary relatedness between bacteria. Fortunately, evolutionary relationships between microbes can be studied by molecular means. Molecular advancements that have come about since the late 1960's have taken microbial taxonomy forward in great strides (Staley, 2006). These advancements included determining the mol% G + C content of the DNA, DNA-DNA hybridisation (DDH), the analysis of 16S rRNA gene sequences, and the analysis of other 'house-keeping' genes (Labeda et al., 2017; Curtis & Meyers, 2012; Richter & Rósselló-Móra, 2009; Busse et al., 1996). The Average Nucleotide Identity (ANI) between 2 genome sequences is one of the more recent advancements and is considered an alternative 'gold standard' to DDH in distinguishing between bacterial species (Richter & Rósselló-Móra, 2009). In turn, a polyphasic taxonomic approach is used for the identification of new species of bacteria.

1.2.2 Polyphasic taxonomy

The term "polyphasic taxonomy" was coined by Colwell (1970) and, since then, taxonomists have come to realise the importance of including multiple characteristics when grouping microorganisms. Polyphasic taxonomy has become the standard for prokaryote classification (Sutcliffe et al., 2012). The different types of information used in polyphasic taxonomy integrate genotypic, phenotypic and phylogenetic information (Colwell, 1970).

1.2.2.1 Phenetic classification

Before the advent of molecular techniques in classification, morphology, physiology and growth conditions were the only aspects that taxonomists could utilise for classification. These are known as the classical characteristics (Prescott et al., 2008; Rósselló-Móra & Amann, 2001). Grouping organisms based on these phenotypic characteristics is called the phenetic system (Prescott et al., 2008). This includes the use of a plethora of tests to note any observable differences between microorganisms. Phenetic classification is an

inefficient method to classify microorganisms independently of other techniques, because of their overall simple phenotypic profiles, as well as the false assumption that all traits are weighted equally (Prescott et al., 2008). However, the inclusion of experimental techniques, such as enzymatic capabilities, substrate utilisation, and growth conditions did help with finding observable differences (Róssello-Móra & Amann, 2001). The analysis of phenotypic characteristics is a tedious task in the classification of a new taxon due to the time and skill required. Furthermore, the phenotypic characteristics of a new isolate must be determined in parallel with those of its closest phylogenetic relatives (i.e. all strains must be grown under exactly the same conditions) so that the observed characteristics are comparable.

1.2.2.2 Genotypic classification

The elucidation of the structure of deoxy-ribonucleic acid (DNA) and the understanding of the principles behind heredity were important advances in science as a whole. And now, with access to an abundance of DNA sequence data, the analysis of 16S rRNA gene sequences are the centrepiece for the description of a novel bacterial taxon (Tindall et al., 2010). However, other genes are also being used in analysing the relationships between bacterial species.

1.2.2.2.1 16S rRNA gene sequences

The use of 16S rRNA gene sequences to assess the relationships between microorganisms has been one of the most influential methods in microbial taxonomy. This approach, pioneered by Woese and Fox, is now widely accepted with hundreds of thousands of sequences in databases such as GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and the Ribosomal Database Project (https://rdp.cme.msu.edu/) (Prescott et al., 2008).

The information gained from 16S rRNA gene sequence data is generally the first indication of whether a bacterium is novel. The current, agreed upon, threshold establishing that a bacterial species is novel is 97% 16S rRNA gene sequence similarity and was proposed by Stackebrandt & Goebel (1994). However, sequence similarity greater than 97% does not imply that one does *not* have a novel taxon. Meier-Kolthoff et al. (2013) demonstrated that the threshold for actinobacterial species specifically, is 98.8%, with a maximum probability of error of 0.5%. That is, there is only a 0.5% probability that two strains that have a 16S rRNA gene sequence similarity of 98.7% or less do not belong to different species. However, other supporting data are needed to prove novelty, such as the phenotypic differences, chemotaxonomic differences, and possibly also analysis of additional genes for greater resolution between an isolate and its closest phylogenetic relatives. These are the types of information that are used in modern polyphasic actinobacterial taxonomy. It is important to note that high quality sequence data must be used to ensure accurate descriptions. However, whole-genome derived and multiple-gene derived relatedness evaluations are better to delineate species (Konstantinidis & Tiedje, 2007). The 16S rRNA gene is too highly conserved to distinguish between different species that are very closely related to each other, as strains of such species have very high 16S rRNA gene sequence similarities (Fox et al., 1992).

1.2.2.2.2 DNA-DNA Hybridisation

DDH, developed in the 1980's, has been the 'gold standard' for describing a novel species, by illustrating that two genomes are dissimilar to one another (Goris et al., 2007; Staley, 2006; Chun & Rainey, 2014). DDH is used when the 16S rRNA gene sequence similarity between bacteria is 97% or higher because a stable, detectable, DNA hybrid will not form if the DNA molecules are too different (Meier-Kolthoff et al., 2013; Tindall, et al., 2010; Prescott et al., 2008). The premise of DDH is that, in a mixture of denatured genomic DNAs from two bacterial strains, the single-stranded DNA (ssDNA) from the two strains will form a DNA hybrid at a hybridisation temperature of about 25°C below the average melting temperature (T_m) of the two genomic DNAs if there is less than 15% base mispairing. Non-complimentary strands remain single stranded (Stackebrandt & Goebel, 1994). Due to strains from related species having similar base compositions, a higher hybridisation temperature will lead to only the similar/same strands forming double-stranded DNA (i.e. the conditions for base pairing are very stringent). At hybridisation temperatures 10°C or 15°C below the T_m, only almost identical DNA molecules should hybridise (Prescott et al., 2008). DDH experiments are largely based on relative binding ratio (RBR) methods where the results are given as a percentage hybridisation. A percentage hybridisation of 70% is considered the threshold for distinguishing between species (Wayne et al., 1987). Thus, two strains that have a DNA relatedness by DDH of ≥70% are considered to belong to the same genomic species and two strains that have a DNA relatedness of <70% by DDH are considered to belong to different genomic species. DDH is used in conjunction with 16S rRNA gene phylogenetic analyses and phenotypic comparisons to describe a new species of bacterium.

Despite DDH being the 'gold standard' for many decades, there are some problems associated with using the technique. It is a time consuming and labour intensive procedure, because of the amount of high quality DNA required for the process. Another main drawback of the technique is that an incremental database cannot be established for future reference (Goris et al., 2007).

1.2.2.2.3 Nucleic acid base composition

Comparing the DNA base compositions between two genomes is considered one of the simplest techniques to determine similarity. The T_m of a DNA molecule is considered a direct measure of the G + C content. DNA with a higher G + C content will denature at higher temperatures due to the presence of more hydrogen bonds between the bases (Prescott et al., 2008). Within prokaryotic genera, the G + C content does not tend to differ by more than 10%, despite great variations between genera (Prescott et al., 2008). Currently, the methods of choice for determining G + C content are based on high-performance liquid chromatography (HPLC) (Tindall et al., 2010). However, Kim et al. (2015) identified a high level of discrepancy between the experimental-based and genome sequence-driven methods of G + C content determination. The G + C range for strains within the same species is generally confined to less than 1mol%. Experimental-based methods were guilty of G + C content falling well out of this acceptable range of 1% (Kim et al., 2015).

1.2.2.2.4 Multilocus Sequence Analysis (MLSA)

The 16S rRNA gene sequence has been heavily relied on for bacterial classification, despite its shortcomings (Curtis & Meyers, 2012). These shortcomings are that the 16S rRNA gene is only a small portion of the genome, it does not encode a protein, and has a slow evolution rate. Thus, any insertions and deletions (indels) have a much greater distorting effect on the phylogenetic relationships (Curtis & Meyers, 2012). Additionally, the 16S rRNA gene does not provide sufficient resolution for differentiation between closely related species (Curtis & Meyers, 2012). Thus, an MLSA approach is used to provide a more accurate and robust representation of the evolutionary relationships between bacteria. MLSA also overcomes the challenges of DDH by not being as laborious and time consuming, whilst also generating information that can be stored in a database (Curtis & Meyers, 2012). MLSA uses multiple, translated, 'housekeeping' genes that are concatenated in frame and then compared to other concatenated sequences using phylogenetic analysis. Housekeeping genes are genes essential to cell growth. This allows one to identify different species within a genus, based on clades within a tree and, because there are several genes involved in the comparison, there is greater resolution (Gevers et al., 2005). The genes selected for MLSA should fit the following criteria: they must be i) ubiquitous; ii) well separated from each other within the genome; iii) only occur as a single copy per genome; iv) be over 900 bp in length; v) be insusceptible to Horizontal Gene Transfer (HGT) or recombination; and vi) have at least two regions of high conservation that can be amplified and sequenced using specific PCR primers (Adékambi et al., 2011). The genetic distance between concatenated sequences can be calculated and gives an indication of the novelty of a strain. For example, Curtis & Meyers (2012) showed that a 4099-nt concatenated gene (*gyrB*-*rpoB*-*recA*-*relA*-*atpD*) genetic distance of ≥0.04 correlates to DDH values of less than 70% between all type strains in the *Kribbella* genus. Based on this method, if a new *Kribbella* strain has a genetic distance of ≥0.04 to all *Kribbella* type strains, it represents a novel species in the genus.

1.2.2.2.5 Average Nucleotide Identity (ANI)

Average Nucleotide Identity (ANI) is determined by comparing the genome sequences of two bacterial strains. The problems in delineating bacteria at the species level, which are observed when using the 16S rRNA gene, are avoided when using ANI. This is due to the high level of resolution that one can achieve when the entire genome is being compared (Konstantinidis & Tiedje, 2005; Konstantinidis & Tiedje, 2007). In addition, analysis using ANI is less prone to being affected by HGT events (Konstantinidis & Tiedje, 2005).

A pairwise analysis of DDH and ANI illustrated a strong linear correlation, such that the 70% DDH threshold corresponded to 95% ANI, with an R^2 value of 0.93 (Konstantinidis & Tiedje, 2005; Konstantinidis & Tiedje, 2007; Goris *et al*., 2007). Different species can even be identified at genetic similarity percentages as high as 98-99% ANI (Konstantinidis & Tiedje, 2004). Thus, ANI is a robust method to measure the evolutionary distance between organisms.

With the decreasing price of DNA sequencing, the use of whole genomes for comparison is becoming more prevalent, because the number of whole genome sequences available is growing. Moreover, the mol% G + C content of an organism is available after genome sequencing, which has historical value in bacterial taxonomy (Chun & Rainey, 2014).

1.2.2.2.6 Molecular signatures

Molecular signatures (amino acid signature sequences and indels) have recently been identified following the increased availability of DNA sequences of genes other than the 16S rRNA gene. These molecular characteristics are uniquely shared amongst different groups of microorganisms and are thus important for defining evolutionary relationships. These molecular characteristics are called conserved signature indels (CSIs), which are lengths of gene or protein sequence that are unique to specific groups of organisms (Gao & Gupta, 2012). The rationale behind this is that an identified CSI is most parsimoniously explained by the change in the genetic code occurring in a common ancestor, which was then vertically passed on. Gao & Gupta (2012) identified three indels in three genes (cytochrome-c oxidase subunit 1, CTP synthetase, and glutamyl-tRNA synthetase) that were distinctly found in the phylum *Actinobacteria*. More recently, Meyers identified amino acid signatures in DNA gyrase subunit B (GyrB) (2014) and recombinase A (RecA) (2015) in genera in the family *Streptosporangiaceae*.

1.2.2.3 Chemotaxonomic classification

Chemical taxonomy (chemotaxonomy) involves the identification of chemical markers in a cell (i.e. diamino acids in the peptidoglycan, whole cell sugar pattern, polar lipids, respiratory isoprenoid quinones and cellular fatty acids). There are many chemotaxonomic features that can be assessed, but only a few will be discussed here:

1.2.2.3.1 Diamino acids in the peptidoglycan

Diamino acids are essential to the crossing linking of the pepdtidoglycan layer of the cell wall in Gram positive and Gram negative bacteria. The Gram negative bacteria display a uniform distribution of *meso*-diaminopimelic acid while Gram positive bacteria demonstrate great variation in the type of diamino acid in their cell walls. This variation observed in Gram positive bacteria has been vital in their classification. These differences include: amino acid sequence variation of the peptide stems, the mode of cross-linkage between the stems and the type of diamino acid present (e.g. LL-diaminopimelic acid, *meso*-diaminopimelic acid, 2,4-diaminobutyric acid, L-lysine and ornithine) (Busse et al., 1996).

1.2.2.3.2 Whole cell sugar pattern

The analysis of the whole cell sugars is widely used in the classification of Gram positive bacteria. Different groups of bacteria have characteristic whole cell sugar patterns (WCSPs). In some cases, there is no characteristic sugar pattern for a genus (e.g. *Streptomyces*) (Busse et al., 1996).

1.2.2.3.3 Polar lipids

Being the main constituents of bacterial membranes, polar lipids are important for classification. Common bacterial polar lipids are phospholipids, such as phosphatidylethanolamine, phosphatidylserine, phosphatidylmethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine and phosphatidylinositol. There are also glycophospholipids, aminophospholipids, glycolipids, aminolipids and lipids. Polar lipids are observed and identified, using different stains, via their R_f values after thin layer chromatography (TLC). In Gram positive bacteria there are five different phospholipid types (PI-PV) (Busse et al., 1996).

1.2.2.3.4 Respiratory isoprenoid quinones

Constituents of the bacterial cytoplasmic membrane and the mitochondrial membrane, respiratory isoprenoid quinones play an important role in the electron transport chain. The different types of quinones, the length of the isoprenoid side chain, and the number of saturated isoprenoid units are all used to characterise bacteria. In Gram positive bacteria, the characteristic differences between menaquinones are the length of the isoprenoid side chain and the number of saturated isoprenoid units (Busse et al., 1996).

1.2.2.3.5 Cellular fatty acids

Fatty acids are present in the cytoplasmic membrane of all bacteria. There are structural differences between the fatty acids that are vital for the classification of bacteria. These structural differences include the chain length (8 – 20 is the typical number of carbon atoms), whether they are saturated or unsaturated, the branching pattern, the presence of cyclopropane moieties, and whether there is a hydroxyl group on the second or third carbon. The cellular fatty acid profile of bacteria is dictated by the growing conditions and thus it is very important to standardise the growing conditions used. A side-by-side analysis between all strains in the study should always be done (Busse et al., 1996).

1.2.3 Moving forward in taxonomy

One of the issues that bacterial taxonomists still need to develop further is the concept of a bacterial species (Staley, 2006). The current species definition has caused much debate with some believing that the definition is 'pragmatic' and covers what is considered to be necessary to characterise a novel species. However, others believe that the current species definition is too conservative and does not truly highlight the prokaryotic diversity in nature (Rosselló-Mora & Amann, 2001). Rosselló-Mora & Amann (2001) laid out a comprehensive review of the species concept for prokaryotes, which is definitely a starting point. Their suggestion is that a phylo-phenetic species definition is introduced as follows: "*a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity with respect to many independent characteristics, and is diagnosable by a discriminative phenotypic property*". Thompson et al. (2013) suggested a genomic species definition. They proposed the replacement of phenotypic tests with genome information, because one can deduce the phenotypic properties based on

the genes present. The problem with this approach is that it infers that genes present will be expressed, which is not true. Thus, Thompson et al. (2013) did note the complexity of metabolism and admitted that culture-based experiments may still be required. In the future, however, such culture-based experiments will not be as predominant as they are in today's characterisation process. It should also be noted that some believe that the concept of a species does not apply to bacteria, because of extensive HGT.

Due to the increased availability of whole genome sequences, taxonomists are predicting that we will move away from the single gene phylogenetic analyses that are widely used at present (Sutcliffe et al., 2012). Instead, whole genome comparisons will complement the polyphasic taxonomic approach of species classification, where taxonomy will be dependent on genome signatures (Thompson et al., 2013). The *Antonie van Leewenhoek Journal of Microbiology* is already encouraging authors to submit the genome sequence of the type strain when describing a novel species, demonstrating the emphasis being placed upon genome sequences (http://static.springer.com/sgw/documents/1521975/application/pdf/Description+of+novel+taxa+I4A+Jan18+update.pdf). Genome sequencing will encompass the high-quality 16S rRNA gene sequencing that is currently required when classifying a novel species and it will provide the DNA G + C content, which has been historically relevant in bacterial taxonomy (Chun & Rainey, 2014). Using the entire content of the genome will provide a truer and more robust measure of the relationships between bacteria (Verma, et al., 2013).

1.3 Antibiotics

Antibiotics are chemical compounds that interfere with some essential process or structure within the bacterial cell, while having as minimal effect on the host as possible. The term antibiotic was first coined in 1942 by Waksman and translates directly to "against life''. These small compounds act against microbial life in two ways: bacteriostatic and bactericidal. Bacteriostatic antibiotics prevent bacterial growth, while bactericidal antibiotics kill bacteria (Yoneyama & Katsumata, 2006). The main targets for antibacterial antibiotics are: i) cell wall biosynthesis, ii) protein synthesis and iii) DNA replication and repair (Walsh, 2000). Natural products have been the main source of antibiotics (~80%), either as the main ingredient or as the inspiration for the active compound (Harvey, 2007). During the early period of antibiotic research, it was found that natural product compounds were useful for more than just treating bacterial and fungal infections, because some of them had antitumor activities too. Since the golden era of antibiotic discovery, the scope of research concerning natural products has dramatically increased (Bérdy, 2005).

1.3.1 Natural products

Natural products are chemical compounds that are derived from either the primary or secondary metabolism of living organisms (Bérdy, 2005). Their use has been documented throughout historical folklore to treat

various ailments (Dias et al., 2012). Some of the earliest recordings of natural product use in medicine date back to 2600 Before the Common Era (BCE), where around 1000 plant-derived substances were used to treat ailments in Mesopotamia (Cragg & Newman, 2013).

In contrast to primary metabolites, secondary metabolites are chemically diverse, low molecular weight compounds that have various targets and modes of action (Bérdy, 2005). Natural products have been the greatest contributors to the drug industry in the history of medicine (Watve et al., 2001). The 'golden era' of antibiotics saw interest in the penicillin, griseofulvin and gramicidin antibiotics. The discovery of penicillin from the fungus *Penicillium notatum* is believed by many to be the main event which ignited the drive for the discovery of novel and diverse antibiotics from naturally occurring microorganisms (Cragg & Newman, 2005). However, after the discoveries of streptomycin, chloramphenicol, the tetracyclines and the macrolides, *Streptomyces* took centre stage (Bérdy, 2005). Between 1983 and 1994, 39% of the 520 approved drugs were either natural products or derivatives of them. Additionally, around 60-80% of antibacterial and anticancer agents were natural product derivatives (Harvey, 2000). Thus, one can see that natural products have contributed considerably to the medical field.

Between the 1970s and 1990s, we saw a waning in the efficiency of antibiotic research. This was due to the increasing costs of research and the rediscovery of known compounds or analogues of known compounds (Bérdy, 2005). Thus, natural products fell out of the limelight in the 1990's in favour of other modern technologies such as *in silico* screening, computer-assisted design of small-molecule ligands, combinatorial chemical synthesis, and combinatorial biocatalysis (Watve et al., 2001). This is emphasised by the image in Figure 2. The figure illustrates the apparent lack of discovery of novel antibacterial agents since the late 1980s. It is suspected that this "Discovery Void" is in fact due to Big Pharma withdrawing from research in the area and not due to a lack of innovation (Silver, 2011).

However, interest in natural products has been revived. It has become ever more apparent that only a small fraction of an incredibly diverse pool of biological diversity has been explored, especially when it comes to bacteria (Watve et al., 2001). This means that there is vast pool of molecular diversity that is waiting to be tapped. This structural diversity, that is inherent to natural products, is thus being recognised once again. A prime example for the discovery of novel antibiotics is the marine environment. The world's oceans cover more than 70% of the earth's surface, and are thus home to a vast and diverse resource of potential drugs (Cragg & Newman, 2013). The marine environment has already been demonstrated as a rich source for bioactive compounds with thousands of new metabolites being reported since the 1980's (Cragg & Newman, 2013). Other extreme and unexplored environments are also enjoying attention.

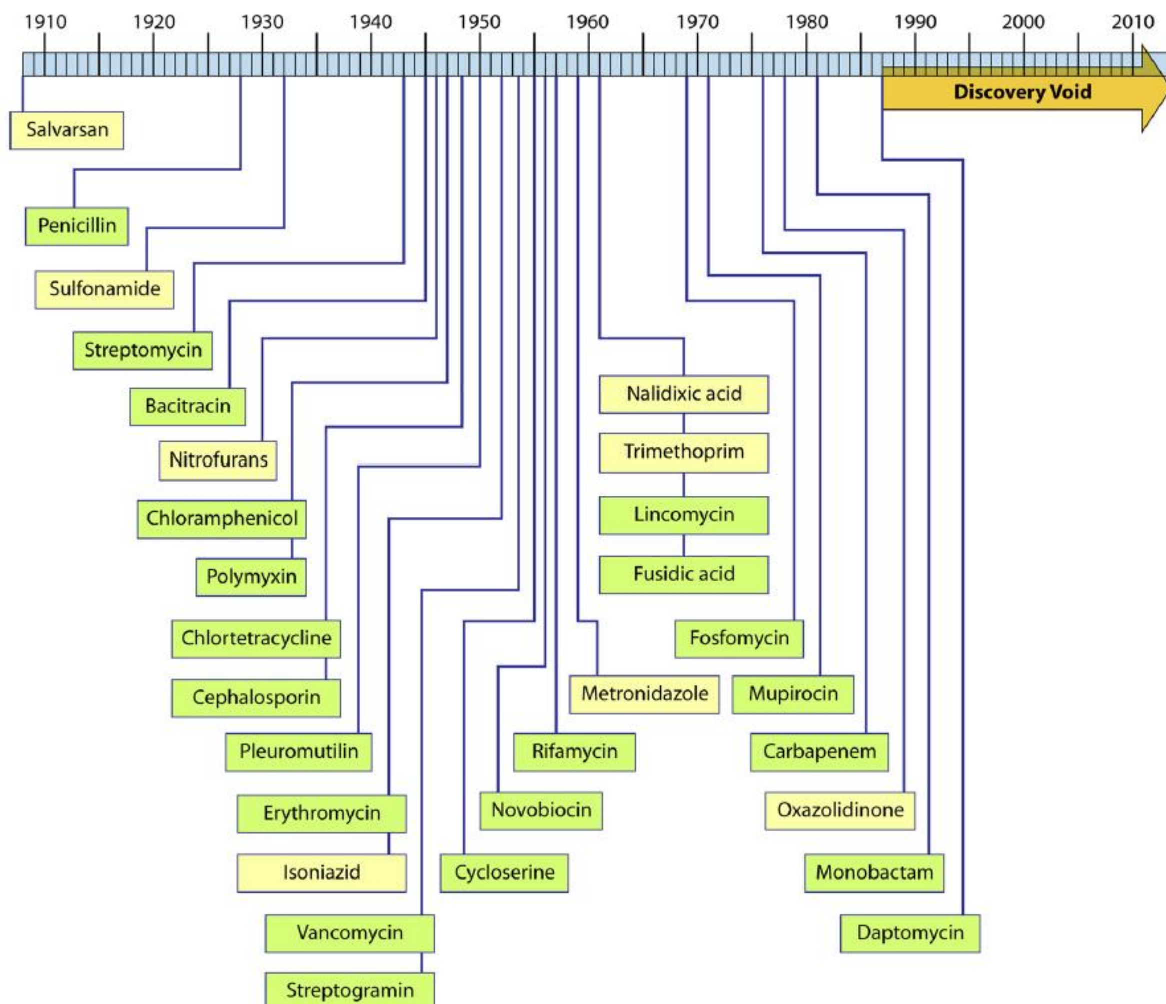

Figure 2: The “Discovery void. Figure taken from Silver (2011).

One of the main advantages of looking for natural products as drug leads is that the active compounds that exhibit drug-like properties are often small molecules. Thus, they are cheaper to develop than other biotechnological products or products from combinatorial chemistry (Harvey, 2000). It is incredibly important to try and access this untapped natural product diversity, because the unchecked use of antibiotics since their introduction in the 1940’s has led to a strong selection pressure for antibiotic resistance (Yoneyama & Katsumata, 2006). The constant pressure of antibiotics on bacterial populations, and the misuse of antibiotics, has led to the need for the continuous discovery of novel antibiotics.

1.3.2 Antibiotic resistance

Antibiotic resistance is a major health problem that effects people from all walks of life and presents a challenge to the effective treatment of both community and hospital-acquired infections (Genilloud, 2017). Upon the introduction of antibiotics around the 1950s to treat microbial infections, the rise of antibiotic resistance during therapy was observed (Davies, 1994). Multidrug resistant bacteria are one of the major causes for treatment failure today (Davies,1994) and we see drug resistant pathogens being associated with all 15 antibiotic classes (Levy & Marshall, 2004). It has also been noted that there is an ever-decreasing rate of novel antibiotic discovery (Kwon et al., 2012).

We are also presented with a conundrum: the increased use of antibiotics will lead to the increased speed of antibiotic resistance development (Yoneyama & Katsumata, 2006). This conundrum is further highlighted by the fact that clinically significant antibiotic resistance typically shows up within months to years of the introduction of a new antibiotic (all dependent on the type of antibiotic and how it is administered). For example, evidence of antibiotic resistance to penicillin appeared within two years of its initial administration (Walsh, 2000). The rapid onset of resistance is a major problem due to the lengthy process required for a new drug to be discovered and introduced to the market (Walsh, 2000). Bacteria already have an advantage in the antibiotics 'arms race' because of their inherent nature: the bacterial life cycle has a rapid generation time with high bacterial count. Along with the rate of mutation of ~1 in 10^7 within a bacterial count of 10^{10}, one can expect mutations to occur frequently. If these mutations confer antibiotic resistance to an individual bacterium, that individual could thrive in the host by being able to grow and take up the space left by the antibiotic sensitive bacteria that have been killed by the antibiotic. Thus, antibiotic resistance will have developed. Antibiotic use itself is a driving factor promoting antibiotic resistance. Additionally, sub-therapeutic levels of drug administration all but guarantees the development of drug resistance (Walsh, 2000). Therefore, it is incredibly important for individuals taking antibiotics to see the course through. The blame for antibiotic resistance does not just lie with antibiotic users failing to finish courses or the bacteria themselves and their inherent ability to rapidly evolve, but also lies with doctors for over-prescribing antibiotics (i.e. prescribing an antibiotic when it is unnecessary to do so). Many doctors give in to their patients' demands and prescribe antibiotics for the treatment of ailments that cannot be cured with antibacterial drugs (such as viral infections). The Centers for Disease Control and Prevention estimated that annually, ~50 million of ~150 million prescriptions were unnecessary in the United States of America (USA) (Levy, 1998). Despite this, bacteria would still have developed antibiotic resistance, but the rate of it could have been curbed.

1.3.2.1 Bacterial strategies for antibiotic resistance development

Bacterial resistance arises due to the presence of resistance genes, which can encode a number of functions such as: i) ejecting antibiotics from the cells with 'efflux' pumps, ii) degrading antibiotics via the production of enzymes, iii) altering/inactivating the antibiotic compounds and/or iv) the structure of the drug target can be altered such that the antibiotic cannot recognise it and bind to it anymore (Walsh, 2000; Levy, 1998). These genes conferring resistance can be present on the bacterial genome (acquired by mutation and/or vertical gene transfer) or on small pieces of circular, extrachromosomal DNA, called plasmids (acquired by HGT). (Levy, 1998).

1.3.2.2 Strategies to overcome resistance

Current drugs generally target the same pathways essential to bacterial life (see the beginning of section 1.3). Thus, one popular strategy to combat antibiotic resistance is to find bioactive compounds that have different targets to the currently used antibiotics (Yoneyama & Katsumata, 2006). This can be achieved by using the prowess of genome sequencing (Genilloud, 2017). Rosamond & Allsop (2000) compared the genomes of *Haemophilus influenzae*, *Mycoplasma*, *Streptococcus pneumoniae*, *Streptococci*, *Chlamydia pneumoniae*, *Klebsiella*, and *Pseudomonas aeruginosa* to 4289 *Escherichia coli* genes, and found that there were 246 conserved genes. Sixty-eight (68) of these genes were not found in humans and 18 were deemed essential to the bacteria. It was finally deduced that three of the essential genes were suitable candidates as drug targets for new respiratory-tract antibiotics (Rosamond & Allsop, 2000).

There are a couple things to consider when using a genomics based approach to identify potential drug targets: i) the gene must be essential, which can be determined by gene inactivation studies; and ii) the target gene must be expressed during the infection process in the lifecycle of the microbe (McDevitt & Rosenberg, 2001). Potential targets identified using the genomics bases approach for novel drug leads include aminoacyl-tRNA synthetases, polypeptide deformylase, fatty acid biosynthesis, DNA replication, protein secretion, cell division, peptidoglycan biosynthesis, signal transduction, aromatic amino acid biosynthesis and isoprenoid biosynthesis pathways, amongst others (McDevitt & Rosenberg, 2001). There is still a long way to go using this strategy, but it has provided a sense of optimism for the future of antibiotic discovery.

In addition to looking for potential drug targets using a genomics approach, one can also look at the antibiotic biosynthetic potential of naturally occurring microbes to produce different types of antibiotics. Analysis of microbial genomes has already revealed that there is a rich antibiotic biosynthetic potential, especially within the actinomycetes (Genilloud, 2017). Screening the genomes allows for the rapid identification of the antibiotic biosynthetic potential of an isolate (Wood et al., 2007) and thus its potential for further work.

It is all well and good to have identified potential drug targets and/or leads for novel drugs based on biosynthetic potential, but we need the actual antibiotic compounds themselves to combat the drug resistant pathogens. One of the main strategies put into place by the pharmaceutical companies to develop new drugs is the development of new antibiotics that are modified versions of existing ones. However, this approach has proven to be difficult, because changing the chemical groups leads to altered pharmacokinetics. These changes and their consequences are difficult to predict, such as increasing activity but losing stability (Yoneyama & Katsumata, 2006). Researchers are also looking at the synthetic development of novel structural classes of antibiotics. A few examples include the oxazolidinones, linezolids, and glycolipodepsipeptides (Walsh, 2000). By synthesising new classes of antibiotics, one can, in practice, have the final say concerning the activity of the compound. Lastly, the search for novel, naturally occurring antibiotics is not over. As pointed out by Genilloud (2017), actinomycetes are still a good source for the discovery of novel antibiotics. To enrich the isolation of compounds with novel chemical diversity, many researchers are mining underexplored environments. This promotes the isolation of novel microbes (actinobacteria specifically in many cases), which increases the chances of finding chemically diverse bioactive compounds. However, current cultivation based methods for isolating microbes fail to isolate most of the microbes in the environment (Genilloud, 2017). Thus, we fail to characterise numerous compounds that could be active against the multi-drug resistant pathogens that we face today, because we cannot grow the organisms that produce them. Consequently, new cultivation techniques are needed to enable the isolation of the biological and chemical diversity that researchers have hitherto been unable to access. This does not imply that current techniques will not allow the isolation of new microbes and their suite of bioactive molecules, but rather that researchers have a lower chance of isolating them using current techniques. One of the main sources for the isolation of novel antibiotics is microbes, and more specifically the actinobacteria.

1.3.2.3 *Mycobacterium tuberculosis*: a case of antibiotic resistance

Drug resistant strains of *Mycobacterium tuberculosis* are prevalent in South Africa and cause either multidrug-resistant tuberculosis (MDR TB) or extensively drug-resistant TB (XDR TB). MDR TB strains are resistant to at least two of the most potent TB drugs: isoniazid and rifampin. XDR TB strains are resistant to isoniazid, rifampin, any fluoroquinolone, and at least one of the 3 injectable second-line drugs (https://www.cdc.gov/tb/topic/drtb/default.htm). The End TB strategy set in place by the WHO has an overall aim to end the global TB epidemic. They hope to have a 90% reduction in TB related deaths and an 80% reduction in the incidence of TB by 2030, in comparison to 2015 (http://www.who.int/tb/en/).

According to the 2016 WHO Global Tuberculosis report (http://www.who.int/tb/en/), South Africa was named as one of the seven countries contributing to 64% of the global incidence of new TB cases (10.4 million TB cases). In the African region, the incidence of individuals infected with the human immunodeficiency virus (HIV) and TB is the highest globally, where 81% of TB patients are also HIV-positive. In Southern Africa, the proportion of TB patients who live with HIV exceeds 50%. Consequently,

TB is the leading killer of HIV-positive individuals, where one in three HIV deaths are TB related. This emphasises the need for novel antibiotics with superior antitubercular activity.

1.4 Aim of this study

Historically, microorganisms have been an abundant source for natural product discovery, as mentioned previously, with actinobacteria being at the forefront of this arms race against antibiotic resistant pathogens. Today we need novel antibiotics with new mechanisms of action and superior bioactivity. This general goal of isolating novel actinobacteria to screen for antimycobacterial activity was the overall focus of the present study.

The aims of this study were to isolate environmental actinobacteria (based on colony morphology) from two compost heap samples and the soil and 'worm tea' of a vermiculture farm. These isolates were tested for antimycobacterial activity against the test bacterium *Mycobacterium aurum* A+. *M. aurum* A+ has been shown to have a similar antibiotic susceptibility profile to *M. tuberculosis* (Namouchi et al., 2017; Phelan et al., 2015; Gupta & Bhakta 2012; Chung et al., 1995). Isolates that exhibited strong bioactivity against *M. aurum* A+ were selected for further analysis by sequencing of the 16S rRNA gene to determine genus identity. These isolates were also tested for their antibiotic biosynthetic potential, at the genetic level, using the polymerase chain reaction (PCR) and primers specific to the core genes of known antibiotic biosynthetic gene clusters. The extraction and preliminary analysis of the active compounds (via TLC) was attempted on strains that exhibited both strong antimycobacterial activity and that have the potential to be novel species based on phylogenetic analyses of the 16S rRNA gene. The particular focus of the study was *Kribbella* strain SK5, which produces strong antimycobacterial activity and is believed to be a novel species in the genus *Kribbella*.

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CHAPTER 2

SCREENING ENVIRONMENTAL ACTINOBACTERIA FOR ANTITUBERCULAR ACTIVITY AND MICROBIAL DIVERSITY

2.1 Summary

One hundred and seventy-five (175) presumptive actinobacteria were isolated from two compost soil samples and a vermiculture farm. These sources were situated in suburban gardens in the southern suburbs of Cape Town, Western Cape Province, South Africa. Following further morphological examination of the isolates, 24 were deemed not to be actinobacteria or were too contaminated for further work. Screening for antimycobacterial activity commenced with 137 of the putative actinobacteria, using the test bacterium *Mycobacterium aurum* A+, which is a non-pathogenic bacterium shown to have a similar antibiotic susceptibility profile to *Mycobacterium tuberculosis*. Thirty-five (35) strains demonstrated strong (2001-3000 mm^2) or very strong (> 3000 mm^2) antimycobacterial activity. The antibiotic-producing isolates were selected for 16S rRNA gene amplification and sequencing. Five strains were also chosen due to activity on the border of moderate (1001-2000mm^2) and strong activity. Many additional isolates were selected based on morphological characters that were distinct from the other isolates. Sequence analysis of the PCR-amplified 16S rRNA genes revealed the following biodiversity: six *Micromonospora* strains, one *Kitasatospora* strain, one *Nocardia* strain, 31 *Streptomyces* strains, and one *Tsukamurella* strain. Analysis of the Gyrase Subunit B gene sequence for the *Kitasatospora* isolate revealed that it was more closely related to the genus *Streptomyces*, via the detection of a molecular signature that was only present in the *Streptomyces* species. The phylogenetic position of these strains in their respective genera was assessed by constructing neighbour-joining and maximum likelihood trees. The *Streptomyces* isolates were responsible for the highest recorded activity against *M. aurum* strain A+.

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2.2 Introduction

Microbes are prolific producers of natural products with medicinal and agricultural relevance (Busti et al., 2006). More specifically, actinobacteria of the *Streptomyces* genus are the most well-known antibiotic producers, responsible for producing approximately 45% of the antibiotics discovered to date (Lazzarini et al., 2000). However, a problem presents itself: the re-isolation of known actinobacteria and thus known antibacterial compounds or even the re-isolation of known antibacterial compounds from novel species. Due to the *Streptomyces* genus being prolific and easily cultivated, it has been thoroughly studied and there are 823 species in the genus with validly-published names, according to the List of Prokaryotic Names with Standing in Nomenclature (LPSN) (http://www.bacterio.net/streptomyces.html). Despite this, mathematical modelling by Watve et al. (2001) estimated that only about 3% of the existing compounds that are produced by the *Streptomyces* genus has been discovered. Nonetheless, the undiscovered antibiotic potential will not be easy to access and identify because many environmental actinobacteria cannot be cultured using standard microbiological techniques. Isolating a novel *Streptomyces* species will lead to an increased likelihood of isolating novel bioactive compounds because biological diversity underpins chemical diversity. However, a better approach is to search for novel actinobacteria of the rarer genera because you are more likely to isolate a novel species (and novel species are more likely to produce antibiotics with novel structures than strains of known species are).

Genilloud (2017) highlighted the continued importance of actinobacteria as a source of novel antibiotics and emphasised that new strategies are being, and need to be, developed to isolate novel species that have previously been inaccessible. This will allow researchers to tap into that undiscovered natural product potential. The current understanding is that microbiologists have barely accessed the natural microbial diversity. Less than 1% of naturally occurring microorganisms have been cultivated using conventional microbiological techniques (Hamaki et al., 2005). Thus, using a suite of media of differing nutrient compositions, and/or un-sampled areas, one may increase the chances of dipping into that previously untapped microbial diversity (Hamaki et al., 2005).

In this study, different environmental sources were sampled to get a broad range of actinobacterial isolates. Two different compost soil samples were chosen, because these are known to be rich in microbial diversity. Additionally, a vermiculture farm was sampled (both the soil and the ‘worm tea’) to access a different resource that could be rich in microbial diversity. Vermicomposting is a non-thermophilic process where organic matter is oxidised and stabilised in the presence of earthworms and microflora to produce a product called ‘vermicompost’ in which the nutrients are more readily available to plants due to increased solubility (Kharrazi et al., 2014; Atiyeh et al., 2002). Vermicompost is known for having a large and diverse microbial population (Kharrazi et al., 2014). This makes it an ideal source to isolate novel actinobacteria.

Stable portions of the genetic code are used to assess the relatedness between species. The 5S, 16S and 23S rRNA genes, and the regions between these genes, are amongst the main candidates for phylogenetic analysis (Clarridge, 2004). There is high sequence conservation in these genes making

them ideal markers of evolutionary distance and the relatedness between organisms. However, the rate of change in the genes between organisms, and even within the gene itself, is not uniform (Clarridge, 2004). Other genes can also be used to assess the relatedness between organisms such as the following housekeeping genes: *gyrB* (DNA gyrase subunit B) (Meyers, 2014), *recA* (recombinase A) (Meyers, 2015), and *rpoB* (RNA polymerase beta subunit). These housekeeping genes experience a higher rate of mutation than the 16S rRNA gene and one can observe greater differences in percentage sequence similarity between closely related species. Using these genes to phylogenetically compare microorganisms in conjunction with the 16S rRNA gene, chemotaxonomic and physiological characterisation make for more robust taxonomic classifications of actinobacteria. Meier-Kolthoff et al. (2013) showed that there are general and phylum-specific 16S rRNA gene sequence-similarity thresholds for predicting the likelihood of two strains belonging to the same species. At a probability of error equal to 0.5%, the actinobacterial threshold for distinguishing between species is 98.8% 16S rRNA gene sequence similarity (Meier-Kolthoff et al., 2013). When the percentage sequence similarity exceeds this threshold, other tests such as DDH are required to assess species novelty. Following the isolation of presumptive actinobacterial colonies, the isolates were screened for biological diversity by amplification of the 16S rRNA gene using conserved universal PCR primers, F1 and R5 (Cook and Meyers, 2003). The PCR products were sequenced and compared to the curated EzBioCloud database (https://www.ezbiocloud.net/).

The aim of this chapter was to isolate actinobacteria from environmental sources in search of novel species that may produce secondary metabolites with antimycobacterial activity. The environmental samples were plated on various nutrient media to encompass a wide range of nutrient conditions to isolate a diverse variety of bacteria. Colony morphology was used to identify actinobacteria. Those colonies with interesting morphological characters were chosen, to try and decrease the number of *Streptomyces* isolates. These isolates were screened for antimycobacterial activity using the test bacterium *M. aurum* strain A+. *M. aurum* A+ is a non-pathogenic microorganism, and it grows faster than *M. tuberculosis*. Isolates exhibiting strong or very strong activity against *M. aurum* strain A+ were identified to the genus level by partial 16S rRNA gene sequencing and analysis. Some isolates that did not have strong antimycobacterial activity were also identified to the genus level due to their having morphological features that were intriguing. Phylogenies were constructed to determine the phylogenetic position of each isolate in its genus.

2.3 Materials & Methods

2.3.1 Bacterial cultivation

2.3.1.1 Sample collection

The actinobacteria isolated in this project originated from three different soil samples. One compost soil sample and a vermicompost sample (soil and 'worm tea' separately) were from a suburban garden in Mowbray, Cape Town, South Africa. A second compost soil sample was taken from a suburban garden in Plumstead, Cape Town, South Africa. The two compost samples are thus named the Mowbray compost sample and the Plumstead compost sample.

2.3.1.2 Sample preparation

Half of each sample was treated and the other half was untreated (except the worm tea sample, which was left untreated). The treated samples were heated at 60°C for 1 hour, in sterile glass Petri dishes. The rest of the procedure is the same for the treated and untreated samples. Approximately 0.1g of soil was suspended in 1mL sterile water in a benchtop centrifuge tube and agitated for 5 min using a vortex mixer to dislodge the microorganisms from the soil. The suspension was left for 5 minutes for the soil particles to settle. The supernatant was serially diluted in sterile distilled water to 10^{-4}, and 100µL of the 10^{-3} and 10^{-4} fractions were plated out in duplicate on International *Streptomyces* project medium No. 2 (ISP2) (Shirling & Gottlieb, 1966), Czapek solution agar (CZ) (Atlas, 2004), Middlebrook agar (7H9) supplemented with 10mM glucose (albumin-catalase supplement omitted), and GOT nutrient medium (Tormo et al., 2003). These four media were chosen because they have very different nutrient compositions to allow for the isolation of bacteria with different nutrient preferences. All media were supplemented with nalidixic acid (10µg/mL) and cycloheximide (50µg/mL) to inhibit the growth of Gram-negative bacteria and fungi, respectively. The plated dilutions were incubated at 30°C for up to 30 days. The worm tea sample was handled in the same manner as the supernatant of the vortexed soil samples.

2.3.1.3 Isolation

Presumptive actinobacterial colonies (colonies slightly sunken into the agar and exhibiting aerial mycelium or (in the absence of aerial mycelium) a leathery or wrinkled surface) were sub-cultured at regular intervals throughout the 30 day incubation period. Strains were sub-cultured onto plates of the same medium as that on which they were isolated (without antibiotics). Colonies were sub-cultured using a sterile toothpick. The sub-cultured isolates were incubated at 30°C for up to 7 days, and named according to the medium they were isolated from and numbered chronologically: M - 7H9, C - CZ, G - GOT, and Y - ISP2. The source of the isolate was recorded. Isolates that were incorrectly identified as actinobacteria were later discarded. If necessary, isolates were re-streaked several times to obtain pure cultures. Stock cultures were made from a spore suspension or a broth culture. Spore suspensions were prepared by adding 2-3 loopfuls of spores to 2 mL of sterile water and shaking vigorously to disperse the hydrophobic spores. Stock cultures were prepared by mixing 700µL of spore suspension or broth culture with 300µL 50% (w/v) sterile glycerol in a sterile benchtop centrifuge tube. Stock cultures were stored at -20°C.

2.3.2 Screening for antimycobacterial activity

Testing for antimycobacterial activity was done using the standard overlay method. The isolates that morphologically resembled actinobacteria were screened for antimycobacterial activity against the test bacterium *M. aurum* A+. The isolates were stab inoculated (four per plate) using sterile toothpicks into ISP2, CZ, 7H9, and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) medium #553 (https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium553.pdf) agar plates and incubated at 30°C for 10 days. On the 9th day of incubation, *M. aurum* A+ was inoculated into a 5 mL 2YT broth (see recipe below) for overnight growth (14-18 hours) at 37°C, with shaking. Prior to overlaying the stab inoculation plates, the purity of the *M. aurum* A+ culture was confirmed by Gram stain and streaking 15μL of the culture for single colonies on a 2YT agar plate. The optical density of the *M. aurum* A+ culture was measured using a BECKMAN DU®-64 spectrophotometer. The optical density (OD) was used to calculate the appropriate amount of *M. aurum* A+ that was to be added to the sloppy agar, such that the OD_{600nm} X Volume of culture (μL) = 160. This empirical formula was used to ensure that the amount of test bacterium used in the overlays was consistent between experiments. Overlays were performed using 6 mL sloppy 2YT agar (0.7% agar) inoculated with the appropriate amount of *M. aurum* A+ and poured gently over the stab inoculated plates, making sure not to wash any spores off the actinobacterial colonies. The overlays were incubated at 37°C for 48 - 72 hours. Antimycobacterial activity was observed as a zone of clearing around the colonies where the test bacterium did not grow. The zone of inhibition (ZOI) is equal to the area of the zone of clearing minus the area of the actinobacterial colony. The presence or absence of aerial mycelium on the actinobacterial colonies was recorded before performing the test-bacterium overlays. The area of the ZOI is measured in mm^2 and is also recorded as one of the following: <$100mm^2$ – very weak; 100-$1000mm^2$ – weak; 1001-$2000mm^2$ – moderate; 2001-$3000mm^2$ – strong; >$3000mm^2$ – very strong. If the ZOI was not able to be read accurately due to overlapping zones, the isolates were tested again individually. Recipe for medium 2YT: 16.0g Pancreatic digest of Casein (Tryptone) (Biolab), 10.0g Yeast extract (Biolab), 5.0g NaCl (Saarchem/MERCK), in 1L distilled water, followed by adjusting the pH to 7.0. 15g agar (Biolab) was added to 1L 2YT broth to make 2YT agar. The medium was autoclaved at 15psi for 15 minutes.

2.3.3 Genus identification

2.3.3.1 Genomic DNA extraction

Genomic DNA was extracted as per the method of Wang et al. (1996), with the following alterations. The cell mass was suspended in lysozyme buffer at a concentration of 25mg lysozyme/mL, and incubated at 37°C overnight (14-18 hrs). Additionally, the genomic DNA was re-dissolved in a volume of Tris-EDTA (TE) buffer (pH 7.8) that was dependent on the size of the DNA pellet. Following DNA extraction, the concentration of DNA was measured using a Nanodrop™ spectrophotometer (model ND-1000).

2.3.3.2 PCR amplification of the 16S rRNA and *gyrB* genes

The 16S rRNA gene was amplified via PCR for sequence analysis using the universal bacterial primers F1 (5'-AGAGTTTGATCITGGCTCAG-3') and R5 (5'-ACGGITACCTTGTTACGACTT-3) (I = inosine) (Cook & Meyers, 2003). 'F' denotes a forward primer while 'R' denotes a reverse primer. The *gyrB* gene was amplified via PCR using the following sequence specific primers pairs: GgyrB-F1 (GF1) & 7G-gyrB-R (7GR), GgyrB-F2 (GF2) & KgyrB-R1892 (K-R1892), 7G-gyrB-R (7GF) & GgyrB-R1 (GR1), and 7GF & KgyrB-R (KR) (Meyers, 2014). PCR was performed in 50 µL reactions with the following components: 2 mM $MgCl_2$ (4 mM $MgCl_2$ for *gyrB*), 150µM of each dNTP, 0.5 µM of each primer (1.5 µM of each primer for *gyrB*), 1U Super-Therm *Taq* polymerase (JMR Holdings, USA), and 100 - 500ng/µL of template DNA (500ng/µL for *gyrB*). The PCR programme can be seen in Table 1 below.

Table 1: PCR programme for 16S rRNA and *gyrB* gene amplification.

Step	Temperature (°C)	Time
1 (initial denaturation)	96	2 min
2 (denaturation)	96	30s
3 (annealing)	56	20s
4 (extension)	72	30s
5 (final extension)	72	5 min
6	20	Hold
Steps 2-4 were carried out 30 times before proceeding to steps 5 and 6		

The PCR products were detected on a 0.8% agarose gel, using ethidium bromide (EtBr) at 0.8µg/mL (EtBr stock concentration = 10mg/mL), following electrophoresis at 90-100V in 1 X TAE tank buffer (Sambrook et al., 1989). A *Pst*I digestion of bacteriophage λ DNA was used as the molecular marker. The agarose gel was visualised at 260nm using a BIORAD ChemiDoc™ XRS+.

2.3.3.3 Sequencing and *blastn* analysis

PCR products were purified for sequencing using an MSB® Spin PCRapace kit (STRATEC Molecular, Berlin, Germany), according to the manufacturer's instructions. The purified DNA was diluted to 50ng/µL in elution buffer for sequencing. Sanger sequencing reactions were performed by Macrogen Europe (Amsterdam, The Netherlands) using the universal primers: 518F (5'CCAGCAGCCGCGGTAATACG3') and 800R (5'TACCAGGGTATCTAATCC3') for determining the sequence of the 16S rRNA gene. Sequencing results were edited using Chromas version 2.6.4 (http://technelysium.com.au/?page_id=13). Edited partial 16S rRNA gene sequences were submitted to the EzBioCloud database (https://www.ezbiocloud.net/identify) and analysed using a standard nucleotide-nucleotide Blast (*blastn*) search (Altschul et al., 1997) to identify each isolate to the genus level based on the top hits by percentage sequence similarity. The *gyrB* gene sequences were sequenced using the appropriate primers (dependent on the primers used in the PCR) and edited as above prior to *blastn* analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3.3.4 Phylogenetic analysis of identified strains

2.3.3.4.1 16S rRNA gene phylogeny

For the isolates that were identified as *Streptomyces*, the type strain 16S rRNA gene sequence of each of the top 5 hits was downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/). Where strains had similar *blastn* results, the next top hits in line were selected, as above. For isolates that were identified to a non-*Streptomyces* genus, the type strain 16S rRNA gene sequence of each of the top 10 hits was downloaded from the NCBI database. Where strains had similar *blastn* results, the next top hits in line were selected, as above. Where the genus was comprised of only a few species with validly-published names, the 16S rRNA gene sequences were downloaded for all type strains in the genus. Multiple sequence alignments of 16S rRNA gene sequences were generated using MUSCLE (Edgar, 2004) or ClustalW (Larkin et al., 2007) in the Molecular Evolutionary Genetics Analysis version 6.0 (MEGA6) software (Tamura et al., 2013). Each alignment was edited such that columns containing any insertions or deletions were removed from the alignment. The length of the aligned, edited sequences was recorded. The alignments were then used to construct maximum likelihood (Felsenstein, 1981) and neighbour-joining (Saitou and Nei 1987) phylogenetic trees using the MEGA6 software.

2.3.3.4.2 Gyrase subunit B gene phylogeny

The available *gyrB* gene sequences for members of the *Kitasatospora* genus were downloaded to compare to the *gyrB* gene sequence generated for strain PR3. A multiple sequence alignment and phylogenetic trees were constructed as described in section 2.3.3.4.1.

2.4 Results & Discussion

2.4.1 Isolation

A total of 175 putative actinobacterial strains was isolated from the three different environmental sources. Forty-seven (47) strains were isolated from the vermicompost soil, 21 strains were isolated from the 'worm tea', 68 were isolated from the Mowbray compost soil sample, 37 were isolated from the Plumstead compost sample, and two isolates were contaminants selected from sub-cultured plates. All isolates were selected based on known actinobacterial colony characteristics (see section 2.3.1.3).

After sub-culturing, 24 of these isolates were determined to be non-actinobacterial species, or pure cultures could not be obtained. These 24 isolates were discarded without further analysis.

2.4.2 Screening for antimycobacterial activity

Of the remaining presumptive actinobacterial isolates, 135 were screened for antimycobacterial activity using the test bacterium, *M. aurum* A+ (Table 2). With these, strain 54Y(WT) which was isolated in a previous study (the author's B.Sc.(Honours) laboratory project), was also screened for antimycobacterial activity. Strain SK5, isolated by a student doing an internship in the lab, was also included here due to already-demonstrated strong activity and being a member of the *Kribbella* genus (Table 3). A positive result here implies the potential for an isolate to produce an antibiotic with antitubercular properties (i.e. activity against *M. tuberculosis*). Thirty-two (32) isolates exhibited no antimycobacterial activity on any of the media, 41 exhibited very weak/weak activity (ZOI $<$ 100 mm^2/100-1000 mm^2), 28 exhibited moderate activity (ZOI 1001-2000 mm^2), 16 exhibited strong activity (ZOI 2001-3000 mm^2), and 20 exhibited very strong activity (ZOI >3000 mm^2). In general, most of the isolates in the strong and very strong range of activities morphologically resembled actinobacteria of the *Streptomyces* genus. Isolates of importance are those which exhibited strong (strains C4, C20, M19, M33, PR4, PR10, PR11, PR29, PR35, PR47, Y2, Y11, Y30, Y31, Y38, Y39) or very strong (strains C3, C14, M7, M8, M18, M20, M40, PR3, PR28, SK5, Y5, Y10, Y12, Y14, Y15, Y16, Y20, Y40, Y41, UK2) activity (Table 2). Strains C23, M27, M42, PR24, and PR36 may also be of interest because their activities were on the border of moderate and strong bioactivity (Table 2). It is important to note that the antibiotic activity of each isolate varied depending on the medium on which it was grown. Most of the isolates with strong or very strong activity only exhibited such activity on one of the four test media used. This is likely due to specific nutrient conditions required for antibiotic biosynthesis (Prescott et al., 2008). Interestingly, when activity was recorded as moderate or greater, it was when the isolates were grown on either DSMZ #553 or ISP2 media (88% of all recorded activity greater than 1000 mm^2). This is unexpected because secondary metabolites, such as antibiotics, are typically made when the environmental conditions are challenging (i.e. low nutrient conditions), which nutrient-rich media like DSMZ #553 and ISP2 do not represent (Martin & Demain, 1980; Tormo et al., 2003). This observation may be due to very good growth and thus almost complete utilisation of the nutrients resulting in starvation conditions and thus secondary metabolite production within the ten-day growth period. Another reason could be that nutrient rich conditions promoted growth and thus more cells are contributing to antibiotic production leading to a stronger antibiotic response. A possible reason for a

general weak antibiotic response on medium 7H9 is that glucose is known to interfere with the biosynthesis of many antibiotics (Martin & Demain, 1980). Thus, glucose should either be exchanged for a different carbon source or the medium should be replaced with a different medium.

Strain PR3 is of interest because it had very strong activity on DSMZ Medium #553 and ISP2 (ZOIs of 4852 and 4040 mm^2, respectively), strong activity on 7H9 medium (2778 mm^2), and moderate activity on CZ medium (1238 mm^2) (Table 2). Strain PR28 also demonstrated good activity on all four of the test media: moderate activity on CZ medium (1914 mm^2), strong activity on 7H9 and DSMZ #553 media (2038 mm^2 and 2964 mm^2, respectively), and very strong activity on ISP2 medium (5740 mm^2) (Table 2). These isolates are ideal strains to work with for antibiotic extraction because they produce strong activity on various media with different nutrient compositions. It is noteworthy that *Kribbella* strain SK5 had a very strong zone of activity at 3309 mm^2 (on ISP2 medium), because members of the genus *Kribbella* are not well known for antibacterial antibiotic production. This makes the strain a priority for further study (strain SK5 is discussed in detail in Chapter 3). It is important to note that a lack of antibiotic activity here does not imply that the microbe is not producing bioactive molecules, or that it does not have the biosynthetic capability to produce an active compound. Activity could be induced under other environmental conditions, or against a different kind of test bacterium (Gram negative bacteria, for example).

2.4.3 Genus identification

The following strains were selected for genus identification via sequencing of the 16S rRNA gene and *blastn* analysis due to a strong or very strong bioactivity against *M. aurum* A+: C3, C4, C20, M7, M8, M20, PR3, PR4, PR10, PR11, PR28, PR29, PR35, PR47, Y5, Y10, Y12, Y14, Y16, Y20, Y30, Y31, Y38, Y39. Strain PR10 was also interesting morphologically, exhibiting an orange substrate mycelium and white aerial mycelium. Strains C23, G2, M13, M27, PR24, PR36, and UK1 were also selected for 16S rRNA gene sequencing because they had moderate antibiotic activity and interesting colony morphologies. Strains G3, G4, M26, M28, PR6, PR22, and 54Y(WT) were chosen for sequencing purely due to displaying interesting colony morphologies. Strains G3, M26, M28 and 54Y(WT) were bright orange in colour, slightly wrinkled, and formed a black spore mass. Strain G4 had a slightly wrinkled, ‘dry-looking’, pale orange cell mass. Strain PR22 had pink substrate mycelium and a light pink spore mass. Table 3 shows each strain’s top hit based on an EzBioCloud analysis. Strains C4 and Y31 were not compared to the EzBioCloud database because good quality sequences could not be obtained. A new round of DNA extraction and PCR amplification followed by cloning into a vector is suggested to obtain good quality 16S rRNA sequences.

Table 2: Antimycobacterial activity of environmental actinobacterial isolates against *M. aurum* A+ on DSMZ #553, CZ, 7H9, and ISP2 agar, using the overlay method. The presence (+) or absence (-) of aerial mycelium (AM) was recorded before overlaying with *M. aurum* A+. Zones of inhibition (ZOI) were calculated and assigned an arbitrary measure of the strength of the antimycobacterial activity: VW = Very weak, W - weak, M - moderate, S - strong, VS - very strong activity and n/a – no activity Overlays were incubated at 37 °C for 48-72 hours.

Strain	Medium	AM	ZOI	Activity#	Strain	Medium	AM	ZOI	Activity#
C1	DSMZ #553	+	-	n/a	C16	DSMZ #553	+	-	n/a
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	-	n/a		7H9	+	-	n/a
	ISP2	+	-	n/a		ISP2	+	-	n/a
C2	DSMZ #553	+	-	n/a	C17	DSMZ #553	+	-	n/a
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	-	n/a		7H9	+	-	n/a
	ISP2	+	1737	M		ISP2	-	414	W
C3	DSMZ #553	+	-	n/a	C18	DSMZ #553	+	-	n/a
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	314	W		7H9	+	-	n/a
	ISP2	+	4048	VS		ISP2	+	-	n/a
C4	DSMZ #553	+	-	n/a	C19	DSMZ #553	-	40	W
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	-	n/a		7H9	-	-	n/a
	ISP2	+	2964	S		ISP2	-	-	n/a
C5	DSMZ #553	+	-	n/a	C20	DSMZ #553	-	2325	S
	CZ	+	-	n/a		CZ	+	366	W
	7H9	+	-	n/a		7H9	-	-	n/a
	ISP2	+	-	n/a		ISP2	-	-	n/a
C6	DSMZ #553	+	-	n/a	C21	DSMZ #553	-	-	n/a
	CZ	+	484	W		CZ	+	-	n/a
	7H9	+	-	n/a		7H9	-	-	n/a
	ISP2	+	-	n/a		ISP2	-	-	n/a
C7	DSMZ #553	+	-	n/a	C22	DSMZ #553	+	-	n/a
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	553	W		7H9	+	-	n/a
	ISP2	+	-	n/a		ISP2	+	-	n/a
C8	DSMZ #553	+	-	n/a	C23	DSMZ #553	+	1936	M
	CZ	+	-	n/a		CZ	+	50	W
	7H9	+	462	W		7H9	+	89	W
	ISP2	+	-	n/a		ISP2	+	1993	M
C9	DSMZ #553	+	-	n/a	C24	DSMZ #553	+	-	n/a
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	283	W		7H9	+	-	n/a
	ISP2	+	-	n/a		ISP2	+	-	n/a
C10	DSMZ #553	+	-	n/a	C25	DSMZ #553	+	-	n/a
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	503	W		7H9	+	-	n/a
	ISP2	+	-	n/a		ISP2	+	-	n/a
C11	DSMZ #553	+	-	n/a	C26	DSMZ #553	-	1549	M
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	-	n/a		7H9	+	469	W
	ISP2	+	-	n/a		ISP2	-	-	n/a
C12	DSMZ #553	+	-	n/a	C31	DSMZ #553	-	90	VW
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	-	n/a		7H9	+	-	n/a
	ISP2	+	-	n/a		ISP2	-	-	n/a
C13	DSMZ #553	+	-	n/a	C32	DSMZ #553		-	n/a
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	408	W		7H9	+	-	n/a
	ISP2	+	1131	M		ISP2	-	-	n/a
C14	DSMZ #553	+	3139	VS	G1	DSMZ #553	+	-	n/a
	CZ	+	45	VW		CZ	+	-	n/a
	7H9	+	-	n/a		7H9	+	484	W
	ISP2	+	864	W		ISP2	-	318	W
C15	DSMZ #553	+	89	VW	G2	DSMZ #553	-	-	n/a
	CZ	+	-	n/a		CZ	-	-	n/a
	7H9	+	460	W		7H9	-	-	n/a
	ISP2	-	-	n/a		ISP2	-	1106	M

Table 2 *(continued)*:

Strain	Medium	AM	ZOI	Activity#	Strain	Medium	AM	ZOI	Activity#
G3	DSMZ #553	-	-	n/a	M18	DSMZ #553	-	4287	VS
	CZ	-	-	n/a		CZ	+	-	n/a
	7H9	-	-	n/a		7H9	+	-	n/a
	ISP2	-	-	n/a		ISP2	+	126	W
G4	DSMZ #553	-	625	W	M19	DSMZ #553	-	2489	S
	CZ	-	-	n/a		CZ	+	704	W
	7H9	-	-	n/a		7H9	+	-	n/a
	ISP2	-	574	W		ISP2		-	n/a
M1	DSMZ #553	+	1623	M	M20	DSMZ #553	+	3536	VS
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	441	W		7H9	+	304	W
	ISP2	+	330	W		ISP2	+	1390	M
M2	DSMZ #553	-	1117	M	M21	DSMZ #553	+	-	n/a
	CZ	+	-	n/a		CZ	-	-	n/a
	7H9	+	-	n/a		7H9	-	-	n/a
	ISP2		-	n/a		ISP2	+	163	W
M3	DSMZ #553	+	1513	M	M22	DSMZ #553	+	283	W
	CZ	+	138	W		CZ	-	-	n/a
	7H9	+	679	W		7H9		-	n/a
	ISP2	+	173	W		ISP2	-	-	n/a
M4	DSMZ #553		-	n/a	M23	DSMZ #553	+	352	W
	CZ	+	99	W		CZ	+	-	n/a
	7H9	+	1307	M		7H9	-	-	n/a
	ISP2	-	302	W		ISP2	-	-	n/a
M6	DSMZ #553	+	330	W	M24	DSMZ #553	-	481	W
	CZ	+	453	W		CZ	-	-	n/a
	7H9	+	264	W		7H9	-	-	n/a
	ISP2	+	-	n/a		ISP2	-	-	n/a
M7	DSMZ #553	+	4497	VS	M25	DSMZ #553	-	1085	M
	CZ	+	-	n/a		CZ	-	-	n/a
	7H9	+	-	n/a		7H9	-	-	n/a
	ISP2		-	n/a		ISP2	-	1483	M
M8	DSMZ #553	+	4852	VS	M26	DSMZ #553	-	-	n/a
	CZ	+	-	n/a		CZ	-	-	n/a
	7H9	+	-	n/a		7H9	-	-	n/a
	ISP2		-	n/a		ISP2	-	-	n/a
M9	DSMZ #553	+	439	W	M27	DSMZ #553	-	1697	M
	CZ	+	622	W		CZ	-	-	n/a
	7H9	+	761	W		7H9	-	-	n/a
	ISP2	+	44	VW		ISP2	-	1238	M
M10	DSMZ #553	-	-	n/a	M28	DSMZ #553	-	414	W
	CZ	+	182	W		CZ	-	-	n/a
	7H9	+	69	VW		7H9	-	-	n/a
	ISP2		-	n/a		ISP2	-	1233	M
M11	DSMZ #553	+	-	n/a	M29	DSMZ #553	-	-	n/a
	CZ	-	574	W		CZ	+	-	n/a
	7H9	+	566	W		7H9	+	-	n/a
	ISP2	-	78	VW		ISP2	-	-	n/a
M12	DSMZ #553	+	-	n/a	M31	DSMZ #553	-	94	VW
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	-	n/a		7H9	-	-	n/a
	ISP2	+	-	n/a		ISP2	-	16	VW
M13	DSMZ #553	+	-	n/a	M32	DSMZ #553	-	88	VW
	CZ	+	1549	M		CZ	-	-	n/a
	7H9	+	419	W		7H9	-	57	VW
	ISP2	-	75	VW		ISP2	-	-	n/a
M14	DSMZ #553	+	-	n/a	M33	DSMZ #553	+	-	n/a
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	-	n/a		7H9	+	-	n/a
	ISP2	+	-	n/a		ISP2	+	2766	S
M15	DSMZ #553	+	409	W	M34	DSMZ #553	+	-	n/a
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	-	n/a		7H9	+	389	W
	ISP2	+	169	W		ISP2		-	n/a
M17	DSMZ #553	-	-	n/a	M35	DSMZ #553	-	427	W
	CZ	+	625	W		CZ	-	-	n/a
	7H9	+	-	n/a		7H9	-	-	n/a
	ISP2	+	276	W		ISP2	-	145	W

Table 2 *(continued)*:

Strain	Medium	AM	ZOI	Activity#	Strain	Medium	AM	ZOI	Activity#
M41	DSMZ #553	-	-	n/a	PR19	DSMZ #553	-	-	n/a
	CZ	-	-	n/a		CZ	+	-	n/a
	7H9		-	n/a		7H9	+	-	n/a
	ISP2	+	-	n/a		ISP2	+	1358	M
M42	DSMZ #553	+	1901	M	PR22	DSMZ #553	+	-	n/a
	CZ	+	189	W		CZ	+	-	n/a
	7H9	+	38	VW		7H9	+	-	n/a
	ISP2	-	1901	M		ISP2	+	-	n/a
M39	DSMZ #553	-	-	n/a	PR23	DSMZ #553	-	629	W
	CZ	-	-	n/a		CZ	+	-	n/a
	7H9		-	n/a		7H9	+	-	n/a
	ISP2	-	-	n/a		ISP2	+	588	W
M40	DSMZ #553	+	4035	VS	PR24	DSMZ #553	+	1763	M
	CZ	-	-	n/a		CZ	+	-	n/a
	7H9	-	-	n/a		7H9	+	-	n/a
	ISP2	+	1342	M		ISP2	+	1710	M
PR1	DSMZ #553	+	-	n/a	PR28	DSMZ #553	-	2964	S
	CZ	+	-	n/a		CZ	+	1914	M
	7H9	+	-	n/a		7H9	+	2038	S
	ISP2	+	-	n/a		ISP2	+	5740	VS
PR3	DSMZ #553	+	4852	VS	PR29	DSMZ #553	+	2832	S
	CZ	+	1238	M		CZ	+	-	n/a
	7H9	+	2778	S		7H9	+	-	n/a
	ISP2	-	4040	VS		ISP2	-	-	n/a
PR4	DSMZ #553	+	2196	S	PR32	DSMZ #553	-	1624	M
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	623	W		7H9	+	-	n/a
	ISP2	+	1291	M		ISP2		-	n/a
PR6	DSMZ #553		-	n/a	PR34	DSMZ #553	+	1478	M
	CZ	+	-	n/a		CZ	+	332	W
	7H9		-	n/a		7H9	+	302	W
	ISP2		-	n/a		ISP2	-	115	W
PR7	DSMZ #553	+	-	n/a	PR35	DSMZ #553	+	2675	S
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	-	n/a		7H9	+	374	W
	ISP2		-	n/a		ISP2	+	78	VW
PR10	DSMZ #553	-	-	n/a	PR36	DSMZ #553	-	1817	M
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	2061	S		7H9	+	-	n/a
	ISP2	-	2400	S		ISP2	-	-	n/a
PR11	DSMZ #553	-	285	W	PR37	DSMZ #553		-	n/a
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	2734	S		7H9		-	n/a
	ISP2	-	2213	S		ISP2	+	1002	M
PR12	DSMZ #553	+	-	n/a	PR44	DSMZ #553	+	-	n/a
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	-	n/a		7H9	+	-	n/a
	ISP2		-	n/a		ISP2	+	-	n/a
PR14	DSMZ #553	+	436	W	PR46	DSMZ #553		-	n/a
	CZ	+	389	W		CZ	+	-	n/a
	7H9	+	260	W		7H9	+	947	W
	ISP2	+	214	W		ISP2	-	-	n/a
PR15	DSMZ #553	+	-	n/a	PR47	DSMZ #553	+	2327	S
	CZ	+	-	n/a		CZ	+	192	W
	7H9	+	-	n/a		7H9	+	134	W
	ISP2		-	n/a		ISP2	+	1830	M
PR17	DSMZ #553	+	-	n/a	PR48	DSMZ #553	+	182	W
	CZ	+	-	n/a		CZ	+	870	W
	7H9	+	5	VW		7H9	+	519	W
	ISP2	+	-	n/a		ISP2	+	1483	M
PR18	DSMZ #553	+	-	n/a	Y1	DSMZ #553	-	65	VW
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	5	VW		7H9	+	-	n/a
	ISP2	+	-	n/a		ISP2	-	28	VW

Table 2 *(continued)*:

Strain	Medium	AM	ZOI	Activity#	Strain	Medium	AM	ZOI	Activity#
Y2	DSMZ #553	+	2793	S	Y20	DSMZ #553	+	3661	VS
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	469	W		7H9	+	981	W
	ISP2	+	648	W		ISP3	-	723	W
Y3	DSMZ #553	+	-	n/a	Y21	DSMZ #553	-	-	n/a
	CZ	+	-	n/a		CZ	+	1056	M
	7H9	+	594	W		7H9	+	-	n/a
	ISP2	-	651	W		ISP2	-	-	n/a
Y4	DSMZ #553	+	-	n/a	Y28	DSMZ #553	-	-	n/a
	CZ	+	-	n/a		CZ	-	-	n/a
	7H9	+	-	n/a		7H9	-	-	n/a
	ISP2	-	-	n/a		ISP2	-	1458	M
Y5	DSMZ #553	+	3649	VS	Y29	DSMZ #553	+	-	n/a
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	-	n/a		7H9	+	94	W
	ISP2	+	911	W		ISP2	+	-	n/a
Y6	DSMZ #553	-	-	n/a	Y30	DSMZ #553	+	2765	S
	CZ	+	572	W		CZ	-	-	n/a
	7H9	+	-	n/a		7H9	+	157	W
	ISP2	-	-	n/a		ISP2	+	-	n/a
Y7	DSMZ #553	+	1208	M	Y31	DSMZ #553	+	2772	S
	CZ	+	-	n/a		CZ	-	-	n/a
	7H9	+	-	n/a		7H9	+	235	W
	ISP3	-	-	n/a		ISP2	+	-	n/a
Y8	DSMZ #553	+	-	n/a	Y33	DSMZ #553	-	-	n/a
	CZ	+	-	n/a		CZ	-	-	n/a
	7H9	+	-	n/a		7H9	-	-	n/a
	ISP2	-	-	n/a		ISP2	-	126	W
Y9	DSMZ #553	-	-	n/a	Y34	DSMZ #553	-	-	n/a
	CZ	+	-	n/a		CZ	-	-	n/a
	7H9	+	-	n/a		7H9	-	-	n/a
	ISP2	-	198	W		ISP2	-	-	n/a
Y10	DSMZ #553	+	1650	M	Y35	DSMZ #553	-	-	n/a
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	-	n/a		7H9	+	-	n/a
	ISP2	+	3673	VS		ISP2	-	-	n/a
Y11	DSMZ #553	+	-	n/a	Y36	DSMZ #553	+	1291	M
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	-	n/a		7H9	+	-	n/a
	ISP2	+	2298	S		ISP2	-	90	VW
Y12	DSMZ #553	+	3040	VS	Y37	DSMZ #553	-	-	n/a
	CZ	+	-	n/a		CZ	-	-	n/a
	7H9	+	-	n/a		7H9	-	-	n/a
	ISP2	-	-	n/a		ISP2		-	n/a
Y13	DSMZ #553		-	n/a	Y38	DSMZ #553	+	2407	S
	CZ	+	332	W		CZ	-	63	VW
	7H9	+	-	n/a		7H9	+	231	W
	ISP2		-	n/a		ISP2	+	2327	S
Y14	DSMZ #553	-	3196	VS	Y39	DSMZ #553	+	-	n/a
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	-	n/a		7H9	+	-	n/a
	ISP2	+	2288	S		ISP2	+	2819	S
Y15	DSMZ #553	+	122	n/a	Y40	DSMZ #553	+	-	n/a
	CZ	+	3290	VS		CZ	+	-	n/a
	7H9	+	-	n/a		7H9	+	-	n/a
	ISP2	+	-	n/a		ISP2	+	3246	VS
Y16	DSMZ #553	+	3444	VS	Y41	DSMZ #553	+	4460	VS
	CZ	+	-	n/a		CZ	+	-	n/a
	7H9	+	215	W		7H9	+	157	W
	ISP2	+	179	W		ISP2	+	2778	S
Y18	DSMZ #553	-	-	n/a	Y42	DSMZ #553	+	-	n/a
	CZ	+	294	W		CZ	+	-	n/a
	7H9	+	-	n/a		7H9	+	-	n/a
	ISP2	-	718	W		ISP2	+	-	n/a
Y19	DSMZ #553	-	-	n/a	Y43	DSMZ #553	+	378	W
	CZ	+	537	W		CZ	+	40	VW
	7H9	+	-	n/a		7H9	+	94	VW
	ISP2	-	741	W		ISP2	+	-	n/a

Table 2 *(continued)*:

Strain	Medium	AM	ZOI	Activity#	Strain	Medium	AM	ZOI	Activity#
Y44	DSMZ #553	+	460	W	UK1	DSMZ #553	-	1620	M
	CZ	+	50	VW		CZ	-	-	n/a
	7H9	+	94	VW		7H9	+	521	W
	ISP2	+	-	n/a		ISP2	-	-	n/a
Y46	DSMZ #553	+	-	n/a	UK2	DSMZ #553	+	3793	VS
	CZ	+	20	VW		CZ	+	45	W
	7H9	+	83	VW		7H9	+	66	W
	ISP2	-	1085	M		ISP2	+	2263	S
Y51	DSMZ #553	+	-	n/a	SK5	DSMZ #553	-	-	n/a
	CZ	+	151	W		CZ	-	-	n/a
	7H9	+	120	W		7H9	-	-	n/a
	ISP2	+	1391	M		ISP2	+	3309	VS
54(Y) WT	DSMZ #553	-	-	n/a					
	CZ	-	-	n/a					
	7H9	-	-	n/a					
	ISP2	-	-	n/a					

Strains C23, G4, PR3, and PR10 were selected for additional sequencing using the 800R primer. Strains C23 and PR10 were chosen because the first round of sequencing (with primer 518F) provided partial 16S rRNA gene sequences that had percentage sequence similarities of less than 99% to their corresponding top hits. This was interesting because they both were identified as *Streptomyces* strains (Table 3) and such low sequence similarity is unusual in the genus. Thus, they are potentially novel species. Strain G4 was also selected because the initial sequencing of the 16S rRNA gene with primer 518F identified the isolate as a member of the *Tsukamurella* genus. This is a rare genus with only 14 species with validly-published names (http://www.bacterio.net/tsukamurella.html) and strain G4 may be a novel member of the genus. Strain PR3 was selected because its antimycobacterial activity was very strong based on the agar overlay method (see Table 2), as well as demonstrating antimalarial activity (Chapter 4). Other 'rare' species of actinobacteria identified in this study were 6 *Micromonospora* strains and 1 *Nocardia* strain. *Micromonospora* strains G2, M27, and M28 exhibited moderate antibiotic activity. These strains are thus of interest for further analysis.

As expected, the predominant genus that demonstrated strong or very strong antimycobacterial activity was *Streptomyces*. Strain PR3, preliminarily identified as a *Kitasatospora* strain, falls into this group, because the genus is closely related to *Streptomyces* (Labeda et al., 2017). Even in extreme environments, the members of the *Streptomyces* genus make up most isolates with strong antibacterial activity (Mohammadipanah & Wink, 2016). This result highlights the predominance that the *Streptomyces* genus has in the natural product research field. By screening for antimycobacterial activity prior to genus identification, the chances of identifying a pool of isolates dominated by *Streptomyces* were high. A different approach could be taken where the isolates with interesting morphologies are identified first and then tested for activity to promote the testing of 'rare' genera for antibiotic activity.

Table 3: Identification of the isolates which had strong antimycobacterial activity and/or were morphologically interesting, to the genus level, based on EzBioCloud analyses. %Sequence similarity is the similarity over the query alignment of a partial 16S rRNA gene sequence obtained by Sanger sequencing using the 518Fprimer. ⱡ Strains have 16S rRNA gene sequences longer than 1400bp that were obtained by additional sequencing with the 800R primer. *Strains have 16S rRNA gene sequences shorter than 620bp.

Strain	Top Hit	% Sequence similarity	Sequence query length (bp)
C3	*Streptomyces violaceus*	100	941
C20	*Streptomyces carpinensis*	99.2	759
C23 ⱡ	*Streptomyces cacaoi* subsp. *cacaoi*	100	1405
G2	*Micromonospora aurantiaca*	99.9	924
G3	*Micromonospora aurantiaca*	100	922
G4 ⱡ	*Tsukamurella pseudospumae*	99.6	1452
M7	*Streptomyces althioticus*	100	941
M8	*Streptomyces althioticus*	100	938
M13	*Streptomyces cellulosae*	99.5	941
M20	*Streptomyces violaceus*	100	927
M26	*Micromonospora aurantiaca*	100	936
M27	*Micromonospora aurantiaca*	100	931
M28	*Micromonospora aurantiaca*	99.9	930
M42	*Streptomyces cacaoi* subsp. *cacaoi*	100	943
PR3 ⱡ	*Kitasatospora albolonga*	100	1430
PR4 *	*Streptomyces lasiicapitis*	99.7	614
PR6	*Streptomyces malachitospinus*	99.9	941
PR10 ⱡ	*Streptomyces antibioticus*	99.7	1481
PR11*	*Streptomyces bobili*	99.5	417
PR22	*Nocardia asteroides*	99.9	926
PR24	*Streptomyces olivaceus*	99.6	937
PR28	*Streptomyces neopeptinius*	99.3	943
PR29	*Streptomyces althioticus*	100	941
PR35	*Streptomyces bacillaris*	100	928
PR36	*Streptomyces althioticus*	100	927
PR47	*Streptomyces pratensis*	100	929
SK5 ⱡ	*Kribbella karoonensis*	99.7	1463
Y2	*Streptomyces roseoviolaceus*	99.7	942
Y5	*Streptomyces violaceus*	100	940
Y10	*Streptomyces roseoviolaceus*	100	940
Y11	*Streptomyces violaceus*	100	937
Y12	*Streptomyces althioticus*	100	927
Y14	*Streptomyces althioticus*	99.7	939
Y16	*Streptomyces violaceus*	100	938
Y20	*Streptomyces violaceus*	100	944
Y30	*Streptomyces cacaoi* subsp. *cacaoi*	100	959
Y38	*Streptomyces cacaoi* subsp. *cacaoi*	100	935
Y39	*Streptomyces violaceus*	99.6	730
UK1	*Streptomyces diastaticus* subsp. *ardesiacus*	99.7	931
UK2	*Streptomyces cacaoi* subsp. *cacaoi*	99.8	935
54Y(WT)	*Micromonospora aurantiaca*	100	926

2.4.4 Phylogenetic analysis of identified strains

2.4.4.1 *Micromonospora* isolates

The genus *Micromonospora* is currently comprised of 79 species with validly published names (http://www.bacterio.net/micromonospora.html). The genus was described by Ørskov (1923) and then emended by Gao et al. (2014). *Micromonospora* species are widely distributed in the environment, from rhizosphere and mangrove forest soil (Thawai et al., 2015; Zhang et al., 2015, respectively) to Black Sea sediment (Veyisoglu et al., 2016).

The phylogenetic position of strains G2, G3, M26, M27, M28 and 54Y(WT) in the genus *Micromonospora* was determined by the construction of a 16S rRNA gene sequence phylogenetic tree using the type strains of the genus (Figure 3). These strains were selected because they had moderate antimycobacterial activity (Table 2) and/or interesting morphological features. These interesting morphological characters were suggestive that the isolates were *Micromonospora* species: all the colonies were slightly raised and folded, deep orange in colour, and later formed a mucous-like black spore mass (Genilloud, 2015). All the isolated strains shared the same closest phylogenetic relative: *M. aurantiaca* strain ATCC 27029^{T} (CP002162) (Figure 3). *M. aurantiaca* was also the top hit based on a *blastn* analysis (Table 3). All the strains had between 99.9 and 100% 16S rRNA gene sequence similarity (over 922-936 bp) to *M. aurantiaca*. This suggests that the six isolates are either very closely related species or they are clones of the same *Micromonospora* strain. Longer 16S rRNA gene sequences will need to be obtained to clarify this. Additionally, *gyrB* gene sequence similarity should also be assessed and a phylogeny constructed. The *gyrB* gene exhibits greater sequence variation between species of the same genus, and clones of the same species would be expected to have 100% *gyrB* gene sequence similarity.

Strains G2, G3 and M28 were isolated from the treated "worm tea" portion of the vermiculture farm. Strains M26 and M27 were isolated from the treated soil of the vermiculture farm. Strain 54Y(WT) was isolated from the worm tea of the same vermiculture farm, but a year prior, in the author's B.Sc. (Honours) project. It is noteworthy that the isolates appear in different sections of the farm and, if they are clones of the same environmental strain, this shows that it can survive in both a terrestrial and aquatic environment (soil and worm tea). Furthermore, it would show that the same strain (54Y(WT)) seems to be present in the farm over the space of a year. This could imply that the strain is established in the vermiculture. The isolation plates also suggested that there is a predominance of *Micromonospora* strains in the vermiculture farm. The agar plates were riddled with small orange colonies with black spores, which resembled known *Micromonospora* colony morphology. However, only strains G2, G3, M26, M27 and M28 were confirmed to belong to the genus *Micromonospora*. Yami et al. (2003) also isolated actinomycetes from vermicompost and found that 57% of the isolates were *Micromonospora* strains, 31% were *Nocardia* strains and 12% were *Streptomyces* strains. The results of these two studies suggest that vermicompost is a good source for the isolation of *Micromonospora* strains.

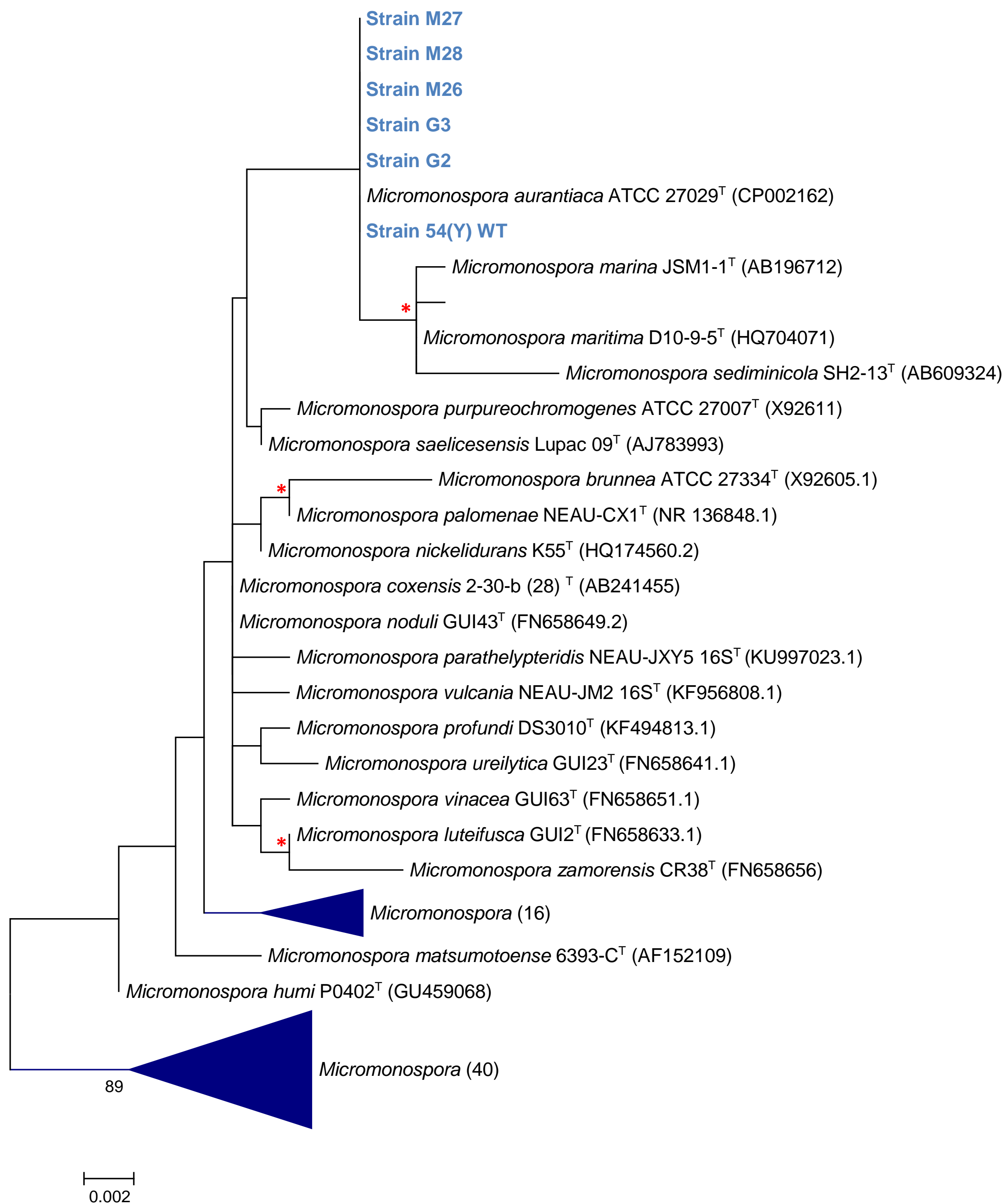

Figure 3: Maximum likelihood tree of the *Micromonospora* genus using partial 16S rRNA gene sequences of 883bp. Bootstrap values were calculated from 1000 resampled datasets. Bootstrap values above 70% are shown. The numbers in parenthesis indicates the number of species in that compressed clade. Asterisks indicate topological conservation between the neighbour-joining tree and the maximum likelihood tree. The scale bar indicates 2 nucleotide changes per 1000 base pairs.

2.4.4.2 *Nocardia* isolate

The first *Nocardia* strain was isolated by Eppinger (1891) from a patient with pneumonia and a brain abscess (McNeil & Brown, 1994). The genus *Nocardia* was formally established by Blanchard (1896) with the description of *Nocardia asteroides*. To date there are over 100 species with validly published names (http://www.bacterio.net/nocardia.html). The genus has grown rapidly from just 16 described species in the year 2000 (Maldonado et al., 2000). *Nocardia* are aerobic, rod-shaped, Gram-positive actinobacteria. Despite being widespread in natural habitats, notably the soil and organic matter (McNeil & Brown, 1994; Orchard et al. 1977), the most attention has been given to the taxonomy of *Nocardia* with clinical significance (Maldonado et al., 2000), such as those nocardiae which cause mycetoma and nocardiosis (McNeil & Brown, 1994).

Strain PR22 was selected for 16S rRNA gene sequencing because its morphology resembled the morphology of known *Nocardia* species. The strain did not have any antimycobacterial activity (Table 2). Strain PR22 was isolated from the Plumstead compost sample. The phylogenetic position of strain PR22 in the genus *Nocardia* was observed via the construction of a 16S rRNA gene phylogenetic tree, using the type strains of some of the *Nocardia* species with validly published names (Figure 4). Strain PR22 groups with *Nocardia neocaledoniensis* SBHR OA6^T (AY282603) and *N. asteroides* DSM 43757^T (AF430019) with 90% bootstrap support (Figure 4). These three strains form a distinct clade within the *Nocardia* genus, with little sequence variation over the 816 nucleotides compared. This phylogeny is in line with the *blastn* analysis of the 16S rRNA gene sequence where *N. asteroides* was the top hit based on percentage sequence similarity (Table 3). As only an 816 bp alignment was used to construct the 16S rRNA gene sequence tree in Figure 4, a longer sequence is needed for a more robust comparison of strain PR22 to the known *Nocardia* species. The *gyrB* gene could also be sequenced to further assess the phylogenetic position of strain PR22 in the *Nocardia* genus. Following this, one could determine whether the strain is worthy for characterisation as a novel species in the genus. No further characterisation of strain PR22 was undertaken, as it exhibited no antimycobacterial activity and working with one of its two closest phylogenetic relatives (*N. asteroides*) requires a Biosafety Level 2 laboratory.

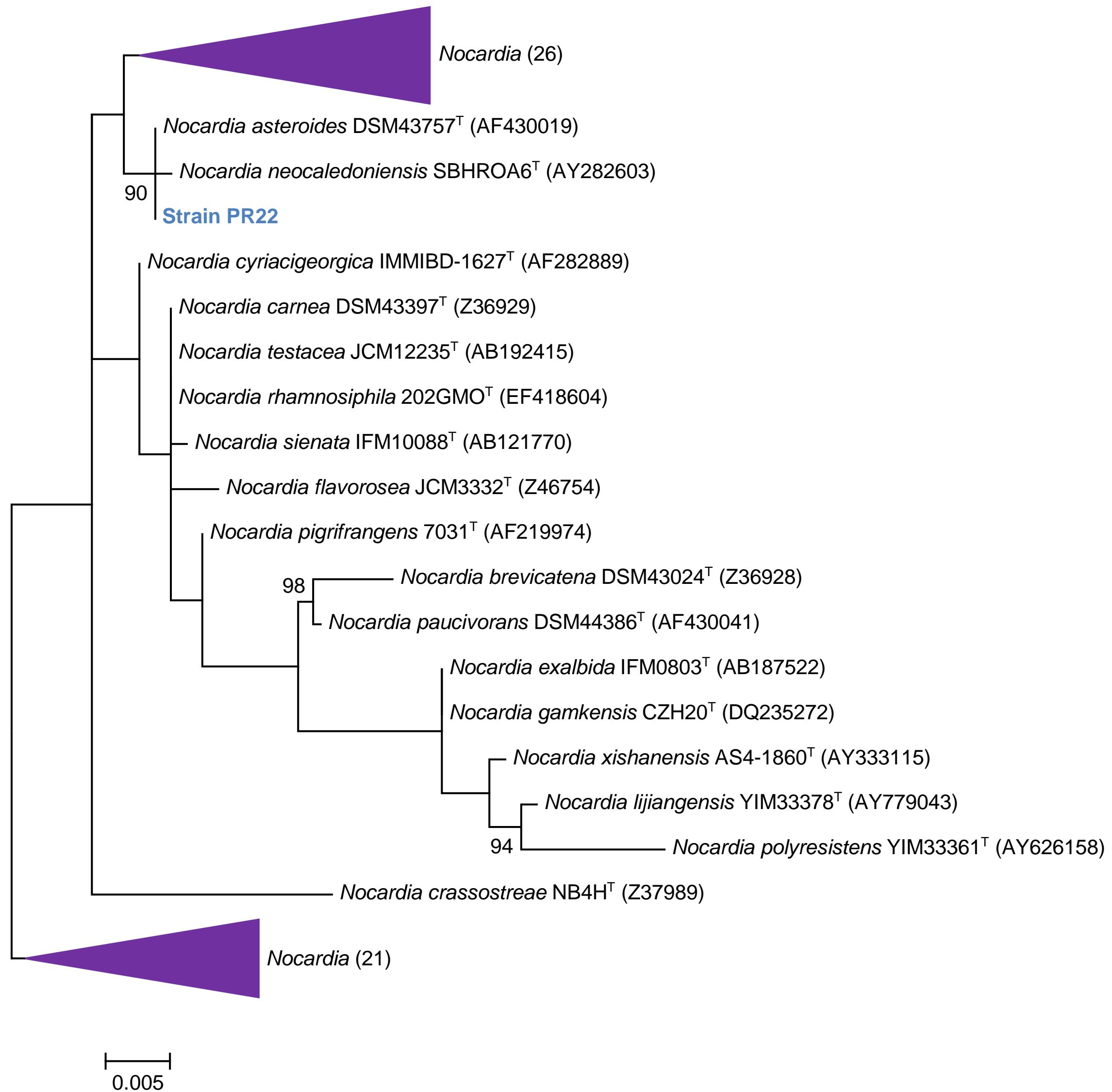

Figure 4: Maximum likelihood tree of 64 species of the *Nocardia* genus using partial 16S rRNA gene sequences of 816bp. Bootstrap values were calculated from 1000 resampled datasets. Bootstrap values above 70% are shown. The numbers in parenthesis indicate the number of species in the compressed clades. Asterisks indicate topological conservation between the neighbour-joining tree and the maximum likelihood tree. The scale bar indicates 5 nucleotide changes in 1000 base pairs.

2.4.4.3 *Streptomyces* isolates

Streptomyces albus, the type species of the genus *Steptomyces*, was first described as *Streptothrix alba* by Rossi-Doria (1891), which was then emended by Krainsky as *Actinomyces albus* before being established as the type species of the genus *Streptomyces* (Waksman & Henrici, 1943). The *Streptomyces* genus is the largest of the actinobacteria, with 823 species with validly-published names (http://www.bacterio.net/streptomyces.html).

Phylogenetic analysis of the *Streptomyces* isolates had to be separated into sections due to the high number of known *Streptomyces* species with validly-published names required for comparison. To determine the position of the isolates in the genus, a phylogeny was constructed using the type strains of *Streptomyces* species with validly-published names. The phylogenetic trees in Figures 5, 6 and 7 were constructed with all the isolates and the top 5 *blastn* hits of each strain (where top hits overlapped, the next top hit was selected).

Figure 5 shows the phylogenetic positions of strains C3, M20, PR6, PR24, UK1, Y5 and Y20, based on partial 16S rRNA gene sequences, with some of the type strains in the *Streptomyces* genus. Strains C3, M20, Y5 and Y20 all group with *Streptomyces arenae* ISP 5293^{T} (AJ399485), *Streptomyces daghestanicus* NRRL B-5418^{T} (DQ442497), *Streptomyces hydrogenans* NBRC 13475^{T} (AB184868), *Streptomyces janthinus* ISP 5206^{T} (AJ399478), *Streptomyces koyangensis* VK-A60^{T} (AY079156), *Streptomyces luteogriseus* NBRC 13402^{T} (AB184379), *Streptomyces massasporeus* NBRC 12796^{T} (AB184152), *Streptomyces purpurascens* JCM 4509^{T} (AB045888), *Streptomyces roseoviolaceus* NRRL ISP 5277^{T} (AJ399484), *Streptomyces violaceus* NBRC 13103^{T} (AB184315), and *Streptomyces violarus* NBRC 13104^{T} (AB184316) with 85% bootstrap support and no sequence variation as indicated by branch length. These 11 *Streptomyces* type strains were all in the top 11 hits based on 16S rRNA gene *blastn* analyses, with 100% sequence similarity over approximately 800 nucleotides. Strains C3, M20, Y5 and Y20 were all isolated from the Mowbray compost soil sample and could be clones of the same environmental *Streptomyces* strain. However, longer 16S rRNA gene sequences could help to distinguish between the strains. In addition, *gyrB* gene sequence analysis would also assist in determining whether these strains are clones of a single strain by determining whether their *gyrB* sequences are identical.

In Figure 5, strain PR6 groups with *Streptomyces malachitospinus* NBRC 101004^{T} (AB249954) and *Streptomyces parvulus* NBRC 13193^{T} (AB184326), albeit with weak bootstrap support (47%). However, the topology is conserved between the neighbour-joining and maximum-likelihood trees (denoted by the asterisk). *S. malachitospinus* was the top hit and *S. parvulus* was the fourth hit by pairwise sequence alignment on the EzBioCloud database with 99.89 and 99.68% sequence similarity, respectively.

Figure 5 shows strain PR24 grouping with *Streptomyces olivaceus* NBRC 3200^{T} (AB184743) and *Streptomyces pactum* NBRC 13433^{T} (AB184398) with weak bootstrap support (54%), but conserved topology (see asterisk). *S. olivaceus* and *S. pactum* were the first and second hits on the EzBioCloud database; both with 99.57% sequence similarity over 937 base pairs of sequence. This isolate could be

promising for further work because of the moderate antimycobacterial activity (Table 2), but the biodiversity of the isolate will need to be assessed using a longer 16S rRNA sequence and *gyrB* gene sequence analysis.

Strain UK1 was a contaminant on one of the sub-culture plates and was selected for further analysis because it exhibited antimycobacterial activity. The strain groups with *S. diastaticus* subsp. *ardesiacus* NRRL B-1773^{T} (DQ026631) with 75% bootstrap support (Figure 5). *S. diastaticus* subsp. *ardesiacus* was also the top hit on the EzBioCloud database (Table 3).

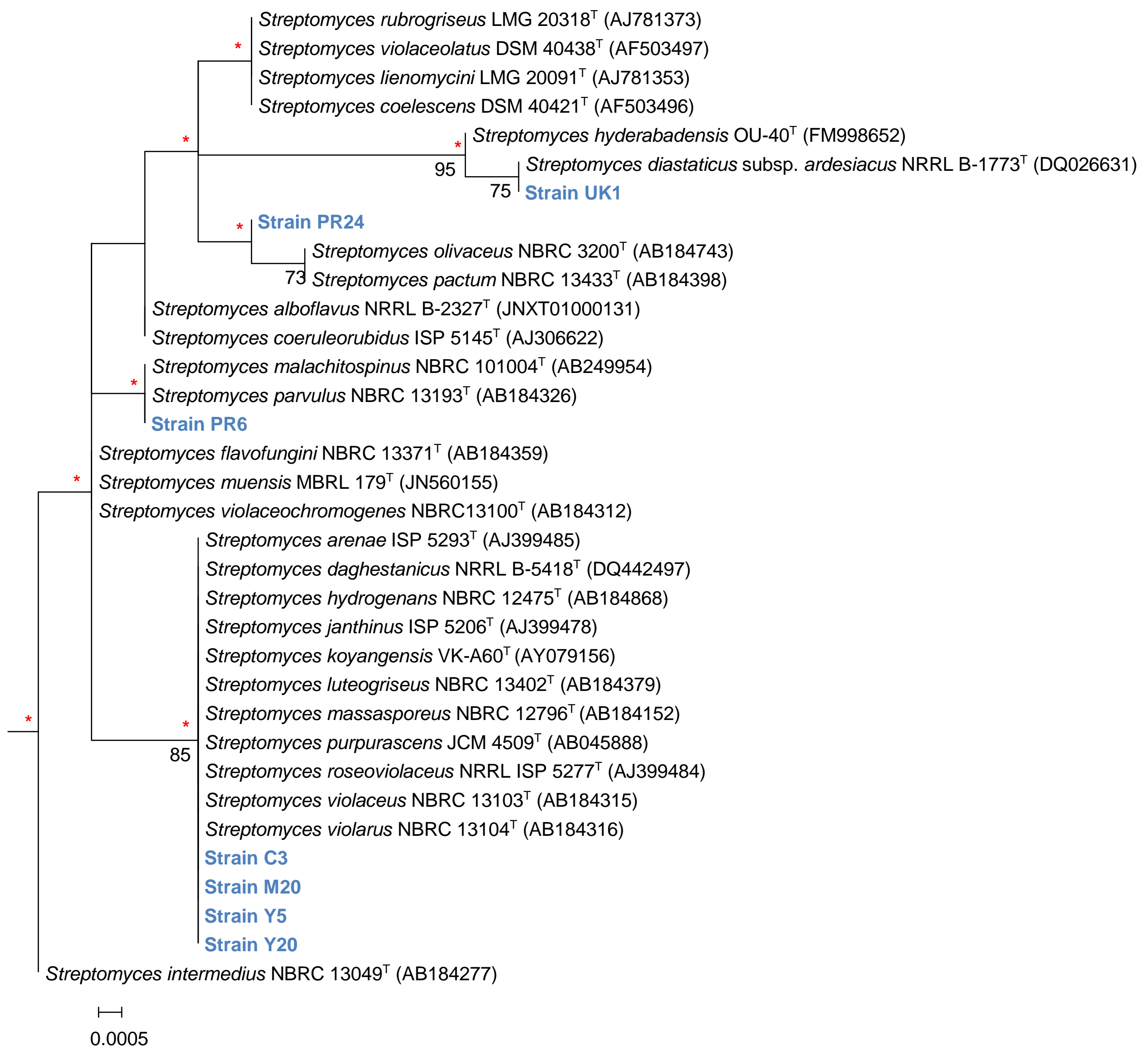

Figure 5: Maximum likelihood subtree tree of *Streptomyces* species using partial 16S rRNA gene sequences of 870bp as the query alignment. Bootstrap values were calculated from 1000 resampled datasets. Bootstrap values above 70% are shown. Asterisks indicate topological conservation between the neighbour-joining tree and the maximum likelihood tree. The scale bar indicates 5 nucleotide changes in 10 000 base pairs. This is a subtree of the full 16S rRNA gene phylogenetic tree of all 104 *Streptomyces* species depicted in Figures 5, 6, and 7.

Figure 6 depicts the phylogenetic positions of the following strains in comparison to selected type strains of species in the *Streptomyces* genus using a partial 16S rRNA gene sequence: C23, M7, M8, M13, PR29, PR36, Y12, Y14 and Y30.

Strains M7, M8, PR29, PR36, Y12 and Y14 group with the following *Streptomyces* species with weak bootstrap support (52%) and no topological conservation in the neighbour-joining tree: *Streptomyces althioticus* KCTC 9752^T (AY999808), *Streptomyces erythrogriseus* LMG 19406^T (AJ781328), *Streptomyces griseoflavus* LMG 19344^T (AJ781322), *Streptomyces griseoincarnatus* LMG 19316^T (AJ781321), *Streptomyces labedae* NBRC 15864^T (AB184704), *Streptomyces matensis* NBRC 12889^T (AB184221), *Streptomyces silaceus* NRRL B-21466^T (LIRJ01000287), *Streptomyces variabilis* NRRL B-3984^T (DQ442551) (Figure 6). Isolates M7, M8, Y12, and Y14 were isolated from the Mowbray compost soil sample while strains PR29 and PR36 were isolated from the Plumstead compost sample. Despite a geographical difference in the source of the samples, the strains still share high sequence similarity. This is not uncommon for members of the *Streptomyces* genus. In the EzBioCloud analysis, these strains shared the same top 9 hits in almost the same order. Further analysis would require longer 16S rRNA gene sequences and repeating the phylogenetic analysis. As mentioned before, analysis of the genetic distances between the *gyrB* gene sequences of the isolates will also help delineate the strains.

Strain M13, isolated from the Mowbray compost sample, groups with the species that are also the top five hits on the EzBioCloud database (Figure 6): *Streptomyces carpinensis* NBRC 14214^T (AB184574) *Streptomyces cellulosae* NRRL B-2889^T (DQ442495), *Streptomyces gancidicus* NBRC 15412^T (AB184660), *Streptomyces prasinosporus* NBRC 13419^T (AB184390) and *Streptomyces pseudogriseolus* NRRL B-3288^T (DQ442541). The sequence comparison was over 941 base pairs, so a longer 16S rRNA gene sequence will assist in determining whether the strain is likely to represent a new species and is therefore worth investigating further. Over 941 nucleotides, the top hit (the type strain of *S. cellulosae*) shared 99.47% of the 16S rRNA gene sequence (Table 3).

Strains C23 and Y30 are in the same clade with *Streptomyces cacaoi* subsp*. cacaoi* NBRC 12748^T (AB184115) with 95% bootstrap support (Figure 6). This agrees with the *blastn* analysis (Table 3). Despite the 100% 16S rRNA gene sequence similarity between the isolates and *S. cacaoi* subsp*. cacaoi* (over 1405 bp), the hits following *S. cacaoi* subsp*. cacaoi* all shared sequence similarity of less than 98.5%. Being producers of a strong antimycobacterial antibiotic makes these strains worthy of further characterisation. It would be useful to compare other genes (e.g. *gyrB*, *rpoB*, *recA, relA,* and *atpD* (ATP synthase F1, beta subunit)) between strain C23 and *S. cacaoi* subsp. *cacaoi* to see if they can be separated as different species. Interestingly, strain C23 was isolated from the soil of the vermiculture farm, while strain Y30 was isolated from the Mowbray compost sample. However, these soil samples are very close to each other in the same suburban garden and the soil from the compost heap is used for the vermicompost. Therefore, these isolates could either be strains of the same species or clones of the same strain. Gyrase subunit B gene sequencing would also shed light on the likelihood that strains C23 and Y30 belong to a new species. A comparison of the *gyrB* gene sequence to that of *S.*

cacaoi subsp. *cacaoi* would allow one to quickly discern whether they are novel species or clones of the same environmental isolate.

Figure 7 shows the phylogenetic position of strains PR10, PR28, PR35 and PR47 in the *Streptomyces* genus based on partial 16S rRNA gene sequences. These strains were isolated from the Plumstead compost soil sample.

Streptomyces antibioticus NRRL B-1701^T (AY999776) is the closest phylogenetic relative to strain PR10 with 98% bootstrap support (Figure 7) and the top hit according to a *blastn* analysis. Strain PR28 is on the periphery of the clade containing *Streptomyces cyaneus* NRRL B-2296^T (AF346475) and *Streptomyces griseoruber* NRRL B-1818^T (LIQS01000280), neither of which were the top hit according to a *blastn* analysis (Table 3). Strains PR35 and PR47 are in the same major clade with 95% bootstrap support, but branch into different sub-clades. The strains are in the same clades as their respective top hits from *blastn* analyses: *Streptomyces bacillaris* NBRC 13487^T (AB184439) and *Streptomyces pratensis* ch24^T (JQ806215), respectively. Strains PR10 and PR28 seem to be the most interesting based on the phylogenetic analysis. Their branch lengths demonstrate sequence variation between them and their closest relatives. Strain PR10 was of particular interest (based on colony morphology and strong antimycobacterial activity) and a longer 16S rRNA gene sequence was generated by additional sequencing using the 800R primer. A *blastn* analysis of the longer sequence (1481 bp, Table 3) revealed that only the top two hits share greater than 98.7% sequence similarity: *S. antibioticus* and *S. griseoruber*. Strain PR10 has the potential to be described as a novel species of *Streptomyces* using the polyphasic taxonomic approach and DDH should be carried out to show that it is not the same species as *S. antibioticus* and *S. griseoruber*.

The first step in taking strain PR28 forward would be to generate a longer 16S rRNA gene sequence to assess how similar it is to the rest of the *Streptomyces* genus. Following this, the *gyrB* gene sequence can also be used to assess the novelty of the isolate by comparison to the *gyrB* gene sequences of its closest relatives determined from the analysis of the 16S rRNA gene sequence.

Except for a few isolates identified to the genus *Streptomyces*, the general trend was for the isolates to group with high sequence conservation to the known *Streptomyces* species. This implies that many of these isolates are not likely to belong to new species. However, to provide support for this conclusion, many isolates will need to have longer 16S rRNA gene sequences generated to allow for a more robust comparison to the known members of *Streptomyces*. The high sequence similarities were to be expected, because the *Streptomyces* genus is known to have very high 16S rRNA gene sequence similarities between closely related species. This high sequence conservation makes it difficult to identify the isolates that one should pursue for characterisation as a new species and in turn their potential to produce chemically diverse natural products. This is one of the many reasons why researchers are turning towards the rarer genera to discover novel actinobacteria and in turn novel bioactive compounds. However, strain PR10 is worthy of characterisation as potentially novel species in the *Streptomyces* genus. Physiological characters, chemotaxonomy, DDH or ANI should be used to characterise the isolate.

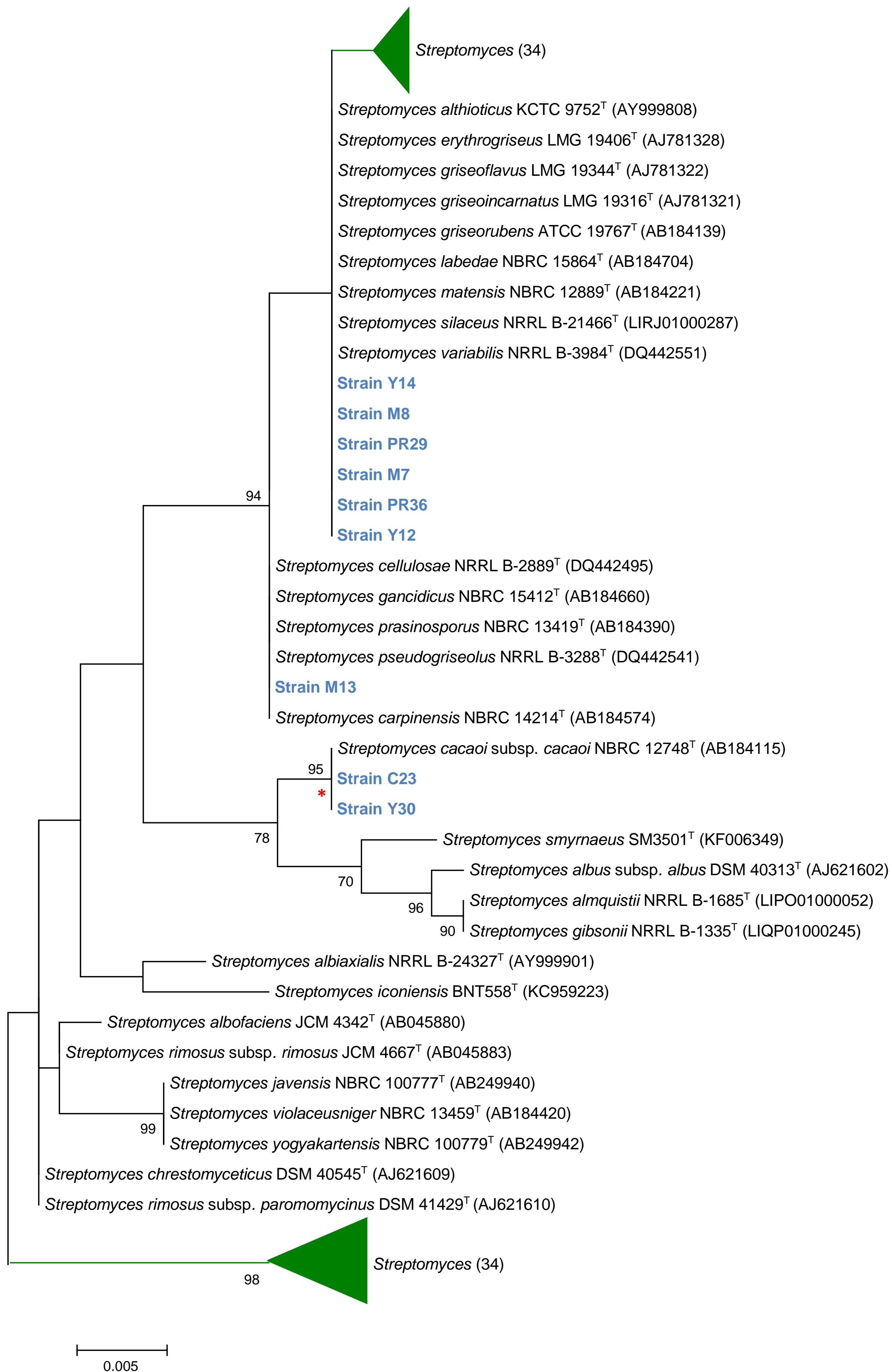

Figure 6: Maximum likelihood tree of *Streptomyces* species using partial 16S rRNA gene sequences of 870bp. Bootstrap values were calculated from 1000 resampled datasets. Bootstrap values above 70% are shown. The numbers in parenthesis indicate the number of species in that compressed clade. Asterisks indicate topological conservation between the neighbour-joining tree and the maximum likelihood tree. The scale bar indicates 5 nucleotide changes in 1000 base pairs.

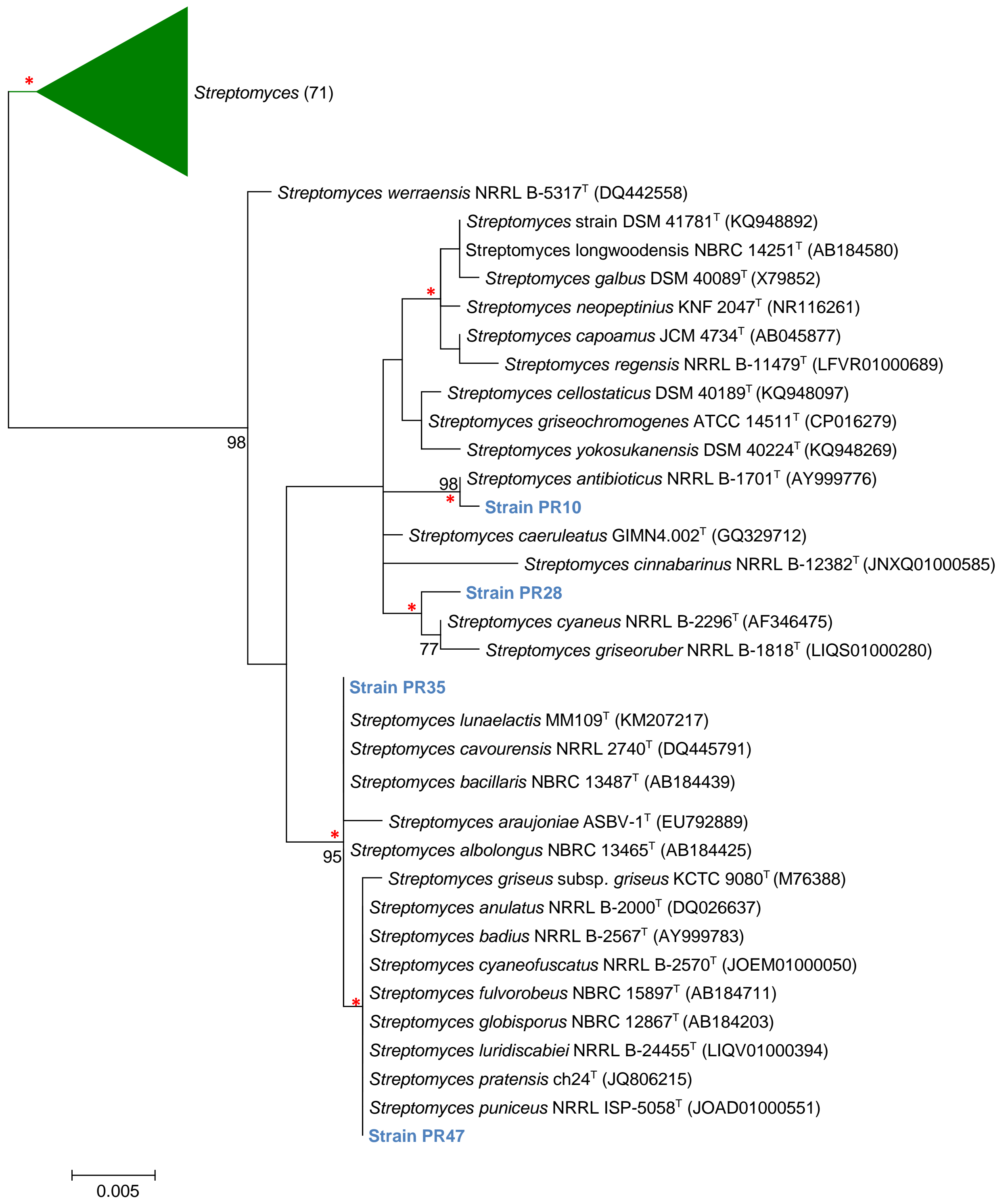

Figure 7: Maximum likelihood tree of *Streptomyces* species using partial 16S rRNA gene sequences of 870bp as the query alignment. Bootstrap values were calculated from 1000 resampled datasets. Bootstrap values above 70% are shown. The numbers in parenthesis indicates the number of species in that compressed clade. Asterisks indicate topological conservation between the neighbour-joining tree and the maximum likelihood tree. The scale bar indicates 5 nucleotide changes in 1000 base pairs.

2.4.4.4 Strain PR3: *Kitasatospora* or *Streptomyces*?

The genus *Kitasatospora* was described by Ōmura et al. (1982) and then subsequently incorporated into the *Streptomyces* genus (Wellington et al., 1992) only to be reinstated as *Kitasatospora* in 1997 (Zhang et al., 1997). Currently the genus has 23 species with validly published names (http://www.bacterio.net/kitasatospora.html) and Labeda et al. (2017) recently suggested the transfer of another nine *Streptomyces* species to the genus *Kitasatospora* based on an MLSA of the following five house-keeping genes: *atpD*, *gyrB*, *rpoB*, *recA*, and *trpB* (tryptophan synthetase, beta subunit). *Kitasatospora* are aerobic, Gram-positive, chemo-organotrophic actinomycetes which form a network of branching hyphae (Groth et al., 2003).

Strain PR3 is a fast-growing actinobacterium that was isolated from the Plumstead compost sample. On ISP2, strain PR3 has a substrate mycelium that is off white to brown in colour and a white spore mass that resembles chalk. The top hit based on an EzBioCloud *blastn* analysis was *Kitasatospora albolonga* NBRC 13465^T (Table 3). However, the rest of the hits in the list were all *Streptomyces* species. Consequently, a 16S rRNA gene sequence phylogenetic tree was constructed using all 32 type strains from the genus *Kitasatospora* with validly-published names and 100 *Streptomyces* type strains with validly-published names to determine the phylogenetic position of strain PR3 (Figure 8). Based on the 16S rRNA gene sequence phylogeny, strain PR3 groups with the *Streptomyces* species, along with *K. albolonga* NBRC 13465^T (AB184425) and *Kitasatospora cinereorecta* NBRC 15395^T (AB184646). In a 16S rRNA gene sequence phylogeny generated with only the sequences from the *Kitasatospora* genus, those strains that grouped with the *Streptomyces* species in Figure 8 also branched off from the main clade of *Kitasatospora* species (Appendix A). Due to the high conservation of the 16S rRNA gene sequence between the two genera, additional information is needed to assign a genus identification to strain PR3.

Labeda et al. (2017) used an MLSA with 5 house-keeping genes to reassess the taxonomic status of *Streptomyces* species which were believed to have been incorrectly classified. From the phylogenetic tree generated, Labeda et al. (2017) proposed the transfer of the following *Streptomyces* species, among others, to the genus *Kitasatospora*: *K. albolonga* (basonym *Streptomyces albolongus*), *K. cinereorecta* (basonym *Streptomyces cinereorectus*) and *K. misakiensis* (basonym *Streptomyces misakiensis*). Interestingly, these transferred species are the ones that group separately from the other *Kitasatospora* species in Figure 8. Thus, the 16S rRNA gene sequence analysis cannot be relied upon to identify strain PR3 to the genus level.

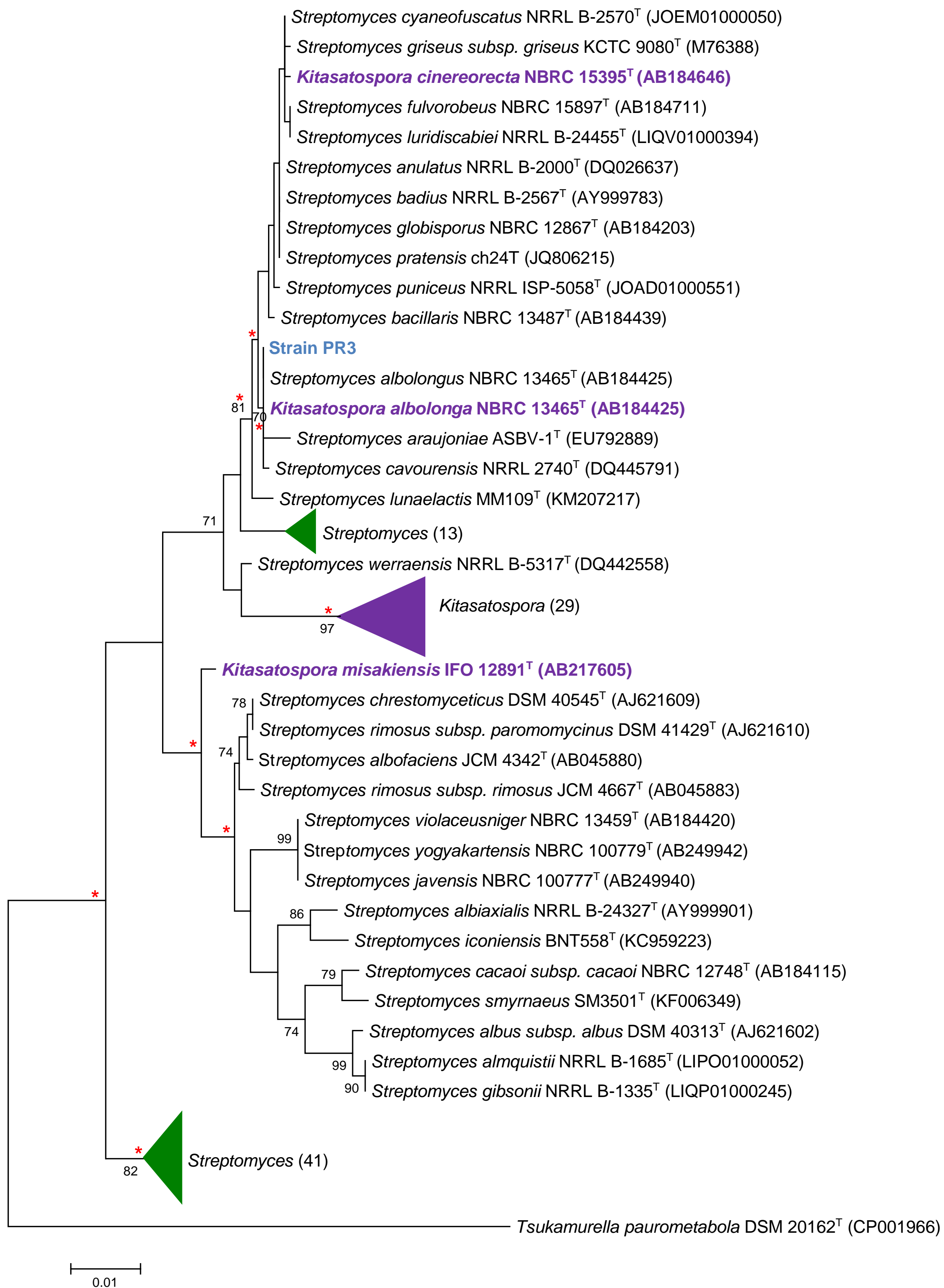

Figure 8: Maximum likelihood tree of the *Kitasatospora* genus and 100 members of the *Streptomyces* genus using partial 16S rRNA gene sequences of 1320bp and rooted with *Tsukamurella paurometabola* DSM 20162^T (CP001966). Bootstrap values were calculated from 1000 resampled datasets. Bootstrap values above 70% are shown. The numbers in parenthesis indicate the number of species in that compressed clade. Asterisks indicate topological conservation between the neighbour-joining tree and the maximum likelihood tree. The scale bar indicates 10 nucleotide changes in 1000 base pairs.

A *gyrB* gene sequence was generated for strain PR3 to compare to the seven available *Kitasatospora gyrB* gene sequences. A ClustalW alignment of *gyrB* gene sequences from strain PR3, *Kitasatospora* species and closely-related *Streptomyces* species was generated (Table 4) and a phylogenetic tree was constructed (Figure 9). ClustalW was used because it produced a better alignment of the sequences than MUSCLE. Table 4 shows a region of the alignment from MEGA6 where the *Kitasatospora gyrB* sequences exhibit two deletions (of 24 and 15 nt), which distinguish them from the *Streptomyces gyrB* sequences. The larger, 24-nt deletion is present in all the *Kitasatospora* sequences and is absent from all the *Streptomyces* sequences used in the alignment. The 24-nt deletion is absent from strain PR3, suggesting that it belongs to the genus *Streptomyces*.

Table 4: Multiple sequence alignment of *gyrB* gene sequences from members of the *Kitasatospora* genus, *Streptomyces* genus, and strain PR3. The alignment was generated using ClustalW in the Mega6 programe.

Kitasatospora azatica KCTC 9699 *gyrB* (JF424118)	GACGAGCGCG------------------------AGGAGCACACCGA---------------CGACGAGG
Kitasatospora cineracea KCTC 19932 *gyrB* (JF424126)	GACGAGCGCG------------------------CCAAGCACCTGGA---------------CGAGGAGG
Kitasatospora cystarginea KCTC 9746 *gyrB* (JF424123)	GACGAGCGTG------------------------CCGAGCACCTGGA---------------CGAGGAGG
Kitasatospora griseola KCTC 9745 *gyrB* (JF424122)	GACGAGCGTG------------------------CCGAGCACGCCGA---------------CGAGGAGG
Kitasatospora kifunensis KCTC 9734 *gyrB* (JF424120)	GACGAGCGCG------------------------AGGAGCACTTCGA---------------CGACCAGG
Kitasatospora mediocidica KCTC 9733 *gyrB* (JF424119)	GACGAGCGCG------------------------CCAAGCACCTGGA---------------GGAGGAGG
Kitasatospora niigatensis KCTC 19933 *gyrB* (JF424127)	GACGAGCGCG------------------------CCAAGCACCTGGA---------------CGAGGAGG
Strain PR3	GACGAGCGCGAGTCGGCCAAGGCCACGGCGGGCGCCGACAGCGCCGAGGCCACCGAGGTCCCCGAGGAGG
Streptomyces lunaelactis MM109^T *gyrB* (KM207219)	GACGAGCGCGAGTCGGCGAAGGCCACCGCCGGTGCTGACACCGTCGAGGAGG---------CGGCCGAGG
Streptomyces cavourensis KCCM 40666 *gyrB* (HQ995508)	GACGAGCGCGAGTCGGCCAAGGCCACGGCGGGCGCCGACAGCGCCGAGGCCACCGACGTCCCCGAGGAGG
Streptomyces bacillaris gyrB KCTC 9018^T (HQ995507)	GACGAGCGCGAGTCGGCCAAGGCCACGGCGGGCGCCGACAGCGCCGAGGCCACCGAGGTCCCCGAGGAGG
Streptomyces globisporus gyrB KCTC 9026^T (HQ995513)	GACGAGCGTGAGTCGGCGAAGGCGACGGCGGGCGCGGACAGCGCCGATCGAGTCGAGCCCGCCGAGGAGG
Streptomyces azureus gyrB NRRL B-2655^T (EU277659)	GACGAGCGCGAGTCGGCGAAGGCCACGGCCGGCGCGGACGAGGCCGGCGAGG---------ACGAGAAGC
Streptomyces pulveraceus gyrB DSM 41657^T (HQ823601)	GACGAGCGCGAGTCGGCGAAGGCGACGGCGGGTGCGGACGTCGCCGAGGTGGCCGAGATCGCCGAGGCGG
Streptomyces gelaticus gyrB DSM 40065^T (HQ823589)	GACGAGCGCGAGTCGGCGAAGGCGACGGTGGGTGCCGAGGTCGCCGAGGTGGCCGAGGTCGCCGAGGCGG
Streptomyces mauvecolor gyrB DSM 41702^T (HQ823596)	GACGAGCGCGAGTCGGCGAAGGCGGTCTCGGGCGCGGACTCCGCGGAGGAGG---------CCGAGGAGC
Streptomyces sindenensis gyrB NBRC 12915^T (HQ995522)	GACGAGCGTGAGTCGGCGAAGGCGACGGCGGGTGCGGACAGCGCCGAGGCGACCGAGGTCCCCGAGGAGG
Streptomyces polyantibioticus gyrB SPR^T (HQ823600)	GACGAGCGGGAGTCGGCGAAGGCCGTCTCCGGGGCGGACTCGGCCGAGGAGT---------CGGAAGTCG

Meyers (2014) showed that molecular signatures, such as indels, can be used to distinguish between genera in the family *Streptosporangiaceae*. The *gyrB* gene alignment (Table 4) shows that strain PR3 contains a molecular signature that is only present in the *gyrB* gene sequences of *Streptomyces* species. This implies that strain PR3 is a member of the *Streptomyces* genus. However, it must be borne in mind that *gyrB* gene sequences are only available for about a quarter of the members of the genus *Kitasatospora*, so the analysis should ideally be repeated to include the entire genus to confirm that the two deletions are common to all members of the genus.

Figure 9 shows the phylogenetic position of strain PR3 based on *Kitasatospora* and *Streptomyces gyrB* gene sequences. Strain PR3 groups with most of the *Streptomyces* clade (*Streptomyces azureus* NRRL B-2655^T is on the periphery) with 75% bootstrap support (Figure 9). The *Kitasatospora* species group together with 100% bootstrap support. This evidence, along with the alignment evidence, suggests that strain PR3 is a *Streptomyces* species. However, chemotaxonomic work will validate which genus strain PR3 belongs to. If strain PR3 is a *Streptomyces* species it will have the following chemotaxonomic characters: LL-diaminopimelic acid as the diagnostic diamino acid in the peptidoglycan, phospholipid pattern type II, no diagnostic sugars, MK-9 (H_6, H_8, H_4) as the major menaquinones, and a DNA G+C content between 69 and 78 mol% (Locci et al. 1989). If strain PR3 is a *Kitasatospora* species, it will have the following chemotaxonomic characters instead: *meso*- **and** LL-diaminopimelic acid, MK-9(H_6,

H_8) as the major menaquinones, phospholipid pattern type II, galactose as a whole-cell diagnostic sugar and a DNA G + C content in the same range as the *Streptomyces* genus (Takahashi, 2017; Li et al. 2009). The major chemotaxonomic differences between the two genera are the presence of galactose as a diagnostic sugar in *Kitasatospora* and the fact that *Kitasatospora* contains both *meso*- and LL-diaminopimelic acid in the peptidoglycan.

An MLSA is also recommended to determine the phylogenetic position of strain PR3 in relation to the members of the genera *Streptomyces* and *Kitasatospora*. This will provide a more robust comparison.

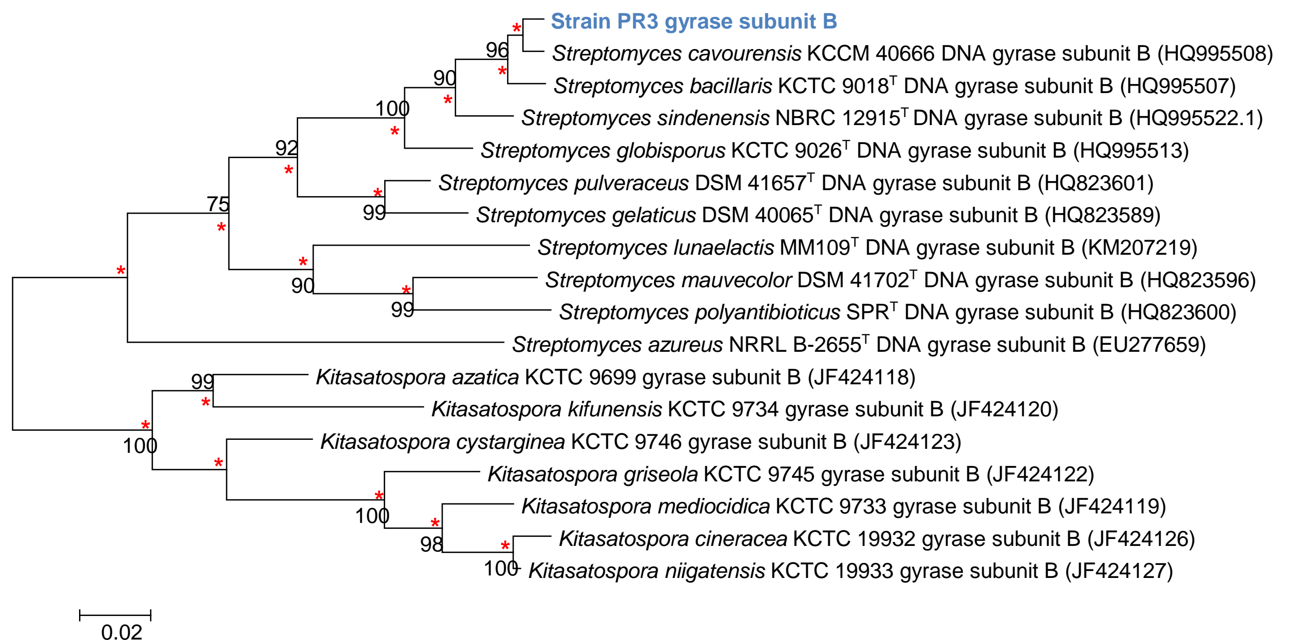

Figure 9: Maximum likelihood tree of *Kitasatospora* and *Streptomyces* species using partial, 1723bp *gyrB* gene sequences. Bootstrap values were calculated from 1000 resampled datasets. Bootstrap values above 70% are shown. Asterisks indicate topological conservation between the neighbour-joining tree and the maximum likelihood tree. The scale bar indicates 20 nucleotide changes in 1000 base pairs.

2.4.4.5 *Tsukamurella* isolate

Members of the *Tsukamurella* genus, established by Collins et al. (1988), are aerobic, Gram-positive, non-spore forming, rod-shaped cells (Nam et al., 2003; Collins et al., 1988). *Tsukamurella* species have commonly been isolated from human patients (Goodfellow & Kumar, 2012; Woo et al., 2009, 2003), but have also been isolated from terrestrial and aquatic sources (Olson et al., 2007; Park et al., 2009; Weon et al., 2010) and animals (Tang et al., 2016).

Strain G4, isolated from the untreated portion of the vermicompost soil sample, is a Gram positive, rod shaped, orange-coloured actinobacterium with a wrinkled and dry-looking colony morphology. The *Tsukamurella* genus is small: there are only 14 species with validly-published names (http://www.bacterio.net/tsukamurella.html). This makes this isolate of keen interest for characterisation as a potentially novel species. The top three hits based on the 16S rRNA gene sequence of 1542bp on the EzBioCloud data base were *Tsukamurella pseudospumae* N1176^T (AY238513), *Tsukamurella spumae* N1171^T (Z37150) and *Tsukamurella sunchonensis* SCNU5^T (AF150494), respectively, all with 99.58% sequence similarity. *T. pseudospumae, T. spumae* and *T. sunchonensis*, were all isolated from the foam of activated sewage sludge (Nam et al., 2004; Nam et al., 2003; Seong et al., 2003). The phylogenetic position of strain G4 was observed with the construction of a 16S rRNA gene tree using all the *Tsukamurella* species with validly-published names (Figure 10). Strain G4 only groups with *T. spumae* of the top three hits from the *blastn* analysis (bootstrap support of 98%), while *T. pseudospumae,* and *T. sunchonensis* group separately (Figure 10).

Only two other *Tsukamurella* species have been isolated from soil, namely *Tsukamurella carboxydivorans* and *Tsukamurella soli. T. carboxydivorans* Y2^T has a 16S rRNA gene sequence similarity of 99.31% with strain G4, while *T. soli* JS18-1^T has a 16S rRNA gene sequence similarity of 96.18% with strain G4. Polyphasic taxonomy and DDH will need to be used to determine whether strain G4 belongs to a distinct genomic species from *T. pseudospumae, T. spumae* and *T. sunchonensis.*

2.4.4.4.1 Should Tsukamurella soli be re-classified?

T. soli strain JS18-1^T (FJ917743), described by Weon et al. (2010), forms a deep branch in the *Tsukamurella* phylogenetic tree constructed using 1401bp of common 16S rRNA gene sequence. This deep branch is immediately suggestive that there is something amiss with the classification of this species. To suggest that *T. soli* does not belong to the *Tsukamurella* genus, one needs to look at the sequence information and chemotaxonomic characters of the strain.

In the paper describing *T. soli*, the levels of sequence similarity reported between the type strain and the rest of the genus was 95.4–96.5 % (Weon et al., 2010). According to Meier-Kolthoff et al. (2013) this is clearly well below the cut-off threshold for classifying a strain as a new species of actinobacterium. Table 5 below highlights this low 16S rRNA gene sequence similarity with a homology matrix created using DNAMAN version 4.13 (Lynnon Biosoft). The column in red shows the low sequence similarity between *T. soli* and the type strains of the rest of the *Tsukamurella* species with validly-published names (Table 5). The next lowest percentage similarity between two *Tsukamurella* type strains is 97.7% (*Tsukamurella serpentis* and *Tsukamurella spongiae*; Table 5, highlighted in blue). The very low 16S rRNA gene sequence similarities between the type strain of *T. soli* and other members of the genus are in the range suggestive of a novel genus. However, sequence comparisons are not enough to draw any concrete conclusions.

Chemotaxonomically, *T. soli* is not very different from the rest of the members of the *Tsukamurella* genus. However, the proportion of fatty acid $C_{18:1\ \omega 9c}$ is unusually low in the type strain of *T. soli* at

14.1%, while in the other *Tsukamurella* type strains the proportion of $C_{18:1\ \omega 9c}$ is 20 to 45.4% (Tang et al. 2016).

This information suggests that *T. soli* could be a member of a different genus, but a more thorough comparison needs to be done to provide support for a proposal to transfer *T. soli* to another genus. According to Barka et al. (2016) the closest genus relative is *Gordonia*. Thus, a chemotaxonomic comparison can be done to identify whether *T. soli* is in fact a member of the genus *Gordonia*. However, *T. soli* is more closely related to all the *Tsukamurella* species before any other genus. Thus, it is more likely that *T. soli* would form a new, closely related, genus possibly named "*Pseudotsukamurella*" or "*Allotsukamurella*". Sequence analysis of the *gyrB* and other housekeeping genes should also be used. An MLSA of all members of the genus and other closely related genera using a set of housekeeping genes (such as *atpD*, *gyrB*, *rpoB*, *recA*, and *trpB*) would be the ideal method, because this would provide a more robust assessment of the phylogenetic position of *T. soli*.

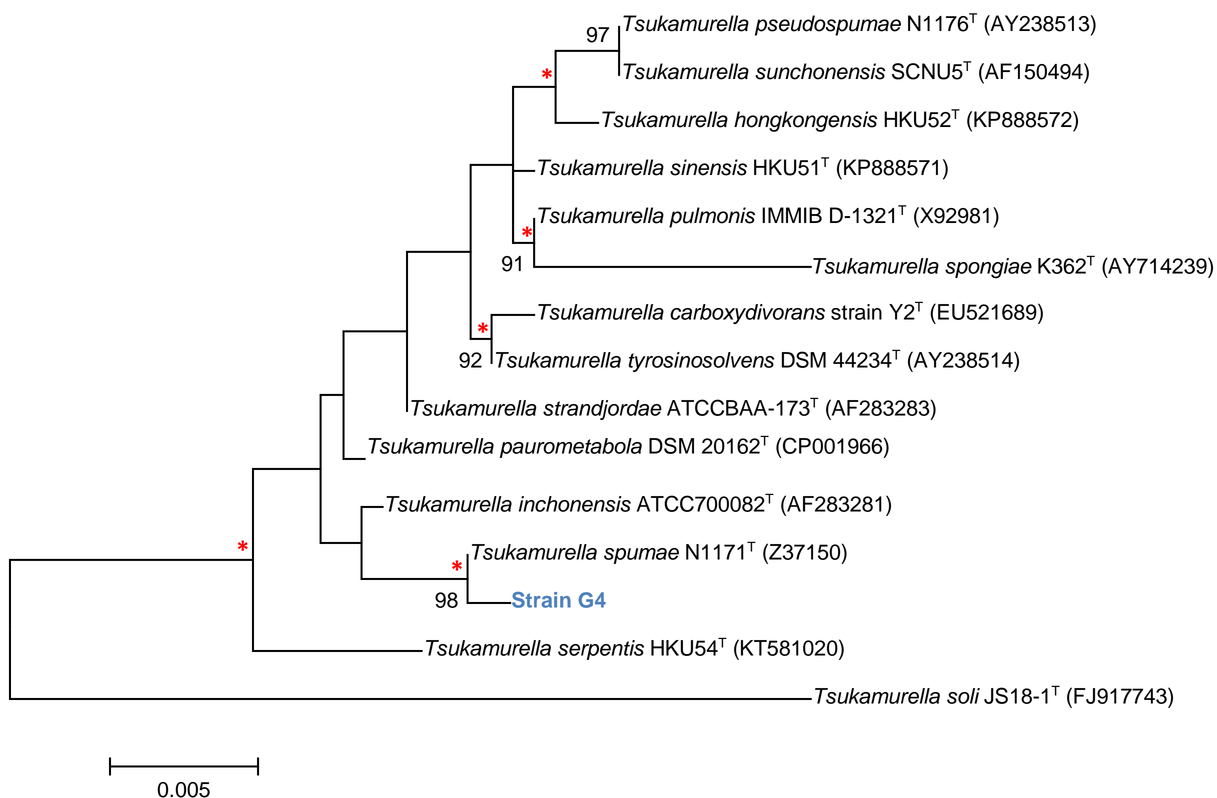

Figure 10: Maximum likelihood phylogenetic tree using the 16S rRNA gene to compare strain G4 to members of the *Tsukamurella* genus. The tree was constructed based on 1401 bp of common sequence. Bootstrap values were calculated from 1000 resampled datasets. Bootstrap values above 70% are shown. Asterisks indicate topological conservation between the neighbour-joining tree and the maximum likelihood tree. The scale bar indicates 5 nucleotide changes per 1000 base pairs.

Table 5: 16S rRNA gene sequence similarity matrix for all the type strains in the *Tsukamurella* genus.

T. soli	**100%**													
T. carboxydivorans	**95.9%**	100%												
T. hongkongensis	**96.2%**	99.1%	100%											
T. inchonensis	**96.1%**	99.3%	98.9%	100%										
T. paurometabola	**96.0%**	99.1%	99.1%	99.4%	100%									
T. pseudospumae	**95.9%**	99.5%	99.4%	99.2%	99.0%	100%								
T. pulmonis	**95.9%**	99.6%	99.4%	99.4%	99.1%	99.6%	100%							
T. serpentis	**96.1%**	98.5%	98.2%	99.0%	98.9%	98.5%	98.6%	100%						
T. sinensis	**96.2%**	99.4%	99.6%	99.1%	99.3%	99.4%	99.6%	98.6%	100%					
T. spongiae	**95.0%**	98.7%	98.5%	98.5%	98.2%	98.7%	99.1%	**97.7%**	98.7%	100%				
T. spumae	**95.9%**	99.3%	99.2%	99.6%	99.2%	99.7%	99.4%	98.9%	99.1%	98.5%	100%			
T. strandjordii	**96.1%**	99.5%	99.1%	99.5%	99.5%	99.4%	99.6%	98.9%	99.4%	98.7%	99.3%	100%		
T. sunchonensis	**95.9%**	99.5%	99.4%	99.2%	99.0%	100 %	99.6%	98.5%	99.4%	98.7%	99.7%	99.4%	100%	
T. tyrosinosolvens	**96.0%**	99.9%	99.3%	99.5%	99.3%	99.6%	99.7%	98.6%	99.5%	98.8%	99.4%	99.7%	99.6%	100%

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CHAPTER 3

ANTIBIOTIC EXTRACTION FROM *Kribbella* STRAIN SK5 AND DESCRIPTION OF STRAIN SK5 AS *Kribbella stellenboschensis* sp. nov

3.1 Summary

Seven actinobacterial isolates (*Kribbella* strain SK5, *Streptomyces* strains PR10, PR28, PR47, UK1 and Y10 and *Micromonospora* strain M27) were tested for the biosynthetic potential to produce ansamycin, glycopeptide, non-ribosomal peptide and Type-II polyketide antibiotics using PCR primers specific to the different antibiotic biosynthetic genes. Strain Y10 demonstrated the potential to produce a non-ribosomal peptide antibiotic. Strain M27 demonstrated the potential to produce ansamycin, glycopeptide, and Type-II polyketide antibiotics. Strains PR10, PR28, PR47, SK5, and UK1 demonstrated the potential to produce Type-II polyketide and non-ribosomal peptide antibiotics. Strains PR3 and Y30 were not screened for their antibiotic biosynthetic potential due to low quality DNA at the time. Following this, strains PR3, SK5, UK1 and Y30 were subjected to antibiotic extraction and their crude extracts were tested for activity against *M. aurum* A+, *Staphylococcus aureus* ATCC 25923, and *Escherichia coli* ATCC 25922. Strains PR3 and Y30 were not screened for their antibiotic biosynthetic potential due to low quality DNA. The cell broth extracts of the *Streptomyces* isolates (strains PR3, UK1 and Y30) dissolved in methanol (MeOH) were active against *M. aurum* A+. Emphasis was placed on the analysis of the crude extract from *Kribbella* strain SK5, which displayed a pattern of activity that was dependent on the growth conditions. The culture broth extract of strain SK5 exhibited activity against *M. aurum* A+ and *S. aureus* ATCC 25923 when strain SK5 was incubated at 22°C or 30°C, with R_f values for the active molecule of 0.37 and 0.72 by 1D TLC when using 100% ethyl acetate (EtOAc) and 100% MeOH as the solvent systems, respectively. The cell mass extract of strain SK5 exhibited activity against *M. aurum* A+ and *S. aureus* ATCC 25923 when strain SK5 was incubated at a fluctuating temperature (~25°C to 30°C) with R_f values of 0.54 and 0.4 by 1D TLC using 100% EtOAc as the solvent system and 0.56 using 100% MeOH as the solvent system. Strains PR3 and UK1 also displayed antimalarial activity against the drug sensitive *Plasmodium falciparum* strain NF54. The cell mass extract and culture broth extract of strain PR3 demonstrated IC_{50} values of 39±5 and 56±5 ng/mL, respectively. The cell mass extract of strain UK1 demonstrated an IC_{50} value of 298±4 ng/mL.

A polyphasic taxonomic approach was used to characterise a soil actinobacterium (strain SK5) that was isolated from Stellenbosch, Western Cape, South Africa. Based on 16S rRNA gene sequence analysis, strain SK5 was assigned to the genus *Kribbella*. The genus identification was confirmed following the identification of key *Kribbella* chemotaxonomic characters, namely the presence of i) MK-9(H_4) as the major menaquinone; ii) LL-diaminopimelic acid; iii) anteiso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{15:0}$ as the main fatty acids; and

iv) phosphatidylcholine in the polar lipid profile. Phylogenetic analysis of the 16S rRNA gene and a multilocus sequence analysis of five concatenated housekeeping genes (*gyrB, rpoB, recA, relA*, and *atpD*) revealed the closest genomic relatives as the type strains of *Kribbella aluminosa*, *Kribbella karoonensis*, *Kribbella pittospori*, *Kribbella shriazensis* and '*Kribbella soli*'. The genetic distance values between the concatenated housekeeping gene sequences for *K. aluminosa*, *K. karoonensis*, *K. pittospori*, *K. shirazensis* and '*K. soli*' were 0.050, 0.053, 0.056 0.045 and 0.030, respectively. Phenotypic comparisons identified differences between strain SK5 and the type strains of the above mentioned *Kribbella* species. The concatenated-gene genetic distance between strain SK5 and the type strain of '*Kribbella sindirgiensis*' was 0.016, but a phenotypic comparison between these closely related strains was not possible, as the type strain of '*K. sindirgiensis*' was not available. Strain SK5 is preliminarily presented as the type strain of a novel species for which the name '*Kribbella stellenboschensis*' sp. nov. is proposed. DDH against '*K. soli*' and '*K. sindirgiensis*' will need to be done to confirm that strain SK5 belongs to a separate genomic species.

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3.2 Introduction

'Rare' actinobacteria are incredibly relevant as sources of novel antibiotics (Lazzarini et al., 2000), because their isolation increases the chances of discovering chemically diverse secondary metabolites. It is for this reason that strong antibiotic producers within the rarer genera of actinobacteria are sought after as sources of bioactive lead compounds for development as pharmaceutical drugs. Members of the genus *Kribbella* are not well known as antibiotic producers, but *Kribbella antibiotica* YIM 31530^T produces an antifungal agent (Li et al., 2004) and *Kribbella sandramycini* ATCC 39419^T (Park et al., 1999) produces an antitumour agent, sandramycin. There has also been the recent description of kribbellosides: new RNA 5'-triphosphate inhibitors isolated from *Kribbella* sp. MI481-42F6, which exhibit antifungal activity (Igarashi et al., 2017).

The genus *Kribbella*, of the family *Nocardioidaceae*, was established by Park et al. (1999) when two strains, previously identified as members of the genus *Nocardioides*, were identified as distinct from the rest of the genus *Nocardioides* based on 16S rRNA gene sequence analysis. The genus description was later emended by Everest et al. (2013). *Kribbella* species are aerobic, Gram-stain positive and generally form colonies that do not have distinctive colouration. They form colonies that are wrinkled and pasty to soft-leathery. If spores are produced on agar plates, the spore mass is often white (Evtushenko & Krausova, 2012). Kribbellae form extensively branched vegetative hyphae, which often undergo varying degrees of fragmentation in both the substrate and aerial hyphae, as well as in liquid culture (Evtushenko & Krausova, 2012). The *Kribbella* genus has the following defining chemotaxonomic features: i) The cell wall peptidoglycan contains LL-diaminopimelic acid; ii) MK-9(H_4) is the major menaquinone; iii) $C_{15:0}$ anteiso, $C_{16:0}$ iso and $C_{15:0}$ iso are usually the predominant cellular fatty acids; iv) phosphatidylcholine (PC) is the major polar lipid; and v) the DNA G + C content is typically 67 – 71.3 mol% (Evtushenko & Krausova, 2012)

At the time of writing, the genus is comprised of 24 species with validly-published names according to the list of prokaryotic names with standing in nomenclature (LPSN) (http://www.bacterio.net/kribbella.html). Recently published species names that have not yet been validated are '*Kribbella podocarpi*' strain YPL1^T (KM382222) (Curtis et al., 2017), '*Kribbella sindirgiensis*' strain FSN23^T (JN896614) (Ozdemir-Kocak et al., 2017a), '*Kribbella soli*' FMN22^T (JN896613) (Ozdemir-Kocak et al., 2017b), and '*Kribbella qitaiheensis*' NEAU-GQTH2-3^T (NR148308) (Guo et al., 2015). The majority of *Kribbella* species have been isolated from soil (Trujillo et al. 2006) with some species being described as endophytes of various plant species (Kaewkla & Franco, 2016; Kaewkla & Franco, 2013; Trujillo et al., 2006; Song et al., 2004; Curtis et al., 2017).

Recent advancements in the genus include the MLSA of five concatenated housekeeping genes to refine the phylogeny of the *Kribbella* genus (Curtis & Meyers, 2012). The MLSA built on the *gyrB* gene work of Kirby et al. (2010) and demonstrated the potential for a concatenated sequence of five housekeeping genes (*gyrB-rpoB-recA-relA-atpD*) to be used in determining the phylogenetic relationships of species within the genus *Kribbella* and underpin taxonomic conclusions. This work by Curtis & Meyers (2012) showed that

the *gyrB*, *rpoB*, *recA*, *relA* and *atpD* genes share 100% gene sequence similarity between the type strain and non-type strains of the same species.

The aims of this chapter are three-fold: i) to determine the antibiotic biosynthetic potential of selected actinobacterial isolates to produce ansamycin, glycopeptide, non-ribosomal peptide and Type-II (aromatic) polyketide antibiotics; ii) to extract the antibiotics from strains PR3, SK5, UK1 and Y30 and analyse the crude extracts for activity against *M. aurum* A+, *Staphylococcus aureus* ATCC 25923, and *Escherichia coli* ATCC 25922 with emphasis on strain SK5; and iii) to determine the taxonomic position of strain SK5 within the genus *Kribbella* using a polyphasic taxonomic approach.

A polyphasic taxonomic approach compares multiple features of a microbe to known species to attain a more robust description of the proposed novel species (Busse et al., 1996; Ludwig, 2007). The different features assessed in this study were genotypic, phenotypic and chemotaxonomic characters. Genotypic analysis allows for the identification of an isolate to the genus level and assessment of potential novelty based on one gene (such as the 16S rRNA gene) or a concatenated sequence of housekeeping genes. The analysis of an MLSA performed by a previous student in the lab (Dr Sarah M. Curtis) provided strong evidence that strain SK5 represents a new species in the genus *Kribbella*. A series of phenotypic tests were used to differentiate strain SK5 from the type strains of the most closely related known species (determined from the MLSA) and chemotaxonomic analyses were performed to confirm that strain SK5 has chemotaxonomic markers that are consistent with membership of the *Kribbella* genus. Strain SK5 was selected for characterization because it demonstrated very strong antibiotic activity against *M. aurum* A+ (Table 2), which is unusual for a member of the genus *Kribbella*.

3.3 Methods & Materials

3.3.1 Antibiotic biosynthetic potential

3.3.1.1 PCR amplification

DNA was extracted as described in section 2.3.3.1.

Primers specific to antibiotic biosynthetic gene clusters were used to assess the antibiotic biosynthetic potential of select isolates to produce Type-II (aromatic) polyketide, ansamycin (Type-I polyketide), glycopeptide, and non-ribosomal peptide (NRP) antibiotics. Primer pairs are listed in Table 6. Type-II (aromatic) polyketides were detected by the amplification of the polyketide synthase gene pair, keto synthase alpha (KSα) and ketosynthase beta (KSβ). Ansamycins were detected by amplification of a section of the 3-amino-5-hydroxy-benzoic acid (AHBA) synthase gene. Glycopeptide antibiotics were detected by amplification of the glycopeptide monooxygenase B (*oxyB*) gene. NRP antibiotics were detected by amplification of conserved motifs of adenylation (A) domains of non-ribosomal peptide synthetase (NRPS) genes.

Table 6: PCR primers used to identify the presence or absence of key antibiotic biosynthetic gene clusters to determine the antibiotic biosynthetic potential of environmental actinobacterial isolates. 'F' denotes a forward primer and 'R' denotes a reverse primer. I = Inosine. Matching colours indicates primer pairs.

Primer	Primer sequence	Expected fragment size (bp)	Reference
ARO-PKS-F **ARO-PKS-R**	5'-GGCAGCGGITTCGGCGGITTCCAG-3' 5'-CGITGTTIACIGCGTAGAACCAGGCG-3'	492 – 630	Wood et al., 2007
ANSA-F **ANSA-R**	5'-CCSGCSTTCACSTTCATCTC-3' 5'-AISYGGAICATIGCCATGTAG-3'	640	Wood et al., 2007
Foxy **Roxy**	5'-CTGGTCGGCAACCTGATGGAC-3' 5'-CAGGTACCGGATCAGCTCGTC-3'	590	Wood et al., 2007
A3F **A7R**	5'-GCSTACSYSATSTACACSTCSGG-3' 5'-SASGTCVCCSGTSCGGTAS-3'	700	Ayuso-Sacido & Genilloud, 2005

PCR was performed in 50 µL reactions with the following components: 2 mM $MgCl_2$, 150µM of each dNTP, 0.5 µM of each primer (1.5µM for NRPS PCR), 1U Super-Therm *Taq* polymerase (JMR Holdings, USA), and ~500ng/µL of template DNA. Table 7 shows the PCR programmes used to amplify the different genes. PCR products were electrophoresed on 0.8% agarose gels containing 0.8µg/mL EtBr. *Pst*I-digested λ DNA was used as the molecular marker. The PCR products were visualised as in section 2.3.3.2.

Table 7: PCR programmes for the amplification of key genes within antibiotic biosynthetic gene clusters.

Step		Temperature (°C)	Time	
			Aromatic, *oxyB*, AHBA	**NRPS**
1.	**(Initial denaturation)**	96	2 min	5 min
2.	**(Denaturation)**	96	45 s	30 s
3.	**(Annealing)**	56 (AHBA), 60 (*oxyB*), 64 (Aromatic & NRPS)	30 s	90 s
4.	**(Extension)**	72	2 min	4 min
5.	**(Final extension)**	72	5 min	10 min
6.	**(Hold)**	20	20 min	20 min
Steps 2 to 4 were repeated for 30 cycles				

3.3.1.2 Sequencing and *blastn* analysis

The same steps were followed as in section 2.3.3.3, with the exception that the sequencing primers used were dependent on the primers used in the PCR and the resulting sequences were compared to the GenBank database using a basic *blastn* search (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

3.3.2 Organic compound solvent extraction

Bacterial cultures were grown in small volumes of broth, prior to upscaling to a larger volume, for more cell mass and thus antibiotic production. Initial cultivation was in 250 mL Erlenmeyer flasks containing 15 mL ISP2 medium at 30°C, with shaking, for four days, or until sufficient cell mass had accumulated. The 15-mL culture was inoculated into a 1 L Erlenmeyer flask containing 100 mL ISP2 broth and incubated for another 10 days at 30°C, with shaking, to allow for the production of antibiotic/s. The purity of the cultures was assessed by Gram stain and streaking for single colonies on ISP2 agar.

The cell mass was separated from the broth by filtering the culture through a paper coffee filter (size 102, House of Coffees). Methanol (MeOH) was added to the cell mass in a beaker such that the cells were completely submerged. The beaker was covered with foil and placed on a shaker (120 rpm) at room temperature for one hour to extract organic compounds from the cell mass into the MeOH. The cell mass was separated from the cell mass extract (in MeOH) by filtering through a paper coffee filter into a 250 mL glass beaker and the cell mass extract was evaporated to dryness in a fume hood. The dried cell mass extract was concentrated by a factor of 19.2 by dissolving in 6 mL MeOH and transferred to 3 X 2 mL microfuge tubes before being evaporated to dryness in a fume hood. The dried cell mass extract was weighed and stored in the microfuge tubes at -20°C. Ethyl acetate (EtOAc) was added to the culture broth at a third of the total culture volume (approximately 30 mL) in a separating funnel. The mixture was shaken vigorously to extract mid-polarity organic compounds from the culture broth into the EtOAc, then left to

stand for one hour to allow the aqueous and organic phases to separate. The aqueous phase was then drained into a glass beaker and the organic solvent layer was poured into a separate glass beaker and covered with foil. The extraction of the culture broth was repeated twice (i.e. three extractions altogether) to ensure maximum organic compound extraction. The three EtOAc extracts were pooled, mixed vigorously and left overnight in the separating funnel. The aqueous phase was discarded and the organic phase was dispensed into a 250 mL glass beaker and evaporated to dryness in a fume hood. The dried culture broth extract was concentrated by a factor of 19.2 by dissolving in 6 mL EtOAc and transferred to 3 X 2 mL microfuge tubes before being evaporated to dryness in a fume hood. The dried culture broth extract was weighed and stored in the microfuge tubes at -20°C. The extracts were tested for activity against different test bacteria by spot bioautography (Betina, 1973).

3.3.3 Spot bioautography

Each of the 2 mL microfuge tubes, containing the dried down and concentrated cell mass and culture broth crude extract fractions (see section 3.3.2) were re-dissolved in 500 µL MeOH (cell mass crude extract) or 500 µL EtOAc (culture broth crude extract). The spot tests were performed by pipetting 5µL of crude extract from each of the concentrated factions, separately, onto a silica TLC plate (Merck 1.05554.0001). The TLC strips were left for approximately 5 min to allow the solvents to evaporate. The tests were repeated with 10µL of crude extract if weak or doubtful activity was initially observed.

Extracts obtained as described in section 3.3.2 were tested for activity against test bacteria *M. aurum* A+, *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 using bioautography. The test bacteria were grown overnight at 37°C, with shaking, in universal glass bottles (standard containers) containing 5 mL 2YT broth. The purity of the cultures was confirmed by Gram stain and streaking for single colonies on 2YT agar. The optical density of the test bacterial cultures was determined as described in section in 2.3.2 and each culture was diluted to an OD_{600nm} of 0.5 with sterile 2YT broth. Sterile non-absorbent cotton wool was used to dab the diluted test bacterial culture onto the silica TLC plates containing the dried extracts. The silica plates were then placed in a sealed plastic container with damp paper towel and left overnight (14-18 hours) at 37°C. Sterile non-absorbent cotton wool was then used to dab on a 0.25% solution of thiazolyl blue tetrazolium bromide (MTT) (Sigma; M2128) that was prepared in phosphate buffered saline (1.78g Na_2HPO_4; 8.50g NaCl; 1l distilled water; pH 7.3). The MTT-treated TLC plate was left at 37°C for one hour or until a colour change was observed. MTT is reduced to an insoluble formazan derivative in the presence of actively respiring cells. Upon reduction, MTT turns from yellow to blue/purple, so the entire TLC is expected to turn blue/purple. A positive result for antibiotic activity is indicated by a lack of colour change at the sites where the extracts were applied to the TLC plate. The silica plates (developed bioautograms) were scanned immediately, as the MTT formazan colour fades over time.

3.3.4 Thin layer chromatography

Five (5) -10 µL of crude antibiotic extract (amount dependent on the antibiotic strength of the extract) was placed on a 1.5 X 8 cm long silica TLC plate and the solvents were allowed to evaporate in a fume hood. A line was drawn 1.5 cm from the end of the silica plate with a pencil to mark where the extract should be placed (the chromatography origin). Concurrently, a TLC chamber was set up with the appropriate organic solvent (the mobile phase) in a 250-mL glass beaker containing solvent saturation pads (Gelman Instrument Company, Ann Arbor, Michigan) as a wick, and foil to cover the beaker (to limit the evaporation of the solvent from the beaker). The volume of solvent added was such that the bottom 0.5cm of the silica TLC plate was submerged in the solvent (e.g. 20 mL solvent in a 250-mL beaker). The TLC chamber was set up 30 min prior to performing the chromatography to develop a solvent-saturated atmosphere in the beaker (to limit the evaporation of the mobile phase directly from the TLC plate during chromatography). 100% EtOAc and 100% MeOH were the two mobile phases used. The silica TLC plates (with the concentrated extracts loaded at the origin) were placed in the TLC chamber and the solvent allowed to run up the plate until it was 0.5cm from the top of the plate. The plate was removed and the solvent allowed to evaporate in the fume hood. Bioautography was performed on the TLC plate as in section 3.3.3.

Two-dimensional (2D) TLC was performed on the extracts using EtOAc as the first-dimension mobile phase and MeOH as the second-dimension mobile phase. An 11.5-by-11.5cm silica TLC plate was used and a 10-by-10cm square was marked with pencil within. Five (5) -10 µL of extract (amount dependent on the antibiotic strength of the extract) was placed on the silica TLC plate at one corner of the 10-by-10cm square and the direction of development was marked with an arrow. A saturated TLC chamber was created, as described above, and the silica TLC plate containing the dried extract was placed in the chamber containing EtOAc for the first-dimensional run until the solvent reached the 10cm mark. The plate was removed and the solvent allowed to evaporate in a fume hood. The TLC plate was rotated 90° and then the second dimension was developed in the TLC chamber containing MeOH as the mobile phase. Bioautography was performed on the TLC plate as described in section 3.3.3. Cerium (IV) ammonium sulphate (CAS) (63g of $Ce(NH_4) SO_4$, initially dissolved in 500ml of 1M H_2SO_4 and made up to a final volume of 1L with deionised water) was dabbed on to a second 2D TLC plate of the same crude extract, using sterile non-absorbent cotton wool, and then heated at 110°C to note how many organic compounds are present (visible as brown spots on the TLC plate).

3.3.5 Testing for antiplasmodial activity

The crude cell mass and culture broth extracts were also screened for antiplasmodial activity against *Plasmodium falciparum* strain NF54 (chloroquine sensitive) by Mr. Daniel Watson (PhD student, Department of Clinical Pharmacology, University of Cape Town). Using a modified method of Trager & Jensen (1976), the asexual erythrocyte stages of *P. falciparum* were maintained as continuous *in vitro*

cultures. The *in vitro* antiplasmodial efficacy was determined by the parasite lactate dehydrogenase (pLDH) assay, using a modified method of Makler et al. (1993). The crude extracts were tested in quadruplicate, twice, using chloroquine and artesunate as the positive controls for antiplasmodial activity.

3.3.6 Characterisation of strain SK5

3.3.6.1 Genotypic characterisation

3.3.6.1.1 16S rRNA gene sequence amplification

Genomic DNA was extracted as described in section 2.3.3.1 and the 16S rRNA gene sequence was amplified via PCR as described in section 2.3.3.2.

3.3.6.1.2 Phylogenetic analysis

The 16S rRNA gene phylogeny was constructed as described in section 2.3.3.4.1.

3.3.6.1.3 Multilocus sequence analysis

Multilocus sequence analysis (MLSA), to compare strain SK5 to all described species in the *Kribbella* genus, was performed by Dr. Sarah M. Curtis as per Curtis & Meyers (2012). Five housekeeping genes were used: *gyrB*, *rpoB*, *recA, relA* and *atpD*. Genetic distances were calculated using the Kimura 2-parameter model in MEGA 6 (Curtis & Meyers, 2012). The *gyrB*, *rpoB*, *recA, relA* and *atpD* gene sequences were determined by Ms Marli de Kock (a former intern in the research group) and the author.

3.3.6.2 Phenotypic characterisation

3.3.6.2.1 Colony morphology and pigmentation

The colour of the aerial and substrate mycelia and the presence of any diffusible pigments were assessed using the following ISP media: inorganic salts-starch agar (ISP4; colour of substrate and aerial mycelia), ISP5 (production of a diffusible pigment), and ISP6 and ISP7 (production of melanin). These ISP media were prepared according to Shirling &Gottlieb (1966) and the cultures were incubated at 30°C for 7 days. General morphological features (aerial mycelium, colony shape and branching pattern of hyphae) was determined on ISP2 agar.

3.3.6.2.2 Physiological characterisation

To determine whether strain SK5 belongs to a novel species, physiological testing was done in parallel with the strain’s closest genomic relatives that were selected based on the 16S rRNA gene and MLSA phylogenetic trees. See Table 8 for the list of type strains used.

Table 8: Type strains of the closely related *Kribbella* species used in the physiological comparison to strain SK5.

Strain	Strain number
Kribbella aluminosa	DSM18824^T
Kribbella karoonensis	Q41^T
Kribbella pittospori	NRRL B-248113^T
Kribbella shirazensis	DSM45490^T
'Kribbella soli'	KCTC 29219^T

For testing compounds as sole carbon sources, a basal mineral salts agar (BMSA) was prepared according to Shirling & Gottlieb (1966). Carbon sources utilised by strain SK5 were determined as per the methods of Shirling & Gottlieb (1966). Carbon sources were filter sterilised separately and then added to the BMSA at a final concentration of 1% (w/v). Strain SK5 and the type strains listed in Table 8 were inoculated onto the agar plates and the presence or absence of growth was recorded every 7 days for 21 days at 30°C. Nitrogen sources utilised by strain SK5 were determined as per the methods of Williams et al. (1989). All nitrogen sources were filter sterilised separately and then added to the BMSA (for nitrogen sources) at a final concentration of 0.1% (w/v). Strain SK5 and the type strains in Table 8 were inoculated onto the agar plates and the presence or absence of growth was recorded after 15 days at 30°C.

The remaining physiological tests were performed as per the methods of Williams et al. (1989). Growth in the presence of inhibitory compounds was determined on Bennett's medium (Atlas, 2004) and observed after 14 days at 30°C. Growth in the presence of NaCl was determined on Bennett's medium with 3%, 4%, 5% and 6% (w/v) NaCl after 14 days at 30°C. Degradation of adenine, allantoin, arbutin, guanine, hypoxanthine, Tween 80, L-tyrosine and xanthine were determined on modified Bennett's medium (Locci, 1989) after 21 days at 30°C. Degradation of urea was determined after 14 days growth at 30°C, based on the methods by Williams et al. (1989). Degradation of gelatin was determined on modified Bennett's medium after 7 days at 30°C. Hydrolysis of pectin was determined after 7 days growth at 30°C, based on the methods by Williams et al. (1989). The pectin hydrolysis medium was prepared as in Locci (1989). Hydrolysis of DNA was determined using Difco DNase medium (prepared according to the manufacturer's instructions) after 7 days growth at 30°C by flooding the plates with 0.25M hydrochloric acid. Growth at 22°C, 30°C, 37°C and 40°C was determined on Bennett's medium after 21 days. Growth at pH 4.3, 5, 7 and 9 was determined after 21 days at 30°C on Bennett's medium.

Catalase and oxidase tests were done to determine whether strain SK5 possesses catalase and can produce cytochrome C oxidase, respectively. The presence of catalyse was determined by the observation of bubbles when cell mass was added to 10 µL of 9% hydrogen peroxide. Cytochrome C oxidase was confirmed to be present when filter paper saturated with 1% N, N, N′, N′-tetramethyl-p-phenylenediamine (TMPD) turned purple after the addition of cell mass.

3.3.6.3 Chemotaxonomic characterisation

3.3.6.3.1 Diaminopimelic acid analysis

A 15 mL ISP2 broth medium was inoculated with strain SK5. After 4 days at 30°C the culture was transferred to a 100 mL ISP2 broth medium in a 1L Erlenmeyer flask. After another 4 days at 30°C, the culture was transferred to a 1L ISP2 broth medium in a 5 L Erlenmeyer flask. The cell mass from the 1 L culture was harvested after 10 days at 30°C by centrifugation at 12 000 rpm for 10 minutes (22 100 X *g*). A Gram stain and streak for single colonies confirmed no contamination. The cell mass was washed twice with approximately 20 mL sterile distilled water each time and then resuspended in 20 mL sterile distilled water in a round-bottom flask. The cell mass was freeze dried and weighed. The DAP isomer was determined using a method from Hasegawa et al. (1983), with the following alterations: dried cells were used instead of colonies from an agar plate and the solvent system was methanol/0.05 M potassium hydrogen phthalate buffer (pH 4) (2:1, v/v; Schön& Groth, 2006). TLC was performed on cellulose plates (MERCK; 1.0552.0001) with a 1% commercial DAP standard (Sigma; D1377-5G).

3.3.6.3.2 Whole-cell sugar pattern analysis

The cell mass was prepared as described in section 3.3.6.3.1, and the whole cell sugar pattern (WCSP) was analysed based on the methods of Hasegawa et al. (1983). TLC was performed on cellulose plates (MERCK; 1.0552.0001) along with two different sugar standards: i) glucose, mannose, and ribose; each at 1% (w/v); ii) galactose, arabinose, and ribose; each at 1% (w/v). The solvent system used was acetate-pyridine-distilled water (100:35:25 v/v).

3.3.6.3.3 Polar lipid analysis

The cell mass was prepared as described in section 3.3.6.3.1 to extract the polar lipids, and their pattern was determined based on the methods of Komagata & Suzuki (1987) and Minnikin et al. (1984). Two-dimensional TLC was performed and the polar lipids were detected using the following reagents: 1.3% molybdenum blue reagent (Sigma; M1942) for phospholipids, 1% (v/v) anisaldehyde (Sigma; A88107) for glycolipids, and 1% (w/v) ninhydrin (Merck; 6762) for aminolipids. The anisaldehyde stain was prepared as per http://lcso.epfl.ch/files/content/sites/lcso/files/load/TLC_Stains.pdf. The ninhydrin stain was prepared in 99% ethanol.

Chromatography for sections 3.3.6.3.1, 3.3.6.3.2, and 3.3.6.3.3 was performed in a TLC chamber prepared 30 minutes in advance, as described in section 3.3.4.

3.3.6.3.4 Menaquinone analysis

The cell mass was prepared as described in section 3.3.6.3.1. The menaquinones were analysed by the Identification Service, DSMZ (Braunschweig, Germany) after extraction using the methods of Komagata & Suzuki (1987) and Minnikin et al. (1984).

3.3.6.3.5 Predominant fatty acids (FAME pattern)

The dried cell mass was prepared as described in section 3.3.6.3.1. The FAME pattern analysis was carried out as a service by the BCCM-LMG, Gent, Belgium.

3.4 Results & Discussion

3.4.1 Antibiotic biosynthetic potential

PCR amplification of conserved regions of core genes in antibiotic biosynthetic gene clusters implies the potential of the strain to produce the antibiotic being screened for. In the following sub-section, the potential for seven strains to produce Type-II polyketide, ansamycin, glycopeptide and non-ribosomal peptide antibiotics is assessed. These strains were chosen because they had moderate to very strong antibiotic activity against *M. aurum* A+ and were interesting phylogenetically (Chapter 2).

The potential for strains M27, PR10, PR28, PR47, SK5, UK1, and Y10 to produce a Type-II polyketide by amplification of a DNA fragment from the tandem KSα and KSβ genes is shown in Figure 11. A band in the size range 492-630bp indicates a positive result. Strain Y10 does not have the potential to produce a Type-II polyketide antibiotic, as indicated by no band on the gel. The amplified product of strain SK5 is smaller than the other strains (~ 340bp), suggesting that this was the result of off-target amplification by the PCR primers and that strain SK5 should be considered to have given a negative result for the presence Type-II polyketide biosynthetic genes. The PCR products with a band were prepared and sent for sequencing (section 3.3.1.2). Upon analysis of the chromatograms, the KSα-KSβ sequences for strains PR10, PR28 and SK5 were messy, suggesting the presence of more than one KSα-KSβ sequence and thus more than one Type-II polyketide antibiotic biosynthetic gene cluster. The PCR was repeated with these strains and the PCR products were cloned into the pGEM-T-easy vector system (as per the manufacturer's instructions) and then more than one clone was sequenced using the ARO-PKS-F primer. This ensured the sequencing of a single KSα-KSβ sequence at a time. Table 9 shows the results of a standard *blastn* search against the GeneBank database using the sequenced PCR amplified KSα-KSβ gene sequences of strains M27, PR10, PR28, PR47, and UK1. Strains PR10 and PR28 each had two cloned KSα-KSβ gene sequences. The two cloned KSα-KSβ gene sequences for strain PR10 sequences were identical (Table 9). The two cloned KSα-KSβ gene sequences for strain PR28 sequences had different top hits and thus represent KSα-KSβ gene sequences from different Type-II polyketide antibiotic biosynthetic pathways (Table 9).

The potential for strains M27, PR10, PR28, PR47, SK5, UK1, and Y10 to produce an ansamycin antibiotic by amplification of the AHBA synthase gene is shown in Figure 12. Only *Micromonospora* strain M27 demonstrated the potential to produce an ansamycin antibiotic due to the presence of a band of approximately 650bp on the gel. No sequence data for the AHBA synthase gene from strain M27 could be obtained.

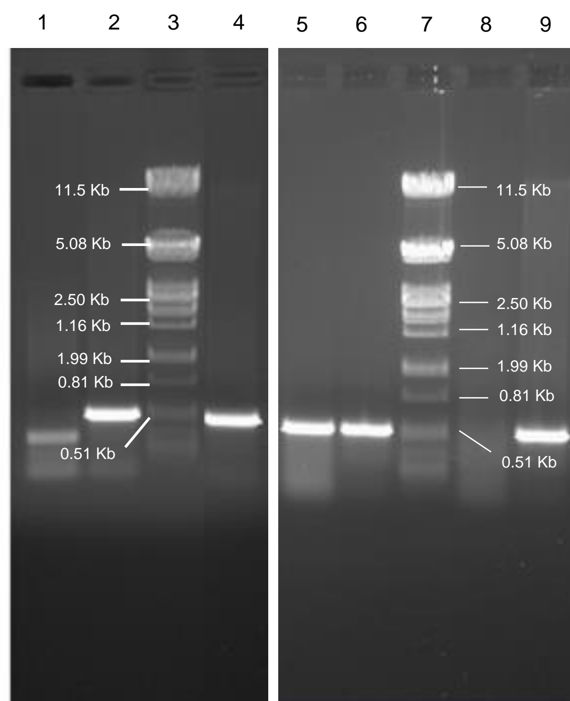

Figure 11: Gel electrophoresis to indicate the antibiotic biosynthetic potential of the strains to produce a Type-II polyketide antibiotic with the detection of the KSα-KSβ genes by PCR using the primer pair ARO-PKS-F & ARO-PKS-F.

1. strain SK5; 2. strain PR47; 3. *Pst*I-digested λ molecular marker; 4. strain PR28; 5. strain UK1; 6. strain PR10; 7. *Pst*I-digested λ molecular marker; 8. strain Y10; 9. strain M27.

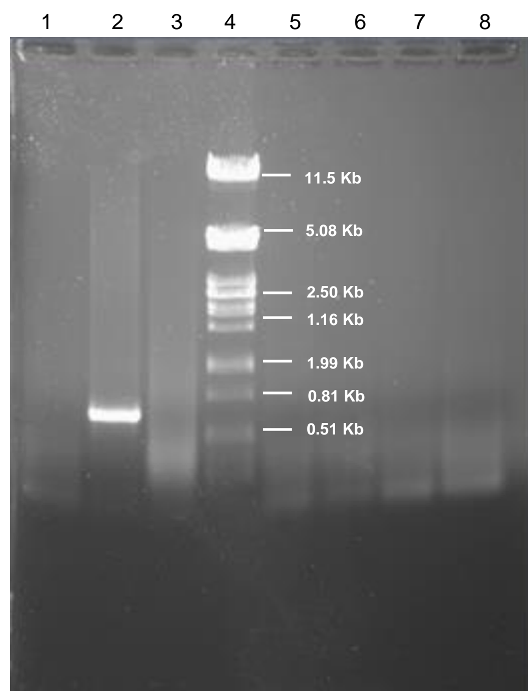

Figure 12: Gel electrophoresis to indicate the antibiotic biosynthetic potential of the strains to produce an ansamycin antibiotic with the detection of the AHBA gene by PCR using the primer pair ANSA-F & ANSA-R.

1. strain UK1; 2. strain M27; 3. strain Y10; 4. *Pst*I-digested λ molecular marker; 5. strain PR28; 6. strain PR47; 7. strain PR10; 8. strain SK5.

The potential for strains M27, PR10, PR28, PR47, SK5, UK1, and Y10 to produce a glycopeptide antibiotic by the amplification of the *oxyB* gene is shown in Figure 13. Only *Micromonospora* strain M27 showed the potential to produce a glycopeptide antibiotic which is demonstrated by a band of approximately 600 bp, indicative of a positive result for the presence of the *oxyB* gene. The double band in Figure 13 could not be eliminated. Changing the PCR protocol led to no PCR product when the annealing temperature was increased and/or the $MgCl_2$ concentration altered. Thus, no sequence data for the *oxyB* gene from strain M27 could be obtained.

The potential for strains M27, PR10, PR28, PR47, SK5, UK1, and Y10 to produce a non-ribosomal peptide antibiotic by amplification of the A domain is shown in Figure 14. Strains PR10, PR28, PR47, UK1, and Y10 show the potential to produce a non-ribosomal peptide antibiotic due to the amplification of a DNA fragment of the expected size (approximately 700bp; Figure 14). PCR products were cloned into the pGEM-T-easy vector system (as per the manufacturer's instructions) and the clones were sequenced using the 17-mer, M13F vector primer (5'-GTAAAACGACGGCCAGT-3'). Table 10 shows the results of a standard *blastn* search against the GeneBank database using the sequenced PCR amplified A domain sequences for strains PR10 (two different clones) and PR28 as well as the predicted substrate specificity of the A domains based on the NRPSpredictor2 software (Röttig et al., 2011; Rausch et al., 2005). The *blastn* top hits for the two cloned sequences of strain PR10 are different, implying the presence of least two different non-ribosomal peptide biosynthesis pathways in this strain (Table 10). However, the substrates that the A domains would bind to, for the two strain PR10 clones, are predicted to be hydrophobic and aliphatic, with scores of 0.93 for clone one and 0.47 for clone two, according to NRPSpredictor2 (Table 10). The latter of the two scores is low and thus inconclusive. For strain PR28, the A domain is predicted to bind to a hydrophilic amino acid, albeit with a low score of 0.21 (Table 10). These substrate specificity predictions of the NRPS A domains should not be relied upon because of the low scores.

An attempt was made to screen strains PR3 and Y30 for their antibiotic biosynthetic potential, but low-quality DNA prevented this. The observed antibiotic activities of these strains (M27, PR10, PR28, PR47, SK5, UK1, and Y10) against *M. aurum* A+ could thus be attributed to one of the antibiotics that they have the demonstrated potential to produce. Knowing the type of antibiotic that a strain has the potential to produce can be useful for determining the appropriate extraction and purification techniques for that class of antibiotics (Wood et al., 2007). However, a positive PCR result only implies the *potential* for a strain to produce a specific type of antibiotic. There are important caveats to consider, such as the following false positives: the entire biosynthetic gene cluster may not be present and thus a functional antibiotic will not be made despite a positive result, or the biosynthetic gene cluster may be silent. There are also false negatives to consider, such as sequence variation in the primer binding site that will lead to a negative result. Additionally, one can only screen for the potential of a strain to produce known classes of antibiotics (Wood et al., 2007).

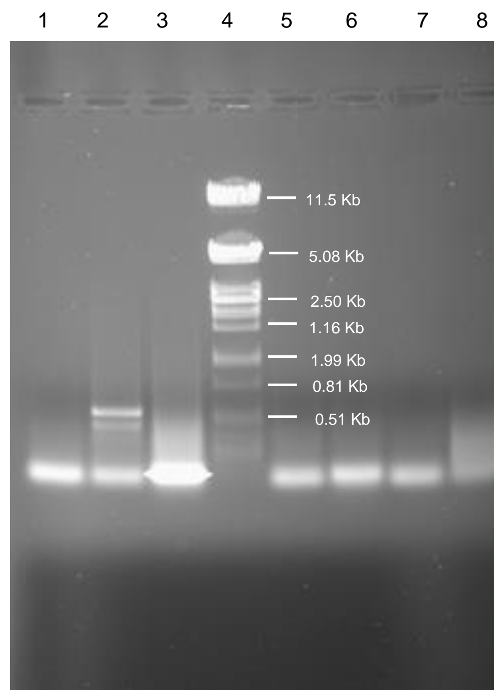

Figure 13: Gel electrophoresis to indicate the antibiotic biosynthetic potential of the strains to produce a glycopeptide antibiotic with the detection of the *oxyB* gene by PCR using the primer pair Foxy/Roxy.

1. strain UK1; 2. strain M27; 3. strain Y10; 4. *Pst*I-digested λ molecular marker; 5. strain PR28; 6. strain PR47; 7. strainPR10; 8. strain SK5.

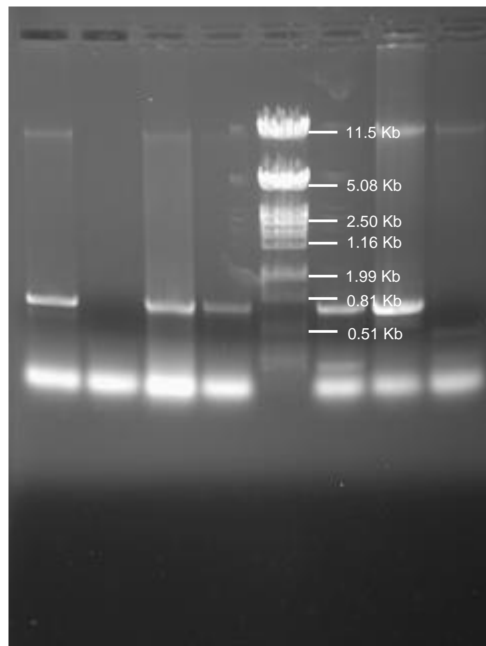

Figure 14: Gel electrophoresis to indicate the antibiotic biosynthetic potential of the strains to produce a non-ribosomal peptide antibiotic with the detection of the A domain by PCR using the primer pair A3F&A7R.

1. strain Y10; 2. strain SK5; 3. strain PR10; 4. strain UK1; 5. *Pst*I-digested λ molecular marker; 6. strain PR28; 7. strainPR47; 8. strain M27.

Table 9: Standard *blastn* search results against the GenBank database for the isolates that showed the potential to produce a Type-II polyketide antibiotic.

Strain	Top Hit	Nucleotide Sequence similarity (%)	No. bp
M27	*Micromonospora aurantiaca* ATCC 27029 (CP002162) - β -ketoacyl synthase	98	425
PR10 (clone 1)	*Streptomyces* sp. 3124.6 (LT670819) minimal PKS ketosynthase (KS/KS alpha) minimal PKS chain-length factor (CLF/KS β)	88	457
PR10 (clone 2)	*Streptomyces* sp. 3124.6 (LT670819) minimal PKS ketosynthase (KS/KS alpha) minimal PKS chain-length factor (CLF/KS beta)	88	459
PR28 (clone 1)	*Streptomyces antibioticus* strain NRRL 3238 oviedomycin biosynthetic gene cluster(KY129858)	82	454
PR28 (clone 2)	*Streptomyces venezuelae* strain NRRL B-65442 (CP018074) ketosynthase chain-length factor	83	453
PR47	*Streptomyces* sp. strain MM19 Type II polyketide synthase 2 (pksII-2) (KX708173)	82	386
UK1	*Streptomyces* sp. CCM_MD2014, (CP009754) β -ketoacyl synthase	99	422

Table 10: Standard *blastn* search results against the GenBank database for the isolates that showed the potential to produce a non-ribosomal peptide antibiotic.

Strain	NRPS A domain specificity	Top hit (*blastn*)	Nucleotide Sequence similarity (%)	No. bp
PR10 (clone one)	Large clusters: gly,ala,val,leu,ile,abu,iva Small clusters: val,leu,ile,abu,iva	*Streptomyces anulatus*ATCC 11523 Actinomycin biosynthetic gene cluster(HM038106)	82	650
PR10 (clone two)	Large clusters: gly,ala,val,leu,ile,abu,iva Small clusters: val,leu,ile,abu,iva	*Streptomyces* sp. strain MM108 non-ribosomal peptide synthetase 3 (nrps3) gene(KX708387)	75	604
PR28	Small clusters: asp,asn	*Streptomyces* sp. ID05-A0470 gene for non-ribosomal peptide synthase(AB432863)	98	753

3.4.2 Solvent extract activity

3.4.2.1 *Streptomyces* species

Streptomyces strains PR28, UK1, and Y30 were selected for solvent extraction of their antibiotics, because they had moderate to very strong antimycobacterial activity (Table 2) and were also phylogenetically interesting (see section 2.4.4.3).

Solvent extraction was performed on both the cell mass (MeOH) and the culture broth (EtOAc) of each strain. The concentrated crude extracts were applied to silica TLC plates and subjected to bioautography (Figure 15).

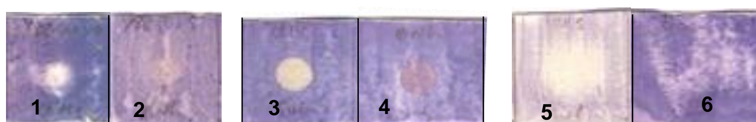

Figure 15: Bioautography of *Streptomyces* strains PR28, UK1, and Y30 cell mass and culture broth extracts against test bacterium *M. aurum* A+. 1. strain Y30 cell mass extract; 2. strain Y30 culture broth extract; 3. strain UK1 cell mass extract; 4. strain UK1 culture broth extract; 5. strain PR28 cell mass extract; 6. strain PR28 culture broth extract.

All three strains had activity in the cell mass extract and not the culture broth extract, implying that the active compounds were located within the cells and not excreted into the broth in high concentrations.

Further study should be done on these crude extracts to assess their potential for future work. Bioautography against other test bacteria can be done to determine the specificity of the active compound/s. In addition, 1D TLC and 2D TLC can be done to determine whether there is more than one active compound present in the crude cell mass extract. TLC will also identify the retention factor (R_f) of the compounds under different solvent conditions, which will assist in purifying the active compound/s.

3.4.2.2 *Kribbella* species (strain SK5): first extraction

Strain SK5 was selected for solvent extraction of its antibiotic/s, because this *Kribbella* isolate exhibited very strong antimycobacterial activity, which is unusual for the genus (Table 2).

Solvent extraction was performed on both the cell mass (MeOH) and the culture broth (EtOAc). The concentrated extracts were applied to silica TLC plates and subjected to spot test bioautography (Figure 16).

Activity was observed in the cell mass extract against *M. aurum* A+, implying that the active compounds are located within the cells and not excreted into the broth in high concentrations (Figure 16). To assess the specificity of the antibiotic compound/s, the activity of strain SK5's cell mass extract and culture broth extract were tested against two other test bacteria, namely *E. coli* ATCC 25922 (Gram negative) and *S. aureus* ATCC 25923 (Gram positive). Both the cell mass and culture broth extracts did not display any activity against *E. coli* ATCC 25922, but the cell mass extract did exhibit activity against *S. aureus* ATCC 25923 (Figure 16). This suggests that strain SK5's activity is specific for Gram-positive bacteria.

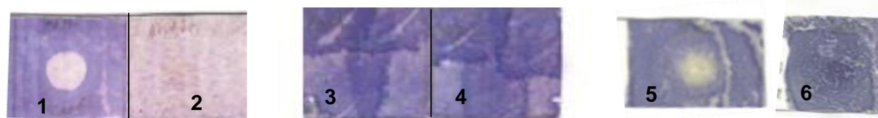

Figure 16: Initial spot test bioautography results for the *Kribbella* strain SK5 cell mass and culture broth solvent extracts against different test bacteria. 1. cell mass extract activity against *M. aurum* A+; 2. culture broth extract activity against *M. aurum*; 3. cell mass extract activity against *E. coli* ATCC 25922; 4. culture broth extract activity against *E. coli* ATCC 25922; 5. cell mass extract activity against *S. aureus* ATCC 25923; 6. Culture broth extract activity against *S. aureus* ATCC 25923.

1D TLC was done on strain SK5's crude cell mass extract to determine how many active compounds were present (Figure 17). This method also provides the R_f value of each active compound, giving valuable information for future work when purifying the active compound/s. However, these results could not be replicated. Each time a crude extract was obtained, one round of spot test bioautography could be done before the extract lost activity, despite care taken to ensure that the extract was not out of the freezer (-20°C) for long periods at a time. This was repeatedly observed over a set of 6 extractions and is indicative of a labile compound. It is important to note that the strain SK5 culture used in this set of extractions was incubated at a time when the 30°C incubator was experiencing fluctuating temperatures. The incubator would occasionally drop to ~25°C throughout the day for the entire incubation period. The temperature did not go above 30°C.

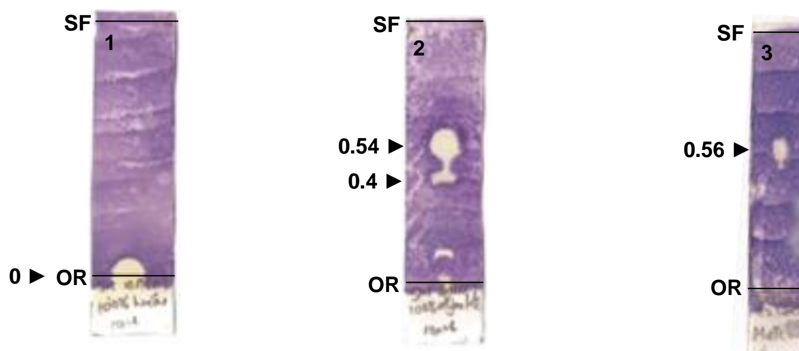

Figure 17: 1D thin layer chromatography, with different solvent systems, on the cell mass extract of strain SK5, which was then subjected to bioautography against the test bacterium *M. aurum* A+. 1. 100% hexane as the mobile phase; 2. 100% EtOAc as the mobile phase; 3. 100% MeOH as the mobile phase.
Arrows mark the active spot with the R_f value displayed at the side.
OR is the origin. SF is the solvent front.

3.4.2.3 *Kribbella* species (strain SK5): second extraction

The activity of strain SK5's crude cell mass and culture broth extracts were assessed once again when the incubator was restored to a constant temperature (30°C). Very interestingly, the location of activity (cell mass vs culture broth) changed when the solvent extraction took place with an SK5 culture that had been grown at a constant temperature of 30°C for the entire incubation period (Figure 18). Antibiotic activity against *M. aurum* A+ and *S. aureus* ATCC 25923 was recorded in the culture broth extract, and no longer in the cell mass extract (Figure 18). As before, activity was not recorded against *E. coli* ATCC 25922 (data not shown). In addition to the change in location of the active compound/s, the activity was stable. This is the first piece of evidence that the compound/s is not the same as what was previously extracted when the incubator thermostat was faulty (because the active compound/s is not labile), which implies that strain SK5 can make more than one antimycobacterial compound. The environment in which the bacteria grow has a huge impact on the biosynthesis of secondary metabolites. The availability of nutrients, nutrient composition, and changes in growth conditions in general can affect the production of antibiotics (Martin & Demain, 1980; Hurley & Bialek, 1974).

Figure 18: Bioautography of the cell mass and culture broth solvent extracts of strain SK5 against test bacteria *M. aurum* A+ and *S. aureus* ATCC 25923. Strain SK5 was incubated at 30°C prior to solvent extraction. 1. culture broth extract activity against *S. aureus* ATCC 25923; 2. cell mass extract activity against *S. aureus* ATCC 25923; 3. culture broth extract activity against *M. aurum* A+; 4. cell mass extract activity against *M. aurum* A+.

Concurrently, strain SK5 was also grown at room temperature (~22°C) prior to solvent extraction, as described in section 3.3.2, to determine whether any changes in activity could be attributed to changes in growth temperature. When strain SK5 was grown at room temperature (~22°C) over the entire incubation period, activity against *M. aurum* A+ and *S. aureus* ATCC 25923 was also recorded in the culture broth extract and not in the cell mass extract (Figure 19). This is the same activity profile that was observed with the crude extract from the strain SK5 culture that was incubated at a constant temperature of 30°C for the entire incubation period (Figure 18).

Figure 19: Bioautography of the cell mass and culture broth solvent extracts of strain SK5 against the test bacteria *M. aurum* A+ and *S. aureus* ATCC 25923. Strain SK5 was incubated at 22°C prior to solvent extraction 1. culture broth extract activity against *M. aurum* A+; 2. cell mass extract activity against *M. aurum* A+. 3. culture broth extract activity against *S. aureus* ATCC 25923; 4. cell mass extract activity against *S. aureus* ATCC 25923.

However, the same location of activity (cell mass vs culture broth) does not mean that the compounds being produced are the same. Thus, 1D TLC and bioautography against *M. aurum* A+ and *S. aureus* ATCC 25923 on both crude culture broth extracts (22°C culture and 30°C culture) were done to note the number of active compounds and their respective R_f values (Figure 20). These TLC data will illustrate whether the active compounds in the different crude culture broth extracts are the same (22°C culture and 30°C culture), as well as whether the activity against *M. aurum* A+ and *S. aureus* ATCC 25923 are caused by the same active compound/s. The 1D TLC profiles across all the solvent runs were almost identical, regardless of where the crude culture broth extract was from (22°C culture vs 30°C culture) or what the test bacterium was (*M. aurum* vs *S. aureus*). The R_f values in a 100% EtOAc solvent system were 0.36-0.37 for all TLC runs, and the R_f values in a 100% MeOH solvent system were 0.73-0.75 for all TLC runs (Table 11; Figure 20). These R_f values are different to those observed in Figure 17, which showed the TLC results of the culture broth extract after the SK5 culture was incubated during fluctuating temperatures. These results imply four things: i) growing the culture at a constant temperature of 22°C or 30°C does not change the number of active compounds produced against *M. aurum* A+ and *S. aureus* ATCC 25923; ii) the same active compound is produced whether the culture incubation temperature is stable at 22°C or 30°C; iii) this active compound (R_f = 0.36-0.37 in 100% EtOAc) is active against both *M. aurum* A+ and *S. aureus* ATCC 25923; and iv) the active compound produced during incubation at a fluctuating temperature is different to that of the active compound produced under a stable incubation temperature, as concluded from the differing R_f values (Table 11). It should be borne in mind that the incubation temperature range when the incubator thermostat was faulty was 25-30°C and that the two constant cultivation temperatures were 22°C and 30°C, as the facilities to incubate at a constant temperature of 25°C were not available. However, given that the same antibiotic was produced at 22°C and 30°C, it seems that it is not the incubation temperature alone that determines which antibiotic is produced, but rather the variation in the incubation temperature over time during cultivation (i.e. the temperature profile). As there is no record of the temporal variation in the incubation temperature while the incubator thermostat was faulty, it would probably be impossible to reproduce the conditions required for strain SK5 to produce the antibiotic with an R_f of 0.56 (100% MeOH).

Table 11: Summarised R_f values of the active compound/s for the cell mass and culture broth extracts from the first and second extractions against test bacteria *M. aurum* A+ and *S. aureus* ATCC 25923. The numbers in brackets indicate the temperature that strain SK5 was incubated at prior to solvent extraction as described in 3.3.2.

Extract	**Test bacterium**	**Solvent system**	**R_f value**
First extraction – Fluctuating incubation temperatures (~25°C to ~30°C)			
Cell mass extract	*M. aurum* A+	100% EtOAc	0.4 & 0.54
Cell mass extract	*M. aurum* A+	100% MeOH	0.56
Second extraction – Stable incubation temperatures			
Culture broth extract (22°C)	*M. aurum* A+	100% EtOAc	0.37
Culture broth extract (30°C)	*M. aurum* A+	100% EtOAc	0.37
Culture broth extract (22°C)	*M. aurum* A+	100% MeOH	0.72
Culture broth extract (30°C)	*M. aurum* A+	100% MeOH	0.73
Culture broth extract (30°C)	*S. aureus* ATCC 25923	100% EtOAc	0.36
Culture broth extract (30°C)	*S. aureus* ATCC 25923	100% MeOH	0.73

It is well known that changes in nutritional factors and growth conditions can lead to varying secondary metabolite production (Tormo et al., 2003). The experiment to change the growth temperature was done to try and determine whether the lower temperature observed during the first few rounds of antibiotic extraction (when the incubator was not maintaining a stable temperature) was responsible for the change in location and identity of the active compounds produced. The R_f values show that the same active compound is made regardless of what the stable growth temperature is. The only difference between the extracts in section 3.4.2.2 and 3.4.2.3 is that they were extracted from cultures that were grown under fluctuating temperature conditions or stable temperature conditions, respectively. Thus, it is possible that the fluctuating temperatures led to a different stress response, which triggered the production of a different antibiotic compound than when the temperature was stable.

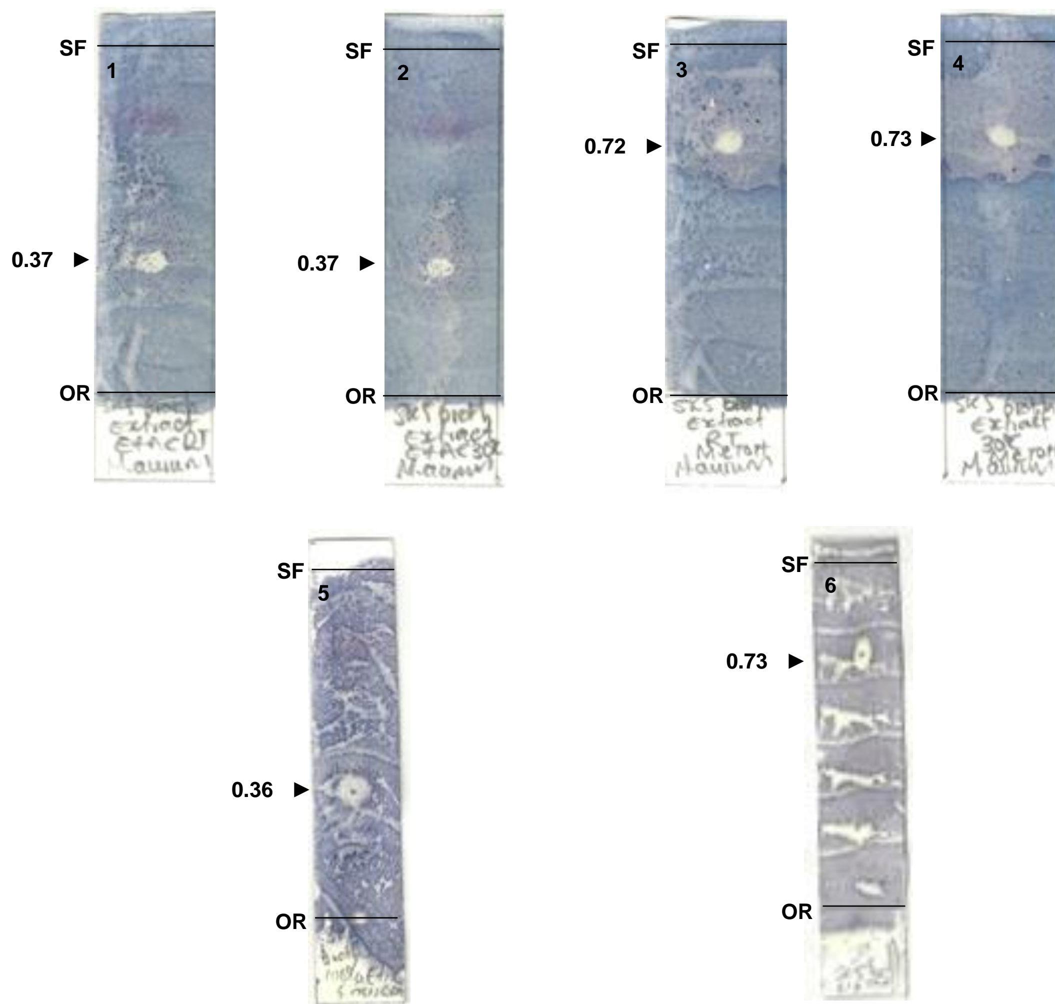

Figure 20: Thin layer chromatography, with different solvent systems, on the culture broth extract of strain SK5, which was subjected to bioautography against the test bacteria *M. aurum* A+ (1 to 4) and *S. aureus* ATCC 25923 (5 & 6). Strain SK5 was subjected to different growth temperatures (22°C or 30°C) prior to extraction which are indicated in the following: 1. 100% EtOAc as the mobile phase – incubation temperature was 22°C; 2. 100% EtOAc as the mobile phase – incubation temperature was 30°C; 3. 100% MeOH as the mobile phase – incubation temperature was 22°C; 4. 100% MeOH as the mobile phase – incubation temperature was 30°C; 5. 100% EtOAc as the mobile phase – incubation temperature was 30°C; 6. 100% MeOH as the mobile phase – incubation temperature was 30°C.
Arrows mark the active spot with the R_f value displayed at the side.
OR is the origin. SF is the solvent front.

Two-dimensional TLC and bioautography with the test bacteria *M. aurum* A+ and *S. aureus* ATCC 25923 were done to determine whether the single active spots observed in the 1D TLC are caused by a single compound or multiple compounds with very similar chromatographic behaviour (Figure 21A, 21B). Furthermore, a CAS stain was performed after a 2D TLC run to determine how many organic compounds are present in the crude extract, thus assessing the extract's purity (Figure 21C). The CAS stain showed that there was no brown spot on the TLC plate corresponding to the antibacterial active spot. This suggests that the active molecule could not be oxidised by CAS. The CAS stain showed the presence of multiple brown spots with different R_f values (Figure 21C). To further purify the active compound, silica column chromatography can be carried out using the solvent systems already established. Preparative HPLC could also be used.

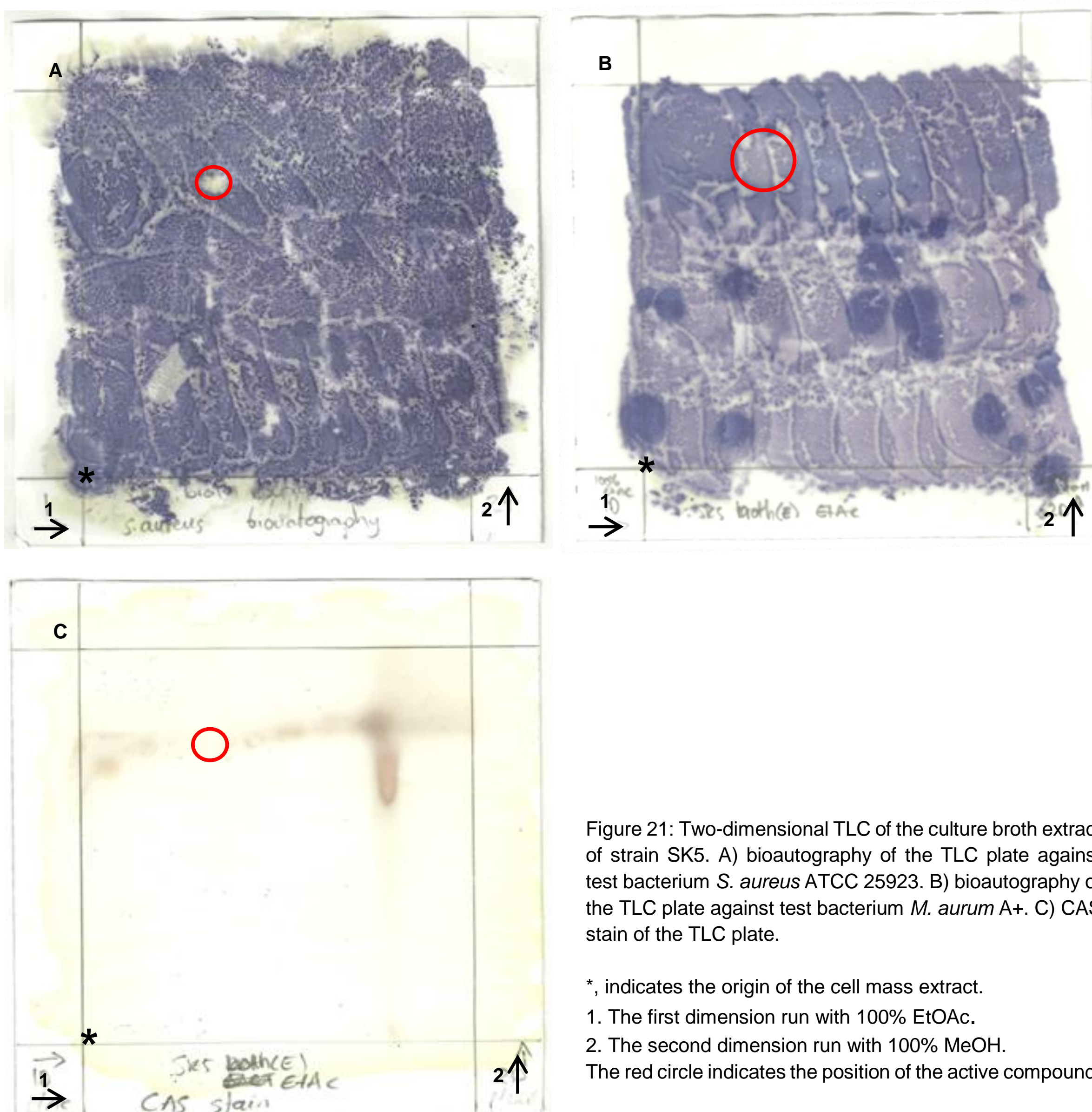

Figure 21: Two-dimensional TLC of the culture broth extract of strain SK5. A) bioautography of the TLC plate against test bacterium *S. aureus* ATCC 25923. B) bioautography of the TLC plate against test bacterium *M. aurum* A+. C) CAS stain of the TLC plate.

*, indicates the origin of the cell mass extract.

1. The first dimension run with 100% EtOAc.
2. The second dimension run with 100% MeOH.

The red circle indicates the position of the active compound.

3.4.2.4 Comparing strain SK5 crude extract to nocardamine

The antibiotic/s produced by *Kribbella* strain SK5 were also analysed by Mr Kojo Acquah (PhD student, Department of Chemistry, University of Cape Town). The antibacterial molecules in the culture broth extract of strain SK5 were purified by HPLC. The structures of the compounds were elucidated using both spectrometric (High Resolution Liquid Chromatography Mass Spectrometry (HR-LCMS)) and spectroscopic (1D and 2D Nuclear Magnetic Resonance (NMR)) Spectroscopy. Several siderophores, including nocardamine and desferrioxamines were purified (personal communication, Kojo Acquah). Nocardamine was of particular interest, because it has been shown to have antimycobacterial activity (Kalinovskaya et al., 2011). Thus, to identify whether the active compound in the crude extract of strain SK5 prepared by the author was nocardamine, side-by-side spot bioautography analysis was done (Figure 22). As reported before, the culture broth extract has activity against both *M. aurum* A+ and *S. aureus* ATCC 25923. Nocardamine, on the other hand, only exhibits activity against *M. aurum* A+ (Figure 22). This implies that the active compound in the culture broth extract is not nocardamine. However, it may be that *S. aureus* ATCC 25923 is less susceptible to nocardamine than *M. aurum* A+ is and thus would require a greater amount of nocardamine for inhibitory activity to be seen. Side-by-side 1D TLC of nocardamine and the strain SK5 crude antibiotic extract, followed by bioatography against *M. aurum* A+, will show whether the R_f value of nocardamine is the same as the R_f value of the active compound in the crude culture broth extract.

If it is shown that the active compound from the culture broth is ***not*** nocardamine, then another round of scaled-up purification will need to be done to start purifying the active compound. Structural analysis will follow to identify whether the compound is novel.

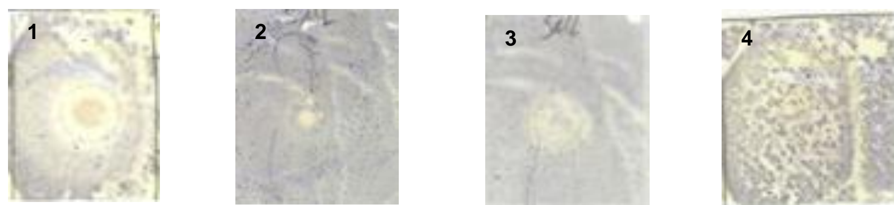

Figure 22: Bioautography of the culture broth extract against 1. *M. aurum* A+ and 2. *S. aureus* ATCC 25923. Spot bioautography of purified nocardamine from the culture broth extract against 3. *M. aurum* A+ and 4. *S. aureus* ATCC 25923.

3.4.3 Testing for antiplasmodial activity

Malaria is a severe disease, caused by the *Plasmodium* parasite, which was responsible for 445 000 deaths worldwide in 2016 (http://www.who.int/malaria/en/). Despite major efforts to reduce the incidence of malaria since the 1990's, the impact of malaria is still severe, particularly amongst children. The WHO recommends artemisinin based combination therapies (ACTs) as the first line of treatment for malaria, because of their efficacy. However, as with *M. tuberculosis*, resistance to the current front-line drugs leads to the dire need

for novel compounds that may be partnered with or replace the current ACTs. It is for this reason that a UCT PhD student in the Department of Clinical Pharmacology, Faculty of Health Sciences (Mr Daniel Watson), is looking at actinobacteria as a source of natural products that can be used to treat malaria.

Three *Streptomyces* strains isolated in this study (PR3, SK5, and UK1) were analysed for antimalarial activity by Daniel Watson (as described in section 3.3.5) against the chloroquine sensitive strain *P. falciparum* NF54. The IC_{50} (ng/mL) was determined for the crude cell mass and culture broth extracts. The IC_{50} is the concentration needed to kill half of the parasite population. It was found that strain PR3 had the strongest antimalarial activity in the cell mass extract (IC_{50} = 39 ± 5 ng/mL) with slightly weaker activity (but still strong) in the culture broth extract (Table 12). Strain UK1 only showed antimalarial activity in the cell mass extract, while strain SK5 exhibited no antimalarial activity in either extract (Table11). The activities of the positive controls used (chloroquine and artesunate) were within the accepted range of activity against *P. falciparum* NF54 (Appendix B).

Table 12: Antiplasmodial activity of select actinobacterial crude extracts against *Plasmodium falciparum* NF54.
n/a: No activity.

***P. falciparum* NF54 IC_{50} (ng/mL)**	**Strain SK5**	**Strain PR3**	**Strain UK1**
Cell Mass (Ethyl Acetate)	n/a	39 ± 5	298 ± 4
Culture Broth (Ethyl Acetate)	n/a	56 ± 5	n/a

The promising activities demonstrated by strains PR3 and UK1 are being investigated by Mr Watson in his PhD studies. The low IC_{50} values are indicative of good hits that have the potential to be drug candidates. Pink et al. (2005) stated that one of the criteria for antiplasmodial drug discovery is that the crude extract should have *in vitro* activity against whole protozoa of ≤ 1µg/mL (≤ 1000ng/mL). Upscaling of growth to extract more material and then purification of the active compound/s is necessary so that concentrated fractions can be tested. The structure/s of these strong active compound/s can then be elucidated. If these compound/s prove to be novel (or are molecules with known structures that were not known to be active against *Plasmodium* species), they can be pursued further as potential drug leads.

3.4.4 Characterisation of *Kribbella* strain SK5

3.4.4.1 Phylogenetic characterisation

3.4.4.1.1 16S rRNA gene phylogeny

The closest relatives of strain SK5 based on an EzBioCloud *blastn* analysis of the 16S rRNA gene sequences were *Kribbella karoonensis* Q41^T (99.72%), '*Kribbella soli*' FMN22^T (99.59%), *Kribbella shirazensis* UTMC693^T (99.57%), *Kribbella swartbergensis* HMC25^T (99.36%), *Kribbella aluminosa* HKI0478^T (99.29%), *Kribbella hippodromi* S1.4^T (98.99%), *Kribbella ginsengisoli* Gsoil001^T (98.82%), *Kribbella jejuensis* HD9^T (98.76%), *Kribbella solani* DSA1^T (98.75%), and *Kribbella pittospori* PIP158^T

(98.74%). '*K. sindirgiensis*' FSN23^T (Ozdemir-Kocak et al., 2017a; JN896614; 98.61%) and '*K. podocarpi*' YPL1^T (Curtis et al., 2017; KM382222; 99.59%), which are in the same clade as strain SK5 (Figure 23), did not appear in the *blastn* search. Interestingly, '*K. sindirgiensis*' has a particularly low 16S rRNA gene sequence similarity to strain SK5 (98.61%).

The phylogenetic position of strain SK5 within the genus *Kribbella* was determined via the construction of a maximum likelihood tree using the 16S rRNA gene sequences (Figure 23). The closest relatives based on the 16S rRNA gene phylogeny were *K. shirazensis*, '*K. soli*', *K karoonensis*, and '*K podocarpi*' with low bootstrap support (58%). *K. jejuensis*, *K. aluminosa*, and '*K. sindirgiensis*' were on the periphery of the strain SK5 clade, however with low bootstrap support (67%). This low bootstrap support implies that the clustering may change when additional strains are added to the tree. The asterisk at the node uniting these strains indicates that the overall topology of the clade is conserved, but the positions of the species within the clade vary between the neighbour-joining and maximum likelihood trees. High sequence similarity is observed in the 16S rRNA gene sequences amongst closely related species. Thus, other genes should also be assessed which have a higher rate of change over time.

3.4.4.1.2 MLSA

To obtain a more robust phylogenetic comparison of strain SK5 to the known members of the *Kribbella* genus, a phylogenetic tree based on the concatenated sequences of five housekeeping genes (*gyrB*, *rpoB*, *recA, relA,* and *atpD*) was constructed (Figure 24).

Strain SK5 grouped with '*K. sindirgiensis*' and '*K. soli*' with very strong bootstrap support (100%) (Figure 24). The genetic distances between the concatenated sequences of the different *Kribbella* type strains were calculated by Dr Sarah Curtis, as described in Curtis & Meyers (2012). Curtis & Meyers (2012) proposed that a genetic distance threshold be used as a species delineation tool. Kirby et al. (2010) first noted that a genetic distance of 0.04 was sufficient to distinguish between species when using the *gyrB* gene. Using the five-concatenated genes, it was noted that all *Kribbella* type strains could also be distinguished by a genetic distance of 0.04 (Curtis & Meyers, 2012). A genetic distance greater than 0.04 implies a DNA relatedness of less than 70% by DDH. The genetic distances revealed that the type strains of '*K. sindergiensis*' and '*K. soli*' are very closely related to strain SK5, with values of 0.016 and 0.024, respectively (Table 13). As these genetic distance values are below 0.04, DDH will be required to determine whether strain SK5 belongs to a different genomic species to '*K. sindergiensis*' and '*K. soli*'. The low genetic distance value of the housekeeping genes between strain SK5 and '*K. sindirgiensis*' is interesting, because the 16S rRNA gene sequence similarity between the two (98.61%) suggested that DDH is not required to distinguish strain SK5 from '*K. sindirgiensis*' because the sequence similarity is less than 98.7% (Meier-Kolthoff et al. 2013). However, due to the low genetic distance value calculated (0.016), DDH will need to be done to confirm that strain SK5 belongs to a unique genomic species. However, because strain SK5 has a concatenated

gyrB-rpoB-recA-relA-atpD sequence that is not identical to any of the type strains of known *Kribbella* species, one can deduce (with reasonable confidence) that strain SK5 represents a novel species in the genus *Kribbella*, because Curtis & Meyers (2012) reported that the *gyrB*, *rpoB*, *recA*, *relA* and *atpD* genes share 100% sequence similarity between the type strain and non-type strains of the same species.

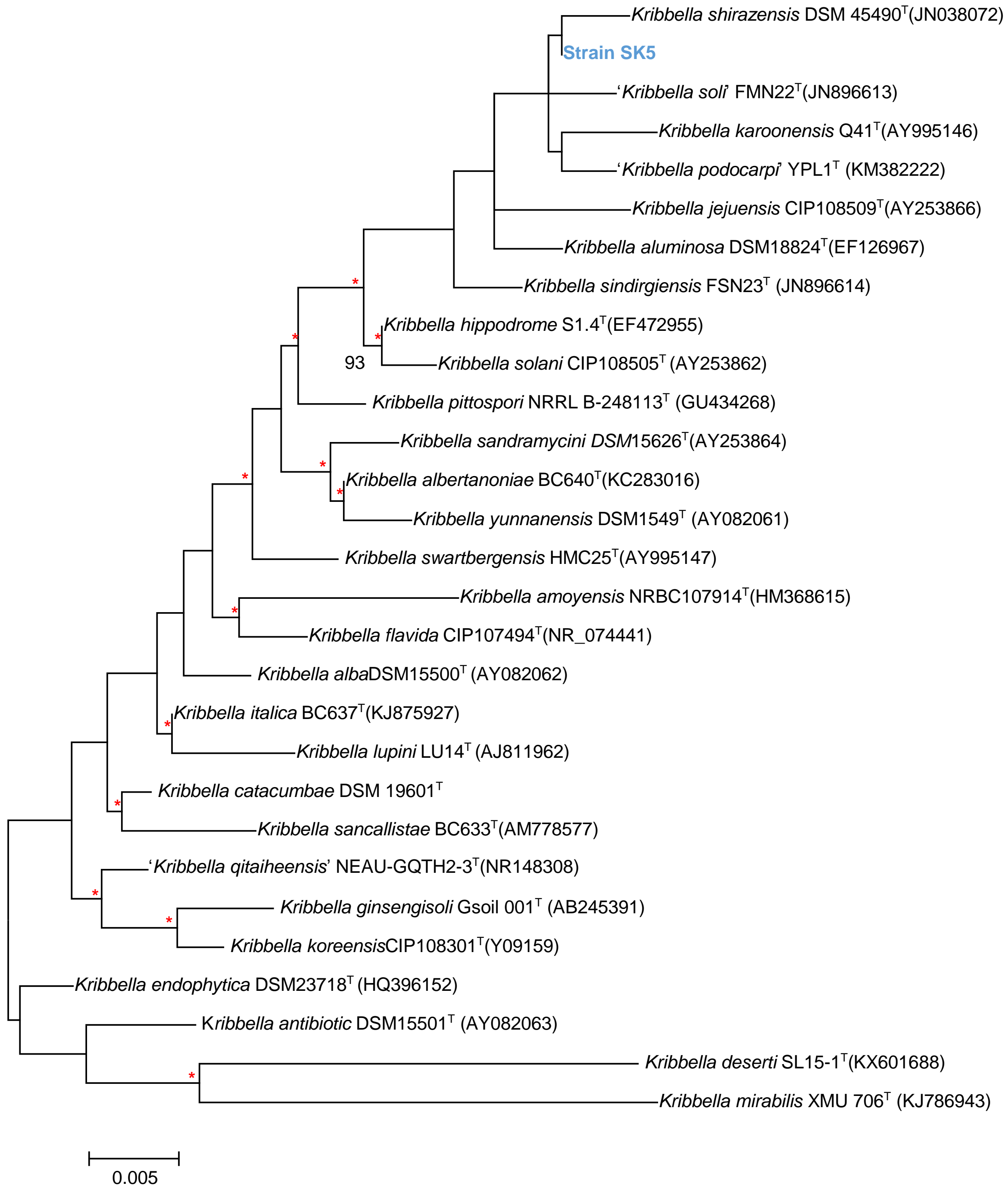

Figure 23: Maximum likelihood tree of the *Kribbella* genus using partial 16SrRNA gene sequences of 1329bp. Bootstrap values were calculated from 1000 resampled datasets. Bootstrap values above 70% are shown. Asterisks indicate topological conservation between the neighbour-joining and the maximum likelihood trees. The scale bar indicates five nucleotide changes in 1000 base pairs.

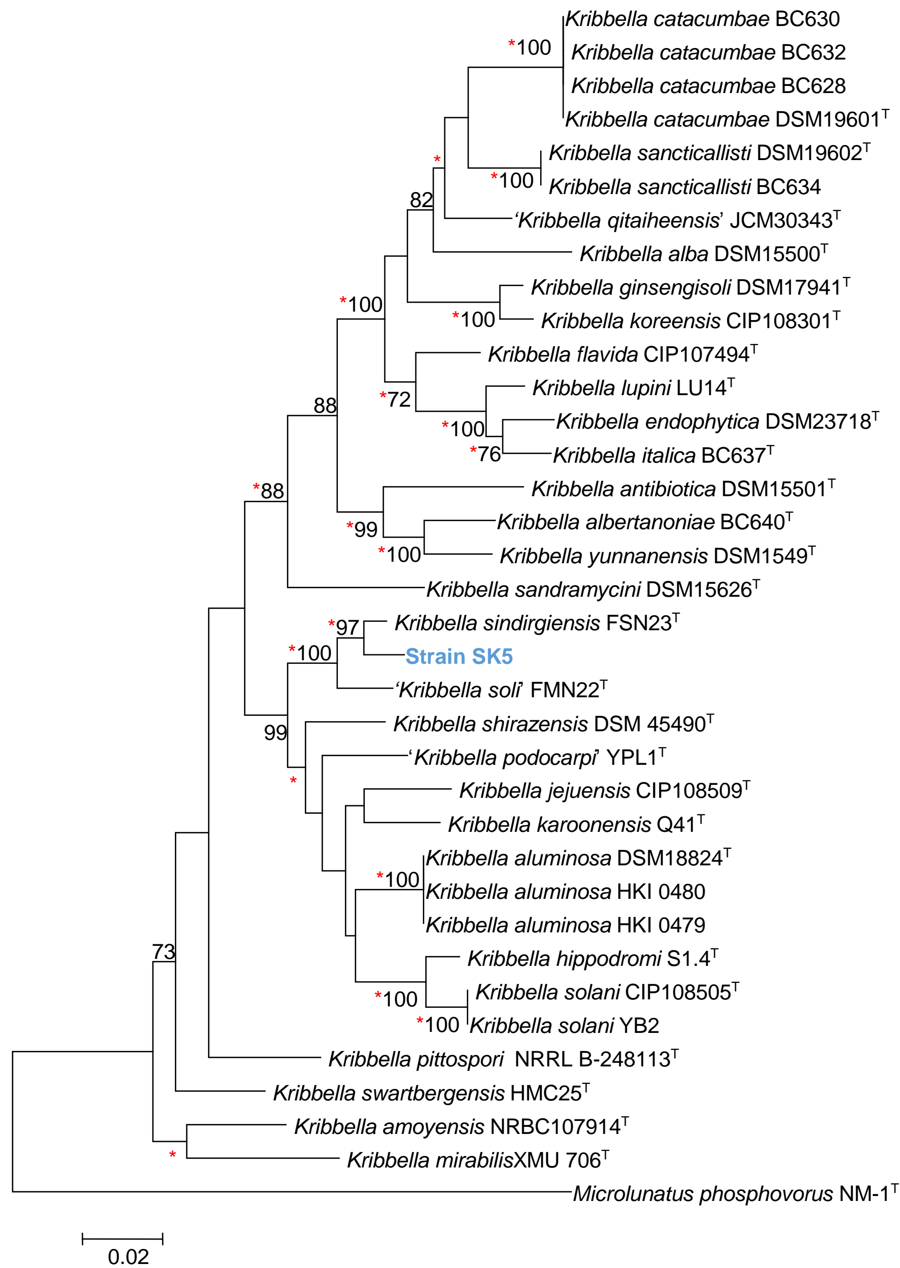

Figure 24: Phylogeny of the *Kribbella* genus using a five-gene concatenated sequence (*gyrB, rpoB, recA, relA*, and *atpD*) based on the maximum likelihood method. The total concatenated sequence length was 4099bp. Bootstrap values were calculated from 1000 resampled datasets. Bootstrap values above 70% are shown. Asterisks indicate topological conservation between the neighbour-joining and maximum likelihood methods. *Microlunatus phosphovorus* NM-1^{T} was used to root the tree. The scale bar indicates 20 nucleotide changes in 1000 base pairs. Tree was constructed by Dr. Sarah Curtis.

Table 13: Genetic distances using the *gyrB-rpoB-recA-relA-atpD* concatenated sequence of 4099bp. Values in red indicate genetic distances below the threshold (0.04). N/A – not applicable

	Strain	**Strain SK5**	**'*K. sindirgiensis*'**
***Kribbella* strain SK5**	strain SK5^T	N/A	**0.016**
Kribbella alba	DSM15500^T	0.087	0.088
Kribbella albertanoniae	BC640^T	0.075	0.074
Kribbella aluminosa	DSM18824^T	0.050	0.052
Kribbella aluminosa	HKI0480	0.050	0.052
Kribbella aluminosa	HKI-479	0.050	0.052
Kribbella amoyensis	NRBC107914^T	0.069	0.066
Kribbella antibiotica	DSM15501^T	0.078	0.082
Kribbella catacumbae	DSM19601^T	0.083	0.083
Kribbella catacumbae	BC628	0.083	0.083
Kribbella catacumbae	BC630	0.083	0.083
Kribbella catacumbae	BC632	0.083	0.083
Kribbella endophytica	DSM23718^T	0.078	0.079
Kribbella flavida	CIP107494^T	0.071	0.072
Kribbella ginsengisoli	DSM17941^T	0.076	0.078
Kribbella hippodromi	S1.4^T	0.053	0.053
Kribbella italica	BC637^T	0.075	0.076
Kribbella jejuensis	CIP108509^T	0.053	0.055
Kribbella karoonensis	Q41^T	0.053	0.054
Kribbella koreensis	CIP108301^T	0.076	0.077
Kribbella lupini	LU14^T	0.074	0.075
Kribbella mirabilis	XMU706^T	0.079	0.077
Kribbella pittospori	NRRL B-248113^T	0.056	0.056
'*Kribbella podocarpi*'	YPL1^T	0.045	0.046
'*Kribbella qitaiheensis*'	JCM30343^T	0.074	0.074
Kribbella sancticallisti	DSM19602^T	0.079	0.076
Kribbella sancticallisti	BC634	0.079	0.076
Kribbella sandramycini	DSM15626^T	0.068	0.066
Kribbella shirazensis	DSM45490^T	0.045	0.044
'*Kribbella sindirgiensis*'	FSN23^T	**0.016**	N/A
Kribbella solani	CIP108508^T	0.055	0.054
Kribbella solani	YB2	0.055	0.054
'*Kribbella soli*'	FMN22^T	**0.030**	**0.024**
Kribbella swartbergensis	HMC25^T	0.057	0.057
Kribbella yunnanensis	DSM15499^T	0.071	0.073
Microlunatus phosphovorus	NM-1^T	0.207	0.206

3.4.4.2 Phenotypic characterisation

Strain SK5 was phenotypically compared to its closest phylogenetic relatives identified in section 3.4.4.1.

K. karoonensis was selected because it was the top hit based on an EzBioCloud *blastn* analysis with 99.72% 16S rRNA gene sequence similarity and it grouped with strain SK5 in the 16S rRNA and concatenated gene trees (Figures 23 and 24) with a genetic distance of 0.053 in the concatenated gene sequence (Table 13). *K. aluminosa*, *K. pittospori*, and *K. shirazensis* were chosen because their phylogenetic positions in Figures 23 and 24 implied that they were closely related species with low sequence variation (denoted by shorter branch lengths). As well as being a close relative in the phylogenetic analyses in Figures 23 and 24, '*K. soli*' has a low genetic distance value of 0.030 when compared to strain SK5 (Table 13). '*K. sindirgiensis*' could not be included because it was not available.

3.4.4.2.1 Colony morphology and pigmentation

Morphologically, strain SK5 and the *Kribbella* species in Table 8 all formed colonies with a wrinkled appearance. In broth culture, strain SK5 and all the comparative *Kribbella* species in Table 8 formed vegetative hyphae that fragmented to form irregular rod-shaped cells.

Members of the *Kribbella* genus tend to have colony colours of off-white, beige, white or pale yellow. However, some *Kribbella* colonies are pale orange or yellow-orange (Evtushenko & Krausova, 2012). Strain SK5, *K. aluminosa*, *K. karoonensis*, *K. shirazensis*, and '*K. soli*' have similar colony colours, namely, beige/off-white colonies with a white spore mass on ISP2, ISP3 and ISP4. *K. pittospori* had the same colony colour on these media, but did not sporulate. No pigmentation was produced by any of the strains on ISP5, ISP6, or ISP7.

3.4.4.2.2 Physiological characterisation

Table 14 shows the phenotypic differences between strain SK5 and its closest relatives.

K. aluminosa, *K. karoonensis*, *K. pittospori*, *K. shirazensis*, '*K. soli*' and '*K. podocarpi*' displayed 2, 5, 13, 6, 3 and 3 phenotypic differences to strain SK5, respectively (Table 14).

Table 14: Phenotypic characteristics of strain SK5 and its closest phylogenetic relatives. 1. strain SK5; 2. *K. aluminosa* DSM18824^T; 3. *K. karoonensis* Q41^T; 4. *K. pittospori* NRRL B-248113^T; 5. *K. shirazensis* DSM45490^T; 6. '*K. soli*' KCTC 29219^T.

++, strong positive; +, positive; +(w), weak positive; -, negative; ^{D}, results differ from the original description of the species. The numbers in brackets indicate the number of days the cultures were incubated.

Test	*1*	*2*	*3*	*4*	*5*	*6*
Utilisation as sole carbon source (21)						
inulin	++	++	-	-	-	-^{D}
sodium acetate	+	+	+	-	-	-
sodium citrate	-	-	+(w)	-	-	-
Utilisation as sole nitrogen source (15)						
DL-α-amino-n-butyric acid	++	++	++	-	+	++
L-cysteine	-	+	+(w)	-	-	-
L-histidine	++	++	++	-	-	++
L-methionine	+	+	+	-	+	+
L-phenylalanine	+	+	+	-	+	++
Growth temperatures & pH (14)						
40°C	-	-	++	-	++	-
pH4.3	++	++^{D}	++	++	-	++
Degradation						
Xanthine (21)	+	+	+	-	+	+^{D}
Adenine (21)	++	++	++	-	++	++
L-Tyrosine (21)	+	++	++	++	++^{D}	++
Urea (14)	++	++	++	-	++	+(w)
Allantoin (14)	+	-	++	-	++	+(w)
Growth in presence of inhibitory compounds (%w/v) (14)						
3%NaCl	++	+	++	-	++	++
4%NaCl	+(w)	+^{D}	+(w)	-	+	-
5%NaCl	-	-	+^{D}	-	-	-
0.3% Phenylethanol	-	-	-	-	+	-
Nitrate Reduction (14)	+	+(w)	+	-	+	+

3.4.4.3 Chemotaxonomic characterisation

The isomer of diaminopimelic acid was determined to be LL-DAP, which is characteristic of the *Kribbella* genus (Figure 25). The polar lipid profile of strain SK5 contains phosphatidylcholine (PC), which is the characteristic polar lipid of the *Kribbella* genus based on the emended genus description by Everest et al. (2013) (Figure 26A). In addition to PC, strain SK5 has the following polar lipid profile: diphosphatidylglycerol (DPG), phosphatidylinositol (PI), four unidentified phospholipids, and eight unidentified amino lipids (Figures 26A and 26B). There were no glycolipids (Figure 26C). The whole-cell sugar analysis revealed the presence of ribose, glucose, and an unidentified pentose sugar (Figure 26D). There are no diagnostic

sugars in the whole-cell sugar pattern for members of the *Kribbella* genus (Everest et al., 2013). The fatty acid profile of strain SK5 is displayed in Table 15. The major fatty acids were anteiso-$C_{15:0}$ and iso-$C_{16:0}$ at 37.55% and 13.72%, respectively. Iso-$C_{15:0}$ was present at 8.65%. As expected for the *Kribbella* genus, anteiso-$C_{15:0}$ is the predominant fatty acid in strain SK5. Analysis of the respiratory quinones for strain SK5 identified MK-9(H_4) as the major component (>90%), while MK-9(H_2) and MK-9(H_6) were minor components.

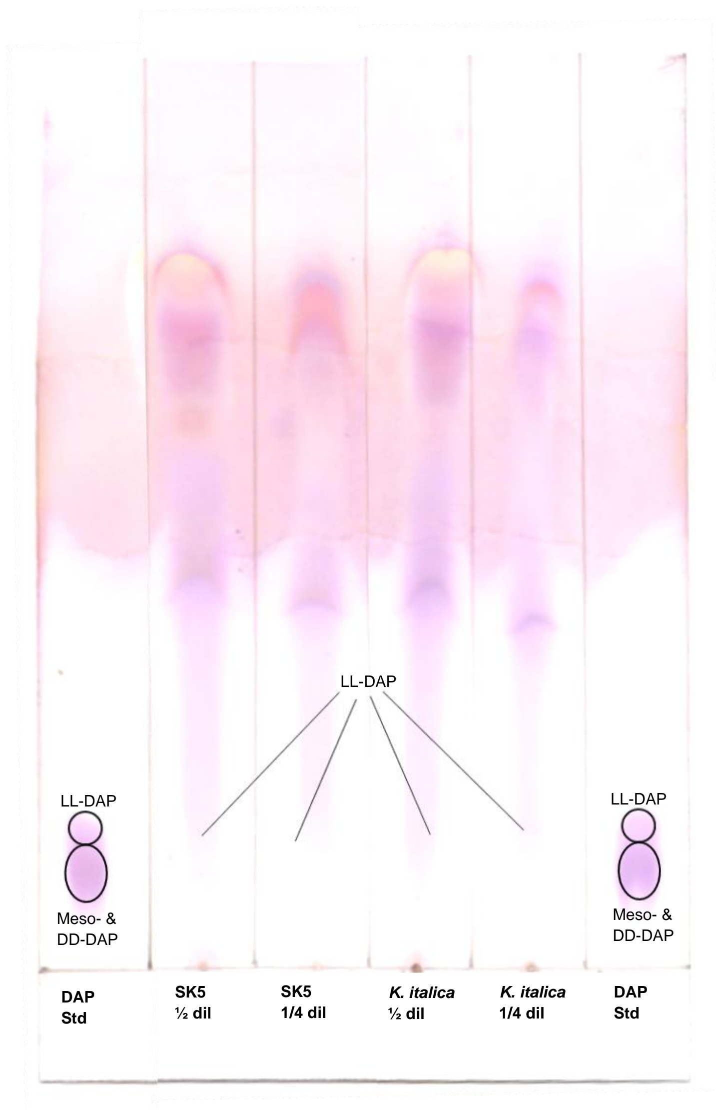

Figure 25: Thin layer chromatography profile to identify the isomer of diaminopimelic acid in the peptidoglycan layer of strain SK5^T. DAP: diaminopimelic acid. Std: Standard. dil: dilution

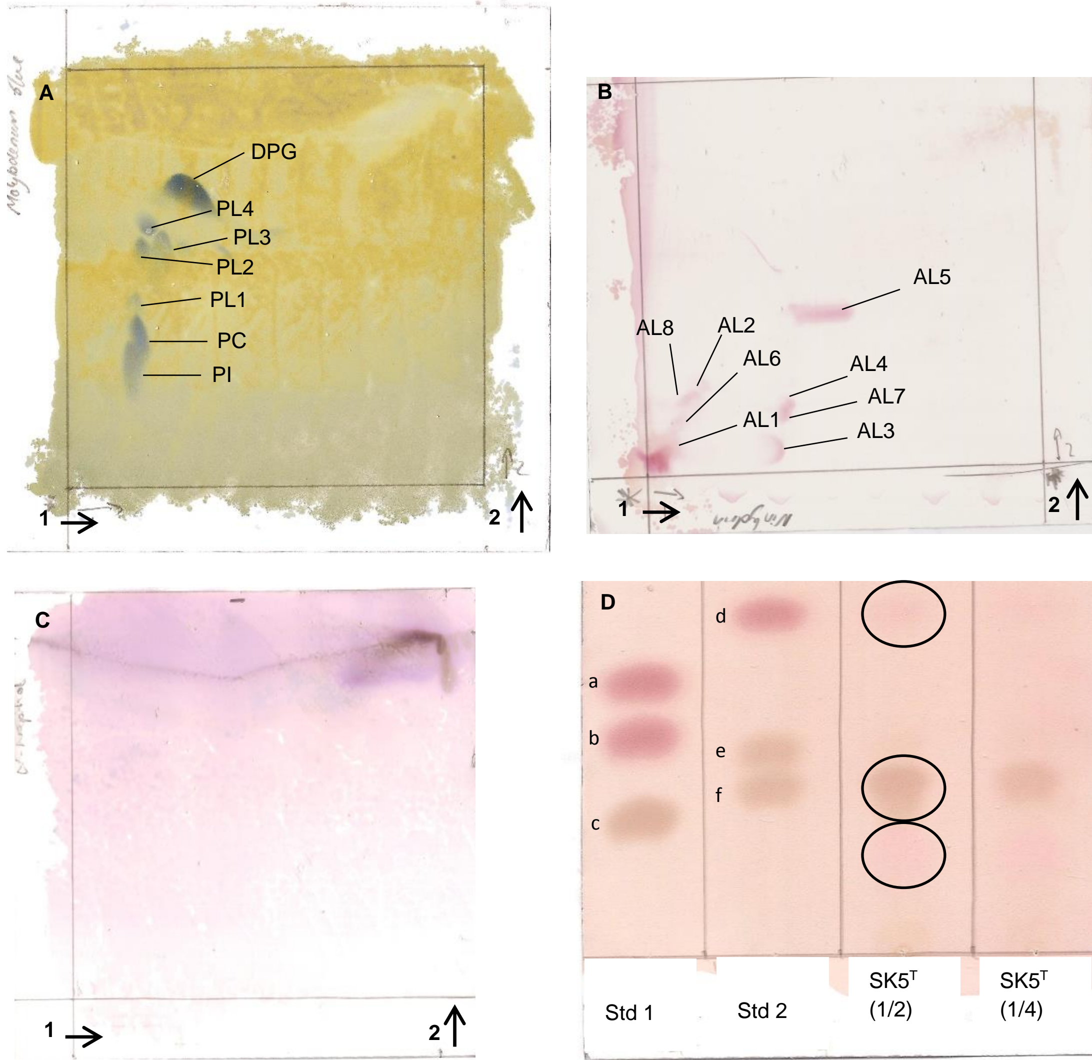

Figure 26: Identification of the polar lipid profile using two-dimensional TLC (A, B, & C) and the whole-cell sugar pattern using one-dimensional TLC (D) for strain SK5. Polar lipids were detected with (A) molybdenum blue, (B) ninhydrin, and (C) *p*-anisaldehyde. Whole-cell sugar pattern (D).

(a) xylose; (b) arabinose; (c) galactose; (d) ribose; (e) mannose; (f) glucose.

Std: Standard.

Table 15: Cellular fatty acid composition (%) of strain SK5. The cells were grown on ISP2 agar for 5 days at 28°C, under aerobic conditions. Fatty acids in red are the major components of the fatty acid profile for strain SK5.

Fatty Acid	Strain SK5^T (%)
$C_{12:0}$	0.14
iso-$C_{13:0}$	0.32
anteiso-$C_{13:0}$	0.26
$C_{14:0}$	0.31
iso-$C_{14:0}$	5.30
anteiso-$C_{14:0}$	1.53
iso- $C_{15:1}$G	0.47
anteiso-$C_{15:1}$A	1.34
iso- $C_{15:0}$	8.65
anteiso- $C_{15:0}$	**37.55**
$C_{15:0\ \omega6c}$	1.07
$C_{15:0}$	-
iso- $C_{16:1}$H	1.28
iso- $C_{16:0}$	**13.72**
Unknown 15.669	0.50
anteiso-$C_{16:0}$	0.80
Sum in feature 3*	4.31
$C_{16:0}$	1.44
iso-$C_{17:1\ \omega9c}$	5.16
Sum in feature 4#	0.33
iso-$C_{17:0}$	3.88
anteiso-$C_{17:0}$	4.34
$C_{17:1\ \omega8c}$	1.33
$C_{17:1\ \omega6c}$	3.65
$C_{17:0}$	1.10
$C_{17:0}$ 10 methyl	0.62
iso-$C_{18:0}$	0.39
$C_{18:1\ \omega9c}$	0.19

*Sum in feature 3 contains $C_{16:1\ \omega7c}$ and/or iso-$C_{15:0}$ 2-OH
Sum in feature 4 contains iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B

The polyphasic taxonomic characterisation of strain SK5 suggests that it represents a novel species in the genus *Kribbella*. Although there are only 2 phenotypic differences between the type strain of *K. aluminosa* and strain SK5, the concatenated-gene genetic distance between strain SK5 and the type strain of *K. aluminosa* is 0.05 (Table 13). A genetic distance greater than 0.04 indicates that DDH is not necessary to prove that the type strain of *K. aluminosa* and strain SK5 belong to different genomic species (Curtis & Meyers, 2012). The recently described species, '*K. sindirgiensis*' (Ozdemir‐Kocak et al., 2017a), has low 16S rRNA gene sequence similarity to strain SK5 (98.61%). However, '*K. sindirgiensis*' is the closest phylogenetic relative based on the concatenated gene phylogenetic tree (Figure 24) and the concatenated-genetic distance between the type strain of '*K. sindirgiensis* 'and strain SK5 is only 0.016 (Table 13). Therefore, the type strain of '*K. sindirgiensis*' will need to be added to the comparative table of phenotypic characteristics, when it becomes available. The concatenated-gene genetic distance between the type strain of '*K. soli*' and strain SK5 is 0.030, which is also below the 0.04 threshold for distinguishing between species in the genus *Kribbella*. Thus, DDH will have to be carried out between the type strain of '*K. sindergiensis*' and strain SK5 and between the type strain of '*K. soli*' and strain SK5 to prove that strain SK5 is a novel species. Although low, the fact that the concatenated-gene genetic distances are above

zero suggests that strain SK5 does belong to a new genomic species, as the type strain and non-type strains of the same species have been shown to have identical gene sequences for the *atpD*, *gyrB*, *recA*, *relA* and *rpoB* genes (Kirby et al, 2010; Curtis & Meyers, 2012).

3.4.4.4 Description of *Kribbella stellenboschensis* sp. nov.

Kribbella stellenboschensis (stell.en.bosch'en.sis. N.L. fem. adj. belonging to Stellenbosch, South Africa, from where the type strain was isolated).

*Kribbella stellenboschensis*is a Gram-stain positive actinobacterium with wrinkled/folded, off-white/beige colonies. Hyphae fragment into rod-shaped elements during growth in liquid culture. Oxidase and catalase positive. Does not produce any diffusible pigments. Grows at 22°C, 30°C and 37°C and at pH 4.3 - 9, but not at 40°C. Grows in the presence of 0.1% phenylethanol, 3% (w/v) NaCl (weakly at 4% (w/v) NaCl), but not in the presence of 5% (w/v) NaCl, 0.1% phenol, and 0.3% phenylethanol. Can utilise adonitol, α (+)-L-arabinose, (+)-D-cellibiose, (-)-D-fructose, (+)-D-glucose, *myo*-inositol, inulin, α-lactose, (+)-D-mannose, (-)-D mannitol, melibiose, raffinose, (+)-L-rhamnose, sucrose, (+)-D-xylose, salicin, and sodium acetate as the sole carbon source, but not sodium citrate. Can utilise DL-α-amino-n-butyric acid, L-arginine, L-asparagine, L-histidine, 4-hydroxy-L-proline, L-methionine, potassium nitrate, L-serine, L-threonine, L-valine and L-phenylalanine as sole nitrogen sources, but not L-cysteine. Can degrade xanthine, adenine, L-tyrosine, hypoxanthine, gelatin, urea, allantoin, Tween 80, arbutin and DNA, but not guanine. Cannot hydrolyse pectin. Can produce H_2S and reduces nitrate to nitrite. The cell wall peptidoglycan contains LL-diaminopimelic acid. The major menaquinone is MK-9(H_4), while the minor components are MK-9(H_2) and MK-9(H_6). The major fatty acids are anteiso-$C_{15:0}$ and iso-$C_{16:0}$. The polar lipid profile is comprised of PC, DPG, PI, unidentified phospholipids and unidentified amino lipids.

The type strain, $SK5^T$, was isolated from a topsoil sample collected in the town of Stellenbosch, Western Cape Province, South Africa.

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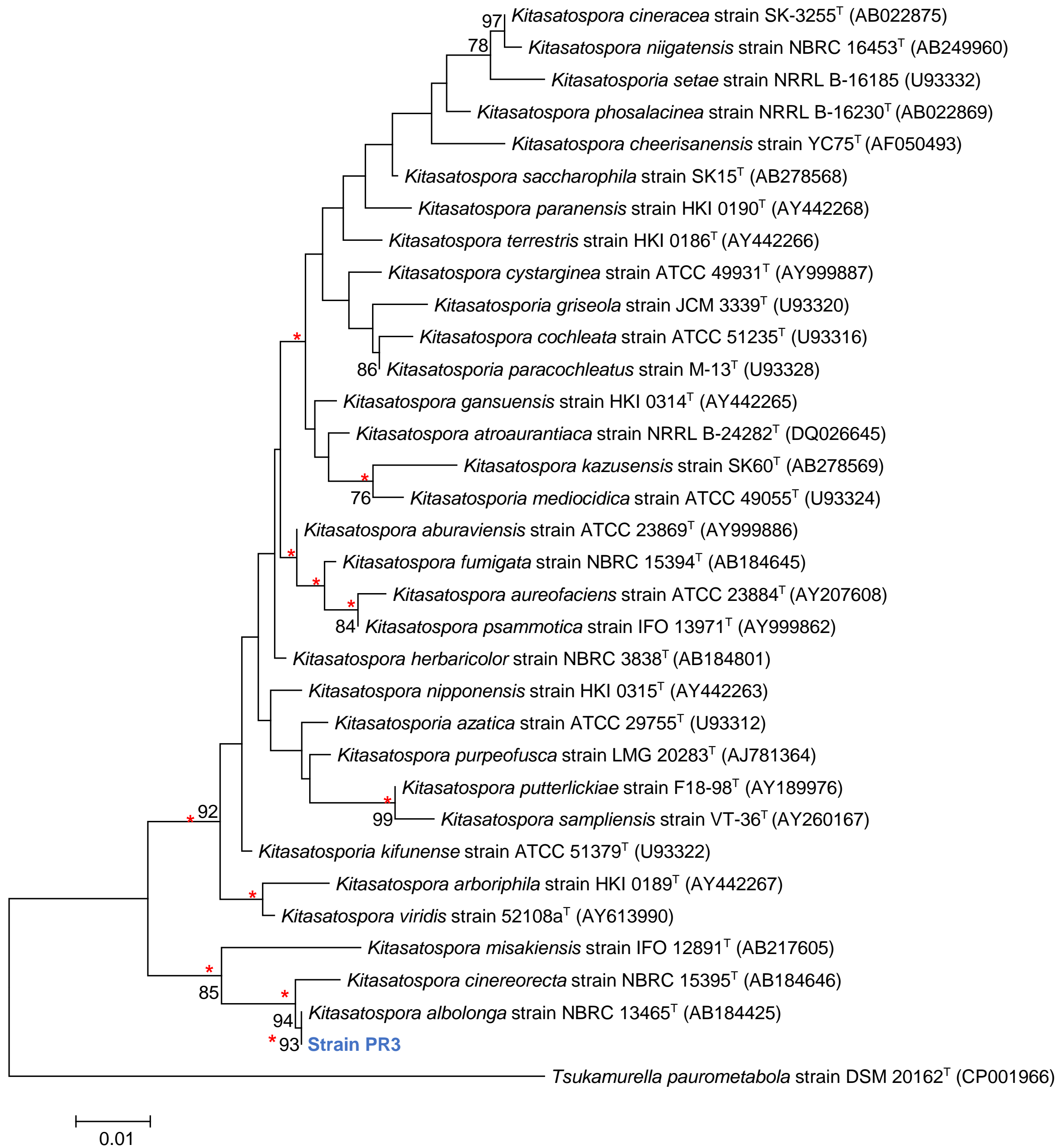
4. Appendices



Appendix A: Maximum likelihood tree of the *Kitasatospora* genus using partial 16S rRNA gene sequences of 1347bp rooted with *Tsukamurella paurometabola* DSM 20162^T (CP001966). Bootstrap values were calculated from 1000 resampled datasets. Bootstrap values above 70% are shown. Asterisks indicate topological conservation between the neighbour-joining tree and the maximum likelihood tree. The scale bar indicates 10 nucleotide changes in 1000 base pairs.

Appendix B: *In vitro* antiplasmodial efficacy of experimental positive controls, chloroquine and artesunate.

Control	IC_{50} (ng/mL)
Chloroquine	4.1 ± 1.2
Artesunate	2.8 ± 1.3

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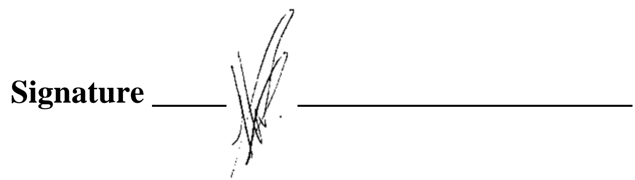

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