Analysis of Actinobacterial Biodiversity in Reservoir Sediment and Cave Soil and Screening of Isolates for Antimycobacterial Activity

by

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LIST OF ABBREVIATIONS

A	–	adenine (DNA base)
ACTs	–	artemisinin-based combination therapies
ANI	–	average nucleotide identity
BLAST	–	Basic Local Alignment Search Tool
BMSAB	–	Bergey's Manual of Systematics of Archaea and Bacteria
bp	–	base pair (DNA)
C	–	cytosine (DNA base)
CB	–	culture broth
CM	–	cell mass
cm	–	centimeter
CS	–	cave soil
CW	–	cave wall
CZ	–	Czapek solution (agar/broth)
dDDH	–	digital DNA-DNA hybridisation
DDH	–	DNA-DNA hybridisation
DNA	–	deoxyribonucleic acid
dNTP	–	deoxyribonucleotide triphosphate
DSMZ	–	Deutsche Sammlung von Mikroorganismen und Zellkulturen (agar/broth)
et al.	–	*et alia* (meaning "and others")
EtBr	–	ethidium bromide
EtOAc	–	ethyl acetate
F	–	forward
g	–	gram
G	–	guanine (DNA base)
gDNA	–	genomic deoxyribonucleic acid
GGDC	–	genome to genome distance calculator
*gyr*B	–	DNA gyrase subunit B gene
HIV	–	human immunodeficiency virus
i.e.	–	*id est* (meaning "it is" or "that is")

IC_{50}	–	half maximal inhibitory concentration
IPTG	–	isopropyl β-D-1-thiogalactopyranoside
ISP	–	international *Streptomyces* project
KS	–	kribbella selective (medium)
L	–	litres
LB	–	luria bertani (agar/broth)
LPSN	–	list of prokaryotic names withstanding in nomenclature
M	–	molar
MB	–	Middlebrook 7H9 (agar/broth)
MDR-TB	–	multidrug-resistant TB
MeOH	–	methanol
mg	–	milligram
min	–	minute
mL	–	milliliter
mM	–	millimolar
mm	–	millimetre
mm^2	–	square millimetre
MTT	–	thiazolyl blue tetrazolium bromide
NBT	–	nitro blue tetrazolium
nm	–	nanometer
nt	–	nucleotide (DNA)
OD	–	optical density
PCR	–	polymerase chain reaction
pH	–	potential Hydrogen
pLDH	–	parasite lactate dehydrogenase
R	–	reverse
*rec*A	–	recombinase A gene
RNA	–	ribonucleic acid
rpm	–	revolutions per minute
rpoB	–	RNA polymerase beta subunit
rRNA	–	ribosomal RNA
RS	–	reservoir sediment
s	–	seconds

sp. nov.	–	– *species nova* (meaning “new species”)
ssDNA	–	single stranded DNA
subsp.	–	subspecies
T	–	thymine (DNA base)
TAE	–	tris-acetate-EDTA
TB	–	tuberculosis
TDR	–	totally drug-resistant TB
TE	–	Tris-EDTA
TLC	–	thin layer chromatography
U	–	units
UV	–	ultra-violet
v/v	–	volume for volume
w/v	–	weight for volume
WHO	–	world health organisation
XDR-TB	–	extensively drug-resistant TB
X-gal	–	X-galactosidase
ZOI	–	zone of inhibition
&	–	and
λ	–	phage Lambda
µL	–	microlitre
Mg	–	microgram
µM	–	micromolar
°C	–	degrees Celcius

ABSTRACT

A total of 56 presumptive actinobacterial strains was isolated from three different samples taken from the Silvermine Nature Reserve (Table Mountain National Park, Cape Town), namely, cave soil, the wall of the cave and sediment from the shallow waters of a reservoir. Twenty nine (29) isolates were successfully identified to the genus level by 16S-rRNA gene analysis: one *Micrococcus* strain, one *Streptacidiphilus* strain, one *Micromonospora* strain and 26 *Streptomyces* strains. The phylogenetic position of each identified strain within its genus was investigated by generating a phylogenetic tree based on its 16S-rRNA gene sequence. Further analysis of the *Streptacidiphilus* strain was conducted based on the *gyrB* gene. Metagenomic analysis was used to further analyse the actinobacterial diversity of the freshwater reservoir sediment from the Silvermine Nature Reserve. A total of 97 16S-rRNA gene clones was obtained from the reservoir sediment sample, RS1, using actinobacterium-specific 16S-rRNA gene primers S-C-Act-0235-a-S-20-F and S-C-Act-0878-a-A-19-R and each clone was identified using the EzBioCloud database. Analysis based on unique phylotypes in the clone library revealed that 80% of the clone library was composed of actinobacterial strains belonging to the orders *Acidimicrobiales, Streptomycetales, Streptosporangiales, Corynebacteriales, Sporichthyales* and the family *Jatrophihabitandaceae* (the remaining 20% was identified as non-actinobacterial strains). The percentage composition of the actinobacterial clonal diversity for each order was as follows: *Acidimicrobiales,* 56%; *Streptomycetales,* 29%; *Streptosporangiales,* 9%; *Corynebacteriales,* 4%; *Sporichthyales,* 1% and family *Jatrophihabitandaceae,* 1%. Rarefaction analysis revealed that the total actinobacterial diversity of the sample was not represented in the clone library. Therefore, further sampling and analysis of the sample site would uncover greater actinobacterial diversity. Thirty seven (37) putative actinobacterial isolates of the 56 that were isolated from the Silvermine Nature Reserve were screened for antimycobacterial activity against the non-pathogenic *Mycobacterium aurum* strain A+ using a standard over-lay method. A total of five identified actinobacterial isolates (*Streptomyces* strains RS6, RS7, RS9, RS13 and RS15) and an unidentified actinobacterium, strain RS4, demonstrated very strong antimycobacterial activity (zone of growth inhibition of over 3000 mm^2). In addition, 15 of the 37 strains were active against *Staphylococcus aureus* ATCC 25923 and three were active against *Escherichia coli* ATCC 25922. *Streptomyces* strains CS1, CS3, CS12, CS18, CS19, CW5, RS3, RS6, RS9,

RS13 and RS15, displaying varying strengths of antimycobacterial antimicrobial activity, were selected for antibiotic extraction from culture broths. The resulting crude extracts were subjected to spot bioautography to test for antibacterial activity. The organic compounds extracted from the cell mass of *Streptomyces* strain CS3 and the broth fraction of *Streptomyces* strain RS3 demonstrated strong activity against *M. aurum* strain A+. Furthermore, the crude extracts of 15 actinobacterial isolates (*Micromonospora* strain RS10 and *Streptomyces* strains CS1, CS3, CS12, CS18, CW2, CW5, RS3, RS6, RS7, RS9, RS13, RS15, RS18 and RS19) were additionally tested for antiplasmodial activity against *Plasmodium falciparum* strain NF54. Seven of these strains showed activity against *Plasmodium* namely, *Streptomyces* strains CW2, CW5, RS3, RS7, RS13, RS15 and RS19. *Streptomyces* strains CW2, CW5 and RS7 displayed the strongest activity against *P. falciparum* strain NF54 with IC_{50} values below the guideline threshold of 1000 ng/mL (strain CW2 culture broth crude extract: IC_{50} 40 ng/mL, strain CW5 culture broth crude extract: IC_{50} 128 ng/mL and strain RS7 culture broth crude extract: IC_{50} 70 ng/mL).

CHAPTER 1

INTRODUCTION

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INTRODUCTION

1.1. ACTINOBACTERIA

The actinobacteria are a group of Gram-positive bacteria that forms one of the largest phyla within the domain bacteria (Ludwig *et al.*, 2012). Actinobacteria have a high guanine and cytosine (G+C) content in their DNA, are unicellular and can be coccoid, rod-shaped or filamentous. The tip extension and branching of the hyphae of filamentous actinobacteria allows them to grow, giving rise to their name. The name actinobacteria arose from the Greek words *aktis* or *aktin*, meaning ray, and *mukes*, meaning fungi, i.e. "ray fungi". Originally, actinobacteria were considered to be a transitional form between bacteria and fungi. Filamentous actinobacteria, often referred to as actinomycetes, produce hyphae, the tips of which may differentiate into spores, similar to fungi. However, the comparison between actinobacteria and fungi is weak. Like all bacteria, actinobacteria have a peptidoglycan cell wall and contain a prokaryotic nucleoid with organised chromosomal DNA (Anandan *et al.*, 2016). Current actinobacterial genomes that have been sequenced are relevant to human studies in medicine, biotechnology and ecology (Ventura *et al.*, 2007). What makes actinobacteria so significant is that they are known to be good producers of various secondary metabolites and bioactive compounds. These natural products include antibiotics and anticancer and antifungal compounds that are valuable in the pharmaceutical industry. Since there is a never-ending need for new drugs derived from natural products due to the development of microbial resistance to existing drugs, one of the most straightforward ways to tackle this problem is to look for novel antibiotics produced by actinobacteria. Actinobacteria can be found in many environmental habitats and are most commonly known to inhabit soil, fresh water, marine habitats and plants (Pernthaler, 2013; Bhatti *et al.*, 2017; Betancur *et al.*, 2017). In these habitats, actinobacteria play a vital role in the carbon cycle and the turnover of organic matter by degrading organic materials, such as chitin, cellulose, polysaccharides, protein, fats and organic acids (Bhatti *et al.*, 2017). This, in turn, replenishes the nutrients in these habitats.

1.1.2. Actinobacterial habitats

The population density of actinobacteria depends on their habitat, the availability of nutrients and climate conditions. Factors such as temperature, pH and moisture all influence the growth of actinobacteria living in particular environments. Actinobacteria have adapted to live in a wide range of terrestrial and aquatic habitats, ranging from mountain soil and plants to the deepest ocean vents and freshwater lakes (Anandan *et al.,* 2016). In the past, the isolation of actinobacteria had been found to be more successful from soils than any other environmental source, particularly alkaline soils and soils rich in organic matter (Barka *et al.,* 2016; Hopwood, 2007). This is where actinobacteria was discovered to form an integral part of the microbial population from the work of early researchers such as Selman Waksman (1931). Researchers are now able to explore and identify actinobacteria from various environmental sources more successfully due to the advancement of science and technology.

1.1.2.1. Terrestrial environments

One of the most significant habitats for actinobacteria is soil. Actinobacteria can be found on the soil surface, as well as deep below ground (Goodfellow & Williams, 1983). Many *Streptomyces* species have been isolated from this habitat and streptomycetes are considered to be the largest component of the culturable actinobacterial population of soil. However, this is only because *Streptomyces* species grow readily on standard laboratory media, making them appear to dominate the soil population (Williams & Vickers, 1988). It was later discovered, through the application of metagenomic analyses, that *Streptomyces* does not necessarily dominate soil habitats, as many non-*Streptomyces* species were detected (Anandan *et al.,* 2016). Actinobacteria living in terrestrial habitats have the potential of producing interesting novel antibiotics with high activity (Oskay *et al.,* 2005). In a mangrove rhizosphere, actinobacterial species of the genera *Streptomyces, Nocardia* and *Micromonospora* were discovered in large numbers (Tan *et al.,* 2009). Extreme environments, such as desert soil, are habitats where only certain species of actinobacteria are able to grow and thrive (Nithya *et al.,* 2015). In the case of actinobacteria residing in desert soil, species are adapted to relatively high temperatures, salt concentrations and radiation (Neilson *et al.*, 2012).

1.1.2.2. Aquatic environments

Historically, it was considered that the primary habitat of actinobacteria was soil, however, molecular-based studies of aquatic habitats have long since changed that perception (Goodfellow & Williams, 1983; Denisova *et al.*, 1999; Zwart *et al.*, 2002). In fact, similar to terrestrial habitats, actinobacteria are also widely spread in aquatic environments, such as rivers, lakes (freshwater) and oceans (marine) (often washed in from the surrounding land) (Crump & Hobbie, 2005; Allgaier & Grossart, 2006; Dhakal *et al*., 2017). It has been proven that actinomycete spores that are washed in from the surrounding land can germinate and grow in aquatic systems, provided that suitable substrates are present and growth conditions are optimal, instead of the microbes specifically adapting themselves to survive in the aquatic environment (Hug *et al.*, 2018). Actinobacteria can contribute more than 50% of the microbial population in the surface waters of freshwater lakes and are considered to be the dominant phylum in lakes across the globe (Glöckner *et al*, 2000; Newton *et al*, 2007; De Wever *et al*, 2008; Humbert *et al*, 2009). Molecular analysis of the 16S-rRNA genes extracted from freshwater lakes found rare actinobacteria that were distinct from actinobacteria residing in soil and marine systems (Rappe´ *et al*., 1999). This suggested that freshwater systems such as lakes can be good sources of novel actinobacteria.

1.1.2.3. Exploring understudied habitats

Actinobacteria residing in the aforementioned habitats, and many other environmental habitats, hold great diversity. However, due to the over-exploitation of actinobacterial species belonging to the genus *Streptomyces* by the pharmaceutical industry and a number of research groups, the search for novel bioactive compounds has been steered towards non-*Streptomyces* species (also referred to as rare actinobacteria) that are found in the lesser studied environments (Figure 1.1). The lesser studied environments include "untapped" aquatic and extreme habitats (Lam, 2006; Mahajan & Balachandran, 2017). Cave habitats also fall into this category, as they contain various mineral formations and many unknown microbes that have evolved due to changes in temperature, humidity and air composition over long periods of time (Culver & Sket, 2000). There is little known about actinobacterial diversity in cave environments, even though many caves have been identified throughout the world (Northup *et al.*, 2001). A previous study by Maciejewska *et al.* (2015) reported the isolation of a novel species, *Streptomyces lunaelactis* sp. nov., from moonmilk speleothem in a cave in Belgium. Another report showed the isolation

of 34 novel species of actinobacteria from subterranean environments (Jurado *et al.*, 2005). These novel actinobacteria were represented in the descriptions of *Agromyces subbeticus* sp. nov. and *Beutenbergia cavernae* gen. nov., sp. nov., which were isolated from caves in Spain and China (Groth *et al.*, 1999a; Groth *et al.*, 1999b). *B. cavernae* is a new genus of actinobacteria containing L-lysine in its cell-wall peptidoglycan. By studying these actinobacterial taxa and their diversity, researchers can discover novel and chemically diverse antimicrobial compounds that could be used in the pharmaceutical industry.

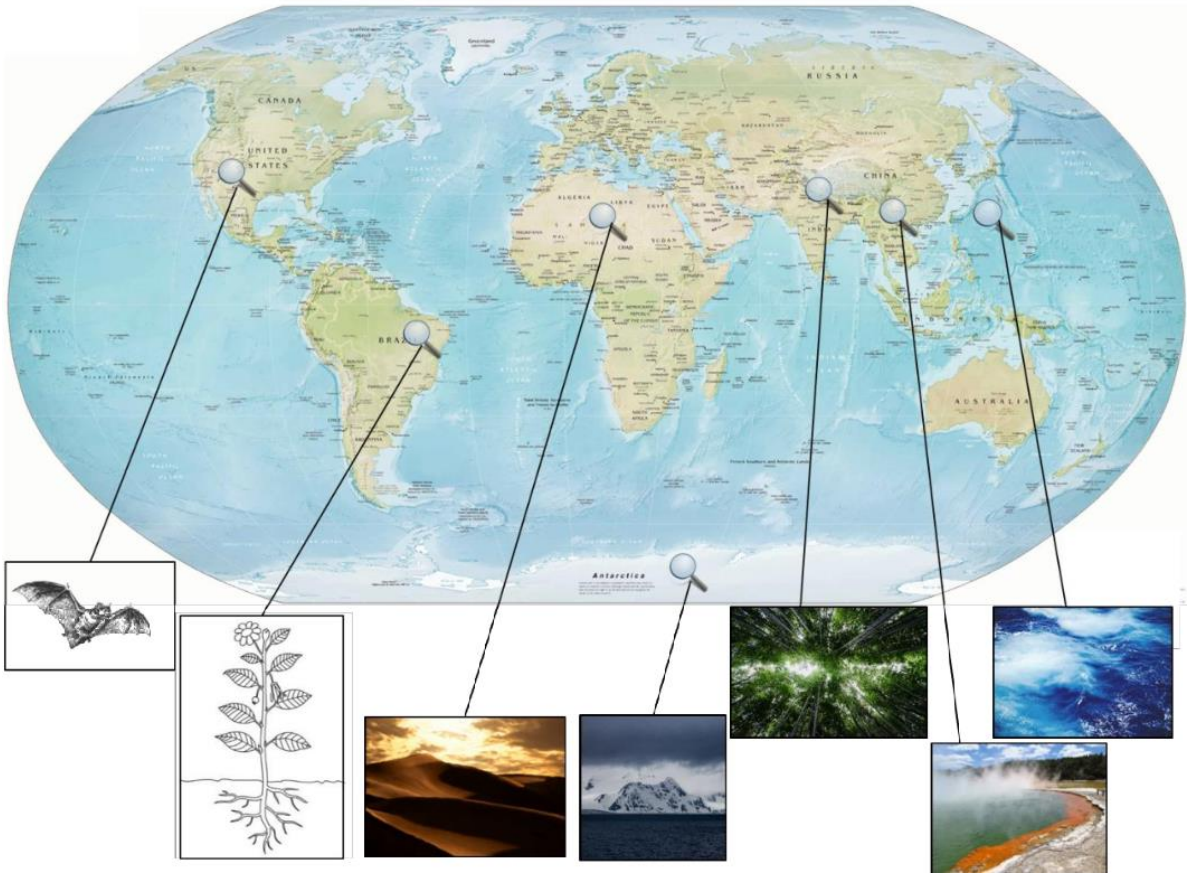

Figure 1.1. Understudied habitats of actinobacteria around the globe have become the focal point for the discovery of new natural products. Oceans, rivers, deserts, mountains, hot springs and plants are only a few examples of habitats that have not been widely exploited in the search for novel bioactive compounds produced by actinobacteria (Hug *et al*., 2018).

1.2. ACTINOBACTERIAL TAXONOMY

Genera belonging to the phylum *Actinobacteria* display rich diversity in terms of their morphology, physiology and metabolism (Ludwig *et al.,* 2012). Taxonomy is an important organising principle in microbiology and is based on classification, nomenclature and identification (Prescott *et al.,* 2002). *Classification* is the arrangement of microbes in specific groups, known as taxa, based on matching characteristics, *nomenclature* is the assignment of names to the taxonomic groups according to published rules and *identification* determines whether an unknown isolate belongs to a validly published taxon (Kämpfer & Glaeser, 2012; Prescott *et al.*, 2002). The full hierarchal taxonomic ranks from highest to lowest are as follows: domain, phylum, class, order, family, genus and species (Prescott *et al.*, 2002). The taxonomy of actinobacteria is essential for the accurate description of their diversity, as it was suggested by Staley (2010) that taxonomy helps to comprehend microbial diversity. Phylogenetic trees based on gene sequences provide a natural structure for describing a taxonomy, taking evolutionary relationships and varying rates of evolution into consideration (Godfray, 2002). The taxonomy of actinobacteria has evolved significantly over the years due to the advancement of scientific methods. The modern practice of taxonomy is referred to as a polyphasic approach. Polyphasic methods utilize various features of a microbe, including phenotypic and chemotaxonomic characteristics together with genotypic and phylogenetic information (Chun & Rainey, 2014; Coenye *et al.,* 2005). Chemotaxonomy, also known as chemosystematics, classifies and identifies organisms according to similarities and differences in their biochemical compositions. Examples of the chemical markers that are analysed within an actinobacterial cell include the diagnostic diamino acid and the whole-cell sugars in the cell wall peptidoglycan and polar lipids, fatty acids and respiratory isoprenoid quinones in the cell membrane. In contrast to chemotaxonomy, molecular classification classifies organisms according to similarities and differences in their genetic material.

1.2.1. Molecular classification

Various studies have been performed using rRNA gene sequences to determine the lineage of actinobacteria and to classify strains as novel taxa (Embley and Stackebrandt, 1994; Koch *et al.*, 1994; Stackebrandt *et al.*, 1995). The use of rRNA gene sequences was pioneered by Woese and Fox (Prescott *et al.,* 2008). A study by Stackebrandt *et al.* (1997) made an important contribution to the molecular-taxonomic characterisation of actinobacteria by revising the

existing taxonomy, at the time, to propose *Actinobacteria* classis nova. The revised classification scheme was based on a phylogenetic analysis of the 16S-rRNA gene. Due to the wide range of DNA sequence data available today, the analysis of the sequences of 16S-rRNA and other genes has become the focus for the identification of novel bacterial taxa (Tindall *et al.,* 2010).

1.2.1.1. 16S-rRNA gene classification

The 16S-rRNA gene formed the basis of the hierarchical taxonomic system of Stackebrandt *et al.* (1997). The class *Actinobacteria* was delineated based on its branching position in 16S-rRNA gene phylogenetic trees and its taxonomy is primarily guided by 16S-rRNA gene sequence relationships (Kämpfer & Glaeser, 2012). The sequencing of the 16S-rRNA gene is generally the first step taken to identify bacteria to the genus level and to identify novel species (Staley, 2006). Novel species are characterised according to various phenotypic differences and genotypic threshold values. These genotypic threshold values can be determined by 16S-rRNA gene sequence similarity and DNA-DNA hybridization (DDH) values (Staley, 2006).

Even though 16S-rRNA gene sequencing forms the backbone of taxonomic analyses, the gene does not distinguish well between closely related species and genera, as it has a slow evolutionary rate and is thus too highly conserved (Staley, 2009). Phylogenetic analyses based solely on 16S-rRNA gene relationships have various limitations that include low phylogenetic resolution at the highest and lowest ranks (Janda & Abbott, 2007), primer mismatches that cause "lost" diversity (Schulz *et al.,* 2017), as well as chimeric sequences produced by PCR ultimately corrupting the topology of trees (DeSantis *et al.,* 2006). This ultimately results in ambiguity. An example of this ambiguity is the taxonomic status of *Kitasatospora* (Omura *et al.,* 1982) within the family *Streptomycetaceae* (Wellington *et al.,* 1992; Zhang *et al.,* 1997). Wellington *et al.* transferred the genus *Kitasatospora* to the genus *Streptomyces* based on a hybridization experiment that used *Streptomyces*-specific 18 bp oligonucleotide probes of 16S-rRNA genes in 1992. This suggested that the genus *Kitasatospora* had a molecular profile that was very similar to that of the genus *Streptomyces.* However, the taxonomic position of *Kitasatospora* and its relatedness to the genus *Streptomyces* has been resolved by re-establishing *Kitasatospora* as a separate genus within the family *Streptomycetaceae* based on phylogenetic analyses using 16S-rRNA genes and 16S-23S rRNA gene spacers and improved molecular analyses (Zhang *et al.,* 1997; Zhi *et al.,* 2009; Girard *et al.,* 2014; Ichikawa *et al.,*

2010). It is now concluded that *Streptomyces* and *Kitasatospora* are distinct genera based on phenotypic and genetic differences. Furthermore, a study by Kim *et al.* displayed how the taxonomic structure of the genera *Streptomyces* and *Kitasatospora* can be analysed using RNA polymerase *β*-subunit gene (*rpoB*) analysis (2004). This demonstrates the importance of additional genetic markers, such as recombinase A (*recA*), gyrase subunit B (*gyrB*), *rpoB* and *ssgB*, and their use for more robust analyses of closely related genera (Girard *et al.*, 2013).

A 16S-rRNA gene sequence similarity of 98.8% (Meier-Kolthoff *et al*., 2013a) has been shown to coincide with the DDH threshold of 70% for differentiating between genomic species of actinobacteria (Wayne *et al.*, 1987). To determine whether two strains belong to distinct genomic species, their DNA relatedness has to be less than 70% (< 70%) by DDH. DDH is therefore required to differentiate between two actinobacterial strains having a pairwise 16S-rRNA gene sequence similarity of more than 98.8% if there is a strong suspicion from other data, such as phenotypic characteristics, that an isolate may be novel. Presently, an actinobacterium with a 16S-rRNA gene sequence similarity of more than 98.8% to its closest relative cannot be claimed as belonging to a novel species without DDH analysis (or analyses based on whole-genome sequences) proving that it belongs to a unique genomic species.

1.2.1.2. DNA-DNA hybridization & average nucleotide identity

DNA-DNA hybridization analysis is used to measure species relatedness when the genomes of different strains are hybridized (Chun & Rainey, 2014). When the DNAs of two genomes are denatured and the single stranded DNA molecules are allowed to hybridize, the amount of base pairing between complementary regions of the single stranded DNA (ssDNA) molecules from the two strains can be measured (Ludwig, 2007). One method for doing this involves binding the ssDNA from one strain to the radioactively labelled ssDNA from another strain and measuring the bound DNA (Prescott *et al.*, 2002). This allows for the amount of newly bound radioactive DNA to be measured to determine the similarity between the DNA sequences (the more radioactive DNA bound, the more closely related are the two strains).

Even though DDH is an effective method for delineating species, it has limitations. These limitations include complex techniques that are time consuming and laborious, sometimes resulting in different outcomes when different methods are used (Richter & Róssello-Móra, 2009) and DDH can have a high error rate (Goris *et al*., 2007). Furthermore, as a new DDH experiment has to be run each time a new bacterial strain is analysed, it is not possible to

establish a cumulative database of DDH data for strains that can be used in future analyses. This has caused researchers to look for alternative methods to DDH for assessing the relatedness between strains. Complete genome sequencing has become popular in the research field, as the cost of whole genome sequencing has become cheaper and a comparison between two genome sequences produces more taxonomic information than any other method. Genome sequencing has also provided more insight into genome evolution (Kirby, 2011). Whole genome sequencing is used to identify genes specific to certain microbes at the genus and family levels. This standardizes taxonomy because it offers more accurate analyses than traditional morphological and biochemical analyses (Konstantinidis & Tiedje, 2007). Average Nucleotide Identity (ANI) is a relatedness index derived from pairwise genome comparisons where a value of 95% between two genome sequences has been shown to correspond to the 70% DDH threshold for distinguishing between genomic species (Arahal, 2014). Furthermore, digital DNA-DNA hybridization (dDDH) has become a popular approach for assessing species relatedness by using a genome to genome distance calculator (GGDC). GGDC displays a higher correlation with wet-lab DDH results compared to other *in silico* methods, without displaying the limitations of wet-lab DDH. GGDC is based on statistical models instead of linear models used by other methods such as ANI, ultimately creating an easier comparison between species as it operates on an identical scale to wet-lab DDH values (Meier-Kolthoff *et al.*, 2013b). dDDH uses the same 70% threshold for defining genomic species as traditional DDH.

Although all of these advances in molecular biology are revolutionizing the field of prokaryotic taxonomy, bacterial taxonomy is restricted to an extent due to the inability to obtain most species in pure culture, in other words they are unculturable under standard laboratory conditions. Metagenomics is an example of a culture-independent approach to overcome the limitation of not being able to cultivate most bacteria on laboratory media.

1.3. METAGENOMICS

Based on analyses using the 16S-rRNA gene, it is estimated that only 1% of microorganisms found in natural habitats are culturable using standard laboratory techniques (Vartoukian *et al.*, 2010). The 99% of microbes that have not been cultivated hold an extensive diversity of enzymes, antibiotics and other bioactive compounds (Nikolaki & Tsiamis, 2013). This could

serve as a prolific source of novel antibiotic compounds, which could be uncovered and assessed using metagenomics and could be developed by the pharmaceutical industry.

Metagenomics is a technique used to study the total genetic pool of large environmental microbial communities in their natural state regardless of whether the members of the community are culturable or not. This is a culture-independent approach that overcomes the barriers of culture-dependent methods and is used to discover and identify rare and novel microbes, including actinobacteria (Lin *et al.,* 2015; Assis *et al.,* 2014). The limitations of culture-dependent methods include that there is a risk of contamination, it is time and resource consuming and the growth of targeted bacteria is dependent on the type of media selected, as not all bacteria require the same nutrients (Figdor & Gulabivala, 2011). Metagenomics provides an extensive, unbiased view of bacterial functions and their diversity within different environments (Brady *et al.,* 2009). DNA is recovered directly from the environmental source (eDNA), PCR amplified, cloned using an appropriate vector and inserted into a culturable bacterium (Daniel, 2004). This allows one to screen the clone library in order to gain access to the hidden genetic sequences of bacteria and their bioactive compounds (Brady *et al.,* 2009). The identification of microbes, such as actinobacteria, can be achieved by using a sequence based approach where specially designed, actinobacteria-specific PCR primers are used to target genes of interest, such as the 16S-rRNA gene (Daniel, 2004). The use of metagenomic approaches together with traditional culture-based methods is increasingly playing a large role in gaining in-depth information about the microbial diversity in different environments (Techtmann & Hazen, 2016).

1.4. BIOACTIVE COMPOUNDS

More than 1 million natural compounds have been isolated around the globe, of which 5% originated from microbes (Demain & Sanchez, 2009). Nature remains the richest and most versatile source for new antibiotics. Actinobacteria are recognized as the major source of natural compounds that play crucial roles in many industries being, for example, excellent producers of a variety of important enzymes that are produced on an industrial scale. The pharmaceutical industry is one example where there is a continuous need for drug development, including antibiotics, such as those seen in Figure 1.2 (Liu *et al.,* 2010). Actinobacteria produce enzyme inhibitors beneficial in the treatment of cancer and immunomodifiers that can enhance immune responses, making them extremely important to our health and well-being. The

dominant pathways for the production of secondary metabolites in actinobacteria are the non-ribosomal peptide synthetase pathway that produces non-ribosomal peptides and the type I and type II polyketide pathways that produce polyketides (Passari *et al.,* 2015).

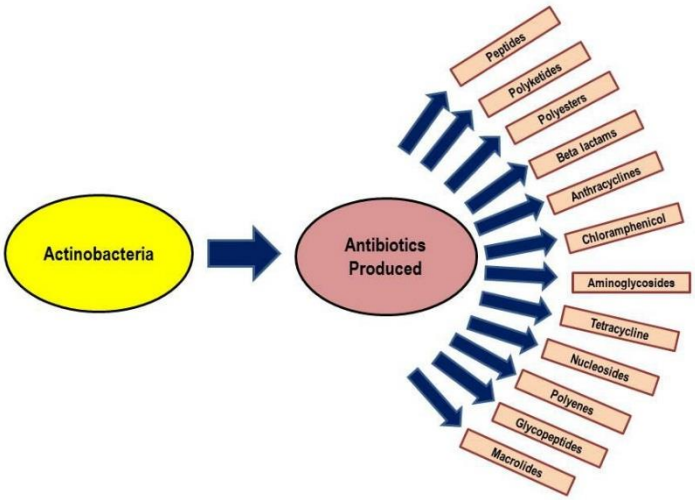

Figure 1.2. Different antibiotics produced by actinobacteria (Hug *et al.,* 2018)

1.4.1. Antibiotic resistance

Problems arise when patients infected with diseases, such as tuberculosis (TB) and the human immunodeficiency virus (HIV), are unfamiliar with medical practices and misuse antibiotics over extended periods (3 – 6 months) (Gelmanova *et al.,* 2007). This misuse of antibiotics occurs when patients do not complete their medical prescriptions and do not take their prescribed medication on time. This results in antibiotic resistance among pathogenic microbes where they acquire various resistance-causing genes within their genomes or suffer mutations in existing chromosomal genes that lead to the development of antibiotic resistance. The acquisition of resistance genes can occur through horizontal gene transfer, which results in the ability to inactivate antibiotics or evade their effects, making these microbial pathogens difficult to inhibit or kill (Wright, 2012). Therefore, novel antibiotics are continuously required to target the drug resistant microbial pathogens capable of causing life-threatening infections in order to inhibit and ultimately destroy them. An example of a microbial pathogen is *Mycobacterium tuberculosis*, which causes TB. TB, together with the HIV and malaria, are the main causes of death from infectious diseases worldwide (Zumla *et al.,* 2013; Riccardi & Pasca, 2014; Ntie-Kang *et al.,* 2014). Multidrug-resistant TB (MDR-TB) is TB that is irresponsive to at least the first-line isoniazid and rifampicin antibiotics – two of the most potent anti-TB drugs. Additionally, extensively drug-resistant TB (XDR-TB) is a form of TB that has further

resistance to some second-line drugs including fluoroquinolones, amikacin, kanamycin and capreomycin. Totally drug resistant TB (TDR-TB), which is resistant to all second-line drugs, has already been reported in some Asian countries (Zumla *et al.,* 2013). TB remains the leading cause of death in South Africa. According to the World Health Organisation (WHO), 22 000 TB related deaths and 56 000 deaths of people with TB-HIV co-infection occurred in 2017 (https://www.who.int/tb/data/en/). Therefore, there is a global need for the discovery of new anti-TB antibiotics with novel mechanisms of action and unique structural features.

Actinobacteria have contributed enormously towards the medical field in drug discovery and drug development where more than 45% of all known antibiotics and up to 90% of antibiotics available on the market today have been obtained from these microbes (Berdy, 2005; Mahajan & Balachandran, 2012). Many commercially important drugs have been produced by species from the genus *Streptomyces*, such as ivermectin, tetracycline, nystatin and anti-TB drugs (Miao & Davies, 2010; Bu *et al.,* 2014; Yassien *et al.,* 2015). Anti-TB drugs produced by *Streptomyces* include streptomycin (first discovered in 1943), D-cycloserine (produced by *Streptomyces garyphalus* and *Streptomyces lavendulae*) (Kumagai *et al.,* 2010). However, the probability of discovering novel antibiotics from *Streptomyces* species has decreased over the years as culture extracts have often yielded plenty of known compounds (Koehn & Carter, 2005). Nevertheless, it is predicted that many *Streptomyces* antibiotics are yet to be discovered (Watve *et al*., 2001). Furthermore, *Streptomyces* species have not been extensively researched for anti-malarial properties, which still makes them relevant and an important part of drug discovery.

1.5. AIMS

The aims of this study were to investigate the actinobacterial biodiversity of three different environmental samples (terrestrial and aquatic) from the Silvermine Nature Reserve, Table Mountain National Park, Cape Town, South Africa. These samples were the sediment from the Silvermine freshwater reservoir, a soil sample from Elephant's Eye cave and a swab sample taken from the Elephant's Eye cave wall. These sample sites were selected as they are considered to be understudied habitats of actinobacteria. The actinobacterial biodiversity of these samples was analysed using both a culture-dependent and a culture-independent approach. The culture-dependent approach was based on standard plate cultivation techniques, DNA extraction, 16S-rRNA gene amplification by PCR and identification of isolates by 16S-

rRNA gene sequence analysis. Phylogenetic trees based on the 16S-rRNA gene sequences were used to analyse the phylogeny of identified actinobacteria at the genus level. The culture-independent approach was based on a metagenomic screening technique where the genomic DNA (gDNA) was extracted directly from the freshwater reservoir sediment and thereafter amplified using actinobacterium specific PCR primers before being cloned and inserted into competent *Escherichia coli* cells. The prepared clone library was sequenced and actinobacteria were identified based on the cloned 16S-rRNA gene sequences.

An additional aim of this study was to screen the isolated actinobacteria for antimycobacterial antibiotic activity against *Mycobacterium aurum* strain A+, which is a non-pathogenic mycobacterium with a similar antibiotic-susceptibility profile to *M. tuberculosis* (Chung *et al*., 1995). The antibiotic spectrum of the actinobacteria was tested further against two test bacteria: another Gram positive bacterium, *Staphylococcus aureus* strain ATCC 25923 and a Gram negative bacterium, *E. coli* strain ATCC 25922. The active antibiotic compounds were extracted from selected actinobacterial isolates and screened for antimicrobial antibiotic activity using a spot bioautography method. Furthermore, selected isolates were also tested for the ability to kill the malaria parasite, *Plasmodium falciparum*, in erythrocyte culture.

The combination of these methods provides some detail about the biodiversity of actinobacteria in the Silvermine Nature Reserve and their ability to produce antimycobacterial and antiplasmodial compounds.

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CHAPTER 2

ACTINOBACTERIAL BIODIVERSITY OF RESERVOIR SEDIMENT, CAVE SOIL AND A CAVE WALL USING CULTURE-DEPENDENT METHODS

SUMMARY

A total of 56 presumptive actinobacterial strains were isolated from three different samples taken from the Silvermine Nature Reserve (Table Mountain National Park, Cape Town), namely, cave soil, the wall of the cave and sediment from the shallow waters of a reservoir. The investigation of 27 of the presumptive actinobacterial isolates was discontinued subsequent to further morphological and PCR analyses, as they were either considered to be non-actinobacteria or their DNA could not be amplified with the 16S-rRNA gene universal primers used in this study. The remaining 29 isolates were successfully identified to the genus level following 16S-rRNA gene amplification, sequencing and analysis. The following non-*Streptomyces* biodiversity was discovered: one *Micrococcus* strain and one *Streptacidiphilus* strain were isolated from the cave soil; one *Micromonospora* strain was isolated from the reservoir sediment. In addition, a total of 26 *Streptomyces* strains was isolated from across the three samples. The phylogenetic position of each identified strain within its genus was investigated by generating phylogenetic trees based on their 16S-rRNA sequences. Further analysis of the gyrase subunit B gene (*gyrB*) sequence and *gyrB* gene phylogenetic analysis of the *Streptacidiphilus* strain was conducted.

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2.1. INTRODUCTION

There is a constant need for novel antibiotics to overcome the serious problem of drug resistance in evolving pathogens. Therefore, the search for novel antibiotics for potential pharmaceutical applications continues (Shah *et al.*, 2017; Takahashi & Omura, 2003). Bacteria have proven to be the most promising source of antibiotics over the years and will certainly remain a vital source of new bioactive natural products for years to come. Microbiology and chemistry went hand in hand in the 1950's and 1960's when bacteria were cultivated and their organic compounds were extracted, resulting in the isolation of large amounts of compounds that displayed antibacterial activity (Aminov, 2010). Among these bacteria were species from the actinomycete genus *Streptomyces* that have provided novel bioactive molecules in abundance: antiprotozoals, antifungals and antivirals in addition to antibiotics (De Lima Procópio *et al*., 2012). *Streptomyces* species have provided over half of the naturally occurring antibiotics discovered to date and continue to be a good source of new secondary metabolites (Li & Vederas, 2009). However, the discovery of novel drugs derived from the bioactive metabolites of *Streptomyces* species has decreased at a steady rate, revealed by a 30% drop in natural product based drugs used in clinical studies between 2001 and 2008 (Li & Vederas, 2009).

Due to the growing demand of medicine for novel antimicrobial compounds, the attention in natural product discovery shifted towards exploring understudied environments, such as marine and extreme habitats (Thumar *et al.,* 2010; Hozzein *et al.,* 2011). Extreme habitats are environments that are considered to be very difficult to survive in due to extreme conditions, such as very high or low temperatures, high pressure or high salt. Examples of extreme habitats include deep ocean vents and volcanoes. The study of rare actinomycete genera (i.e. non-*Streptomyces* genera), together with novel *Streptomyces* species isolated from the abovementioned habitats, have already provided novel antibiotics displaying unique chemical structures (Tiwari & Gupta, 2014). This confirms that natural products produced from actinobacteria are still a promising resource for drug development. Exploring actinobacterial biodiversity broadens the pathway to finding new species that produce chemicals with novel structures, as biological diversity is known to underpin chemical diversity.

The problem of isolating strains that produce bioactive compounds that are already known still remains. Modern day natural product screening strategies show the implementation of improved methodologies for isolating understudied and rare actinobacteria that can overcome

this problem and improve the quality of screened natural products (Lazzarini *et al.*, 2001; Bérdy, 2005). Various enrichment and pre-treatment techniques have been implemented to enhance the isolation of rare actinomycete genera and prevent the growth of any undesirable contaminants (Hayakawa, 2008). This study shows the implementation of media with varying nutrient compositions to accommodate the isolation of a wide range of rare actinobacteria, because different actinobacteria are known to require different nutrients. Additionally, the isolation media were supplemented with antibiotics to prevent the growth of unwanted fungi. Together with the environmental samples taken from an unstudied actinobacterial habitat used in this study, these techniques improve the chances of isolating a diverse range of rare actinobacteria and new *Streptomyces* species that produce antibiotic compounds with novel structures.

Actinobacterial biodiversity is assumed to be reflected by their genomic heterogeneity (Ventura *et al.*, 2007). Various species of actinobacteria are delineated based on their branching position in 16S-rRNA gene phylogenetic trees. The 16S-rRNA gene is among a group of genes that are considered to be ideal for phylogenetic analysis due to their highly conserved sequences (Clarridge, 2004). This makes the 16S-rRNA gene a good candidate for the determination of evolutionary relationships among microorganisms. However, 16S-rRNA gene sequences do not distinguish very well between closely related species (and sometimes genera, e.g. *Streptomyces* and *Kitasatospora*), which causes ambiguity (Barka *et al.*, 2016). Additional genetic markers such as the DNA gyrase subunit B (*gyrB*) (Meyers, 2014), the recombinase A (*recA*) (Meyers, 2015), and the RNA polymerase beta subunit (*rpoB*) genes have, therefore, been used to discriminate between closely related species, because they have a higher mutation rate than the 16S-rRNA gene. Today, a novel species cannot be claimed without 16S-rRNA gene sequence analysis (and possibly also DNA-DNA hybridization (DDH)), although the emphasis is moving to the comparison of whole genome sequences. DDH analysis is necessary to assess the novelty of an isolate if its 16S-rRNA gene sequence similarity to its closest phylogenetic relative exceeds the threshold. The threshold for predicting the probability of two actinobacterial strains belonging to the same species based on 16S-rRNA gene sequence similarity is 98.8% (based on a maximum probability of being wrong of 0.5%) (Meier-Kolthoff *et al.*, 2013). Thus, if the 16S-rRNA gene sequence similarity between two actinobacterial strains is <98.8%, they are likely to belong to distinct genomic species (with a maximum probability of being wrong of only 1 in 200). DDH testing, together with 16S-rRNA gene sequence analysis, chemotaxonomic and physiological characterisation of actinobacteria

provides a more robust taxonomic classification, which is known as polyphasic taxonomic characterisation.

In the work presented in this chapter, the aim was to isolate a wide range of actinobacteria from samples taken from various parts of a nature reserve and to assess their phylogeny. The nature reserve samples were prepared by a dilution series before plating onto various agar media of different nutrient compositions to accommodate the growth of a diverse range of actinobacteria. Actinobacterial isolates were identified by colony morphology before further assessment by Gram staining and light microscopy. All presumptive actinobacterial isolates were identified to the genus level by partial 16S-rRNA gene sequencing analysis after amplifying the 16S-RNA gene with conserved universal PCR primers F1 and R5 (Cook and Meyers, 2003). The phylogenetic position of each confirmed actinobacterial isolate within its genus was determined by generating phylogenetic trees. All of the actinobacterial isolates described in this chapter were screened for antimycobacterial activity (data presented in Chapter 4).

2.2. METHODS & MATERIALS

2.2.1. Actinobacterial Isolation and Identification

2.2.1.1. Sample Collection

Samples of 2g sets of reservoir sediment (RS) and cave soil (CS) were collected from the Silvermine Nature Reserve, Table Mountain National Park in Cape Town, South Africa (34° 5' 26" S, 18° 25' 48" E) in January 2018. The name of the cave where the soil was collected is "Elephant's Eye" cave. In addition to this, a sterile cotton swab dipped in sterile water was used to sample the cave wall (Elephants Eye cave, Silvermine) by gently wiping the cave wall with the cotton swab and streaking the residue directly onto prepared agar plates (section 2.2.1.2). The soil and sediment samples were stored at 4°C in sterile Falcon conical centrifuge tubes for less than 24 hours until processed, while the cave wall (CW) streak plates were incubated at 30°C. The pH of the soil and sediment samples was not tested.

2.2.1.2. Bacterial sample preparation

The soil and sediment samples were prepared by weighing 0.1g of each sample into separate sterile Eppendorf microcentrifuge tubes of known weights. Sterile dH_2O was added to the samples to make up 1mL of soil-water and sediment-water mixture. Each sample mixture was mixed vigorously for approximately 60 seconds by using a vortex to dislodge the bacteria from the soil and sediment particles. The soil and sediment particles were left to settle for 5 minutes before preparing a 10-fold dilution series of the bacterial suspension with sterile dH_2O down to a final dilution of 10^{-5}. Using the spread plate technique, 100µL aliquots of each bacterial suspension dilution was spread onto prepared agar media plates in duplicate.

Various media containing different nutrient compositions were used to accommodate the growth of actinobacteria with different nutrient preferences from the reservoir sediment, cave soil and cave wall samples. The media used were Czapek solution agar (CZ) (Atlas, 2004), Difco MiddleBrook 7H9 agar (MB) containing 10mM glucose (Becton Dickinson, USA), Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) medium no. 553 (DSMZ #553) (DSMZ, 2007), JCM medium no. 61 (JCM61) (Ara & Kudo, 2007), *Kribbella* Selective Medium (KS) (Curtis, 2015) and International *Streptomyces* Project medium No. 2 (ISP2) (Shirling and Gottlieb, 1966). Nalidixic acid (10µg/ml) and cycloheximide (50µg/ml) were used to supplement all actinobacterial isolation media to inhibit the growth of Gram-

negative bacteria and fungi, respectively. All actinobacterial isolation plates were incubated at 30°C for a period of 21 days.

2.2.1.3. Bacterial isolation and purification

All bacterial isolation plates were inspected periodically for actinobacterial growth. Presumptive actinobacterial colonies having wrinkly, leathery surfaces or "volcano-like" shapes and colonies slightly sunken into the surface of the agar displaying aerial mycelium that differentiated into spores were selected and sub-cultured onto fresh agar plates (excluding antibiotics) of the same media they were isolated from. Tough, hard colonies that were difficult to pick up with a sterile loop were picked up using a sterile toothpick and lightly broken into smaller pieces for easier sub-culturing.

Presumptive actinobacterial selection and sub-culturing were performed regularly to avoid the chances of overlooking smaller actinobacterial colonies due to possible overgrowth by non-actinobacteria. Each new sub-cultured colony was given a strain name, named after its source of isolation and numbered chronologically e.g. CS1, CW1, RS1, while the media on which the isolates were found were recorded separately. All sub-cultured plates were incubated at 30°C for 7 - 14 days, after which any impure actinobacterial strains were re-streaked multiple times to achieve pure strains. Any strains that were misidentified as actinobacteria based on colony morphology were discarded at a later stage. Once pure actinobacterial cultures were achieved, 15% (v/v) glycerol stocks of each strain were prepared from either a broth culture or a spore suspension. Broth cultures were prepared by growing the actinobacterial strain in 20mL of liquid medium in a 250-ml Erlenmeyer flask for 4 – 7 days at 30°C until there was sufficient cell mass. Spore suspensions were prepared by inoculating three loopfuls of spores from a plate culture into 2mL of sterile dH_2O in a sterile universal container and mixing vigorously by vortexing in order to disperse the hydrophobic spores. The glycerol stocks were prepared by adding 700µL of broth culture or spore suspension to 300µL of 50% (v/v) sterile glycerol in a sterile Eppendorf microcentrifuge tube. All glycerol stock cultures were stored at -20°C.

2.2.1.4. Genomic DNA extraction

Actinobacterial strains were inoculated in 20mL of liquid medium known to promote optimal growth and incubated at 30°C with shaking for 7 – 14 days until sufficient cell mass was

achieved. The broth cultures were inspected for contamination by Gram staining and by streaking for single colonies onto appropriate agar plates. Glycerol stock cultures were then prepared from the pure broth cultures as mentioned in section 2.2.1.3, before harvesting the cells.

A benchtop centrifuge (Eppendorf Centrifuge 5418) was used to harvest the cells (14000 rpm [16873 X *g*] for 2 min per run) to obtain 200µL of wet cell mass from the broth cultures. A modified version of the phenol extraction method of Wang *et al.* (1996) was used to extract the genomic DNA (gDNA) from the cells. The modifications included re-suspending the cell mass in lysozyme buffer of increased concentration of 25 mg lysozyme/mL, and digesting the cells for an increased time period of 14 - 18 hours at 37°C. Furthermore, the resultant gDNA pellet was re-dissolved in varying amounts of Tris-EDTA (TE) buffer (pH 8.0) dependent on the size of the pellet. Thereafter, the gDNA was stored at 4°C overnight before measuring the DNA concentration using a Nanodrop^{TM} spectrophotometer, model ND-1000.

2.2.1.5. Identification of isolates: 16S-rRNA and *gyrB* gene amplification

The extracted gDNA was amplified using the polymerase chain reaction (PCR) for sequence analysis to identify each isolate to the genus level. The primers used to amplify the 16S-rRNA gene were the universal primers F1 and R5 ("F" denoting the forward primer and "R" denoting the reverse primer), while various pairs of *gyrB* primers were used to amplify the *gyrB* gene for a more robust identification (see Table 2.1).

Table 2.1. All primer combinations used to amplify the 16S-rRNA and *gyrB* genes.

Gene	Forward primer	Reverse primer	Reference
16S-rRNA	F1 (5'-AGAGTTTGATCITGGCTCAG-3')*	R5 (5'-ACGGITACCTTGTTACGACTT-3')*	(Cook and Meyers, 2003)
gyrB	7G-gyrB-F	KgyrB-R	(Kirby and Meyers, 2010)
	GgyrB-F2	KgyrB-R1892	
	GgyrB-F2	7G-gyrB-R	
	7G-gyrB-F	GgyrB-R1	
	GgyrB-F1	7G-gyrB-R	

*I = inosine

PCR amplification of 16S-rRNA genes was performed in 50µL reaction volumes containing 2mM $MgCl_2$, 10X Buffer, 150µM of each dNTP, 0.5µM of each primer, 1U Super-Therm *Taq* polymerase (JMR Holdings, USA), 100 – 500ng/µL of template DNA and sterile dH_20 to make up the final volume. The same PCR components and reaction volumes, as stated above, were used for *gyrB* amplification with the following changes to concentrations: 4mM $MgCl_2$, 1.5µM of each primer and 500ng/µL of template DNA. A standard PCR programme (Cook and Meyers, 2003) was used to amplify the 16S-rRNA genes while another programme was used to amplify the *gyrB* genes (Kirby and Meyers, 2010). Different annealing temperatures were used for the different primer pairs for optimal *gyrB* gene amplification. The PCR programmes were run on a Techne TC-512 gradient thermal cycler and are summarized in Table 2.2.

Table 2.2. 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
	20	Hold

Steps 2 to 4 were carried out for 30 cycles before proceeding to step 5.

*****Annealing temperatures for different *gyrB* gene primer pairs: 7G-gyrB-F & KgyrB-R, 58°C; GgyrB-F2 & KgyrB-R1892, 60°C; GgyrB-F2 & 7G-gyrB-R, 58°C; 7G-gyrB-F & GgyrB-R1, 58°C; GgyrB-F1 & 7G-gyrB-R, 58°C.

The resultant PCR products were electrophoresed on 0.8% (w/v) agarose gels containing ethidium bromide (EtBr) at 0.8µg/mL (10mg/mL EtBr stock) at 90 – 100V in 1 X TAE buffer. A molecular marker of *Pst*I-digested bacteriophage lambda DNA was used and the agarose gel was visualized using the ChemiDoc™ XRS+ Molecular Imager® (Bio-Rad) illuminometer at 260nm.

2.2.1.6. DNA sequencing and analysis

The PCR amplified DNA was purified prior to sequencing using an MSB® Spin PCRapace kit (STRATEC Molecular, Berlin, Germany) according to the manufacturer's protocol. The purified PCR products, diluted to 50ng/µL in elution buffer, were sequenced using the universal primers 518F (5'-CCAGCAGCCGCGGTAATACG-3') and 800R (5'-TACCAGGGTATCTAATCC-3') for the 16S-rRNA gene, while the primers used to determine the *gyrB* gene sequences depended on the primers used in the PCR amplification. Sanger sequencing was performed as a service by Macrogen Europe Inc. (Amsterdam, The Netherlands). The resulting sequence chromatograms were analysed and edited using Chromas version 2.6.4 (Technelysium Pty. Ltd., Australia). Sequences were aligned and assembled in DNAMAN version 4.13 (Lynnon Biosoft). The edited 16S-rRNA gene partial sequences were submitted and compared to a curated database, EzBioCloud (https://www.ezbiocloud.net/), to accurately identify the isolated actinobacterial strains to the genus level (Kim *et al.,* 2012) with the use of a standard nucleotide-nucleotide BLAST search (Basic Local Alignment Search Tool - *blastn*) (Altschul *et al.,* 1997). The edited *gyrB* partial gene sequences were subjected to BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for comparison against the GenBank database. The sequences of the top hits for each actinobacterial strain were downloaded from the databases for phylogenetic analyses.

2.2.2. Phylogenetic analysis

2.2.2.1. 16S-rRNA gene phylogeny

Phylogenetic trees were generated for each actinobacterial strain together with their top hits by sequence similarity. The 16S-rRNA gene sequences of the type strains of the top 5 hits were downloaded from the EzBioCloud database for strains identified to belong to the genus *Streptomyces.* For strains that were identified as non-*Streptomyces* species, the 16S-rRNA gene sequences of the type strains of the top 30 hits were selected for download from the EzBioCloud database. Furthermore, the 16S-rRNA gene sequences of all the type strains in the genus were downloaded for strains that were identified as non-*Streptomyces* species belonging to small genera with only a few validly published names. For strains having identical top hits, the next top hits in line were selected for downloading to avoid repetition of top hits in the generation of the phylogenetic trees. The Molecular Evolutionary Genetics Analysis version 7.0 software (MEGA7; Kumar *et al.,* 2015) was used to create multiple sequence alignments of the 16S-

rRNA gene sequences for each genus using the Muscle alignment algorithm with default settings (Edgar, 2004). The alignments for each genus were then edited to remove any columns containing ambiguous bases, insertions or deletions. The edited alignments were used to generate neighbour-joining (Saitou and Nei, 1987), maximum parsimony (Fitch, 1971) and maximum likelihood (Felsenstein, 1981) trees for each genus. For each analysis, the three generated trees were compared to each other and matching topologies were marked on the maximum likelihood tree, which are shown in the results section of this chapter.

2.2.2.2. *Gyrase subunit* B gene phylogeny

The whole genome sequences of available strains belonging to the *Streptacidiphilus* genus were downloaded from the GenBank database. The partial *gyrB* gene sequence of *Streptacidiphilus* strain CS11 was aligned to the *gyrB* gene sequences of the *Streptacidiphilus* species whose sequences were available in the GenBank database. The alignment was edited and used for the generation of phylogenetic trees as described in section 2.2.2.1.

2.3. RESULTS & DISCUSSION

2.3.1. Bacterial Isolation

Bacterial isolation plates were incubated for up to 6 weeks during which time a total of 56 presumptive actinobacterial isolates were selected across the three environmental samples. Five presumptive actinobacterial isolates were identified from the cave wall (CW) sample, 25 were identified from the cave soil (CS) sample and 26 were identified from the reservoir sediment (RS) sample.

2.3.2. PCR DNA 16S-rRNA Identification

All 56 presumptive actinobacterial isolates displaying interesting colony textures, shapes and colours were cultured in broth to extract, amplify and sequence their DNA for genus identification. Twenty nine (29) of the 56 isolates (five from the cave wall, 10 from the cave soil and 14 from the reservoir sediment) were successfully identified to the genus level and confirmed as actinobacteria after successful DNA extraction, PCR amplification and DNA sequencing. The identification of the remaining 27 isolates was discontinued because either: a) they were suspected to be moulds due to their wider hyphae when viewed by light microscopy (actinobacteria have much thinner hyphae than fungi) or b) unsuccessful PCR amplification.

Nonetheless, the strains of particular interest to identify were strains CS8, CS11, CS16, CS18 and RS10 due their different or unusual colony morphologies and colours. Strain CS8 displayed wrinkled colonies that were slightly sunken into the agar and were bright yellow in colour with no aerial mycelium when cultured on DSMZ #553 medium. Strain CS11 displayed pale yellow to pale brown colonies when cultured on ISP2 medium. Strain CS16 displayed small, wrinkled "dried out" colonies that were pale yellow, appearing almost colourless. Strain CS18 had colonies shaped like asterisks, sunken into the agar and had white aerial mycelium which later sporulated to produce black spores. Colonies of strain RS10 were wrinkled and deep orange in colour with no aerial mycelium and formed a black spore mass directly on the colonies. A summary of all strains identified to the genus level, together with their respective top hits can be found in Table 2.3.

Since strain CS11 was identified as a rare strain of actinobacteria, belonging to the genus *Streptacidiphilus* and underwent further analysis using the *gyrB* gene. The *gyrB* gene was successfully amplified via PCR and sequenced before using *Blastn* analysis on the GenBank

database to determine whether the top hit was the same as that for the 16S-rRNA gene (Table 2.3). This gives more information about its diversity, as there is greater sequence variation in the *gyrB* gene.

Table 2.3. Genus identification of 29 actinobacterial isolates from cave soil (CS), cave wall (CW) and reservoir sediment (RS) samples from the Silvermine Nature Reserve. Identification was based on EzBioCloud analyses of partial 16S-rRNA gene sequences. The identification of the top *gyrB* gene for strain CS11 was based on *blastn* analysis using the GenBank database. Percentage (%) sequence similarity is the similarity to the query sequence of the partial 16S-rRNA gene sequence generated using the 518F primer. For strain CS11, the partial *gyrB* gene sequence was generated using the GF1 primer. ♦Strains with 16S-rRNA partial gene sequences longer than 1200 bp obtained by additional sequencing with the 800R primer. *Strain CS11 partial *gyrB* sequence longer than 1200 bp obtained by additional sequencing with the GF2 primer.

Strain	Top Hit	% Sequence similarity	Query sequence length (bp)
	Cave soil (CS)		
CS1 ♦	*Streptomyces laculatispora*	99.72	1 440
CS3 ♦	*Streptomyces drozdowiczii*	99.58	1 446
CS5 ♦	*Streptomyces brevispora*	100	1 452
CS8 ♦	***Micrococcus aloeverae***	**99.93**	**1 412**
CS11 ♦	***Streptacidiphilus carbonis***	**99.79**	**1 452**
CS11 (*gyrB*) *	***Streptacidiphilus carbonis***	**98.71**	**1 133**
CS12	*Streptomyces yanii*	99.89	941
CS14	*Streptomyces setonii*	100	936
CS16 ♦	***Pilimelia columellifera* subsp. *pallida***	**99.35**	**1 240**
CS18 ♦	*Streptomyces griseus* subsp. *griseus*	99.79	1 458
CS23 ♦	*Streptomyces griseus* subsp. *griseus*	99.79	1 417
	Cave wall (CW)		
CW1 ♦	*Streptomyces beijiangensis*	99.02	1 429
CW2 ♦	*Streptomyces luridiscabiei*	100	1 454
CW3	*Streptomyces setonii*	100	937
CW4 ♦	*Streptomyces drozdowiczii*	100	1 360
CW5	*Streptomyces beijiangensis*	99.79	935
	Reservoir sediment (RS)		
RS3 ♦	*Streptomyces rapamycinicus*	98.94	1 443

RS6 ♦	*Streptomyces sanglieri*	99.52	1 455
RS7 ♦	*Streptomyces geldanamycininus*	99.93	1 423
RS9 ♦	*Streptomyces cyslabdanicus*	99.51	1 420
RS10 ♦	***Micromonospora matsumotoense***	**99.51**	**1 226**
RS13	*Streptomyces xiamenensis*	100	729
RS14 ♦	*Streptomyces galbus*	99.52	1 450
RS15	*Streptomyces yatensis*	99.79	932
RS16 ♦	*Streptomyces pulveraceus*	99.86	1 452
RS17	*Streptomyces yatensis*	99.36	935
RS18	*Streptomyces decoyicus*	100	946
RS19	*Streptomyces yatensis*	99.36	932
RS20	*Streptomyces cyslabdanicus*	99.78	930
RS22	*Streptomyces atratus*	100	936

The majority of the isolated strains were identified as *Streptomyces* species, however, four rare actinobacterial strains were identified belonging to the genera *Micrococcus* (orange text in Table 2.3), *Streptacidiphilus* (green text in Table 2.3), *Pilimelia* (blue text in Table 2.3) and *Micromonospora* (red text in Table 2.3).

2.3.3. Phylogenetic analyses

Phylogenetic trees for all identified actinobacterial strains were constructed based on their 16S-rRNA gene sequences and, in the case of *Streptacidiphilus* strain CS11, an additional phylogenetic tree was constructed based on its partial *gyrB* gene sequence. These analyses provided insight into the phylogenetic position of each isolate within its genus.

2.3.3.1. Genus *Micrococcus*

The genus *Micrococcus* is comprised of only nine species with validly published names (List of Prokaryotic names with Standing in Nomenclature (LPSN)) (http://www.bacterio.net/micrococcus.html; Parte, 2018). The genus was first described by Cohn in the year 1872 and its description has been revised several times before being emended by Wieser *et al.* (2002). *Micrococcus* strains are known to produce convex or raised colonies

with depressed centres that are usually pigmented with various shades of yellow (Kloos *et al,* 1974). Additionally, their cells are Gram-positive cocci which, in some cases, are surrounded by a Gram-negative, mucous-like layer (Kloos *et al.,* 1974). *Micrococcus* strain CS8 was deep yellow in colour, produced colonies that were wrinkled with depressed centres and had Gram-positive, coccoid cells. These interesting characteristics suggested that the isolate could be a *Micrococcus* species (before its identification was confirmed by 16S-rRNA gene identification). Micrococci have been isolated from human skin, as well as from water, dust and soil (Kloos *et al.,* 1974; Zhuang *et al.,* 2003; Kooken *et al.,* 2012). In this study, *Micrococcus* strain CS8 was isolated from a cave soil sample.

A 16S-rRNA gene phylogenetic tree was generated for *Micrococcus* strain CS8, using all nine type strains of *Micrococcus* with validly published names, to determine its phylogenetic position within the genus (Figure 2.1). *Micrococcus* strain CS8 is seen clustering closely with *Micrococcus aloeverae* AE-6^{T} (KF524364) with moderate bootstrap support (75%). Furthermore, the topology of the clade as seen in the maximum likelihood tree matched those of the neighbour-joining and maximum parsimony trees. Given the very high 16S-rRNA gene sequence similarity between strain CS8 and the type strain of *M. aloeverae* (99.93% over 1412 nucleotides), the *gyrB* gene of *Micrococcus* strain CS8 could be sequenced and used to construct a phylogenetic tree based on the *gyrB* gene sequences of those strains for which sequences are available on the GenBank database. The genome sequences of *Micrococcus aloeverae* BCRC 80870^{T}, *Micrococcus endophyticus* BCRC 16908^{T}, *Micrococcus flavus* BCRC 80069^{T}, *Micrococcus luteus* ATCC 4698^{T}, *Micrococcus lylae* NBRC 15355^{T}, *Micrococcus terreus* CGMCC 1.7054^{T} and *Micrococcus yunnanensis* BCRC 80243^{T} are available on GenBank and the *gyrB* gene sequences would therefore have to be extracted before further phylogenetic analysis. This would provide a more robust comparison of *Micrococcus* strain CS8 with the known species of *Micrococcus*. However, the analysis would be restricted to only seven sequences of the *Micrococcus* type strains (out of a total of nine type strains in the genus).

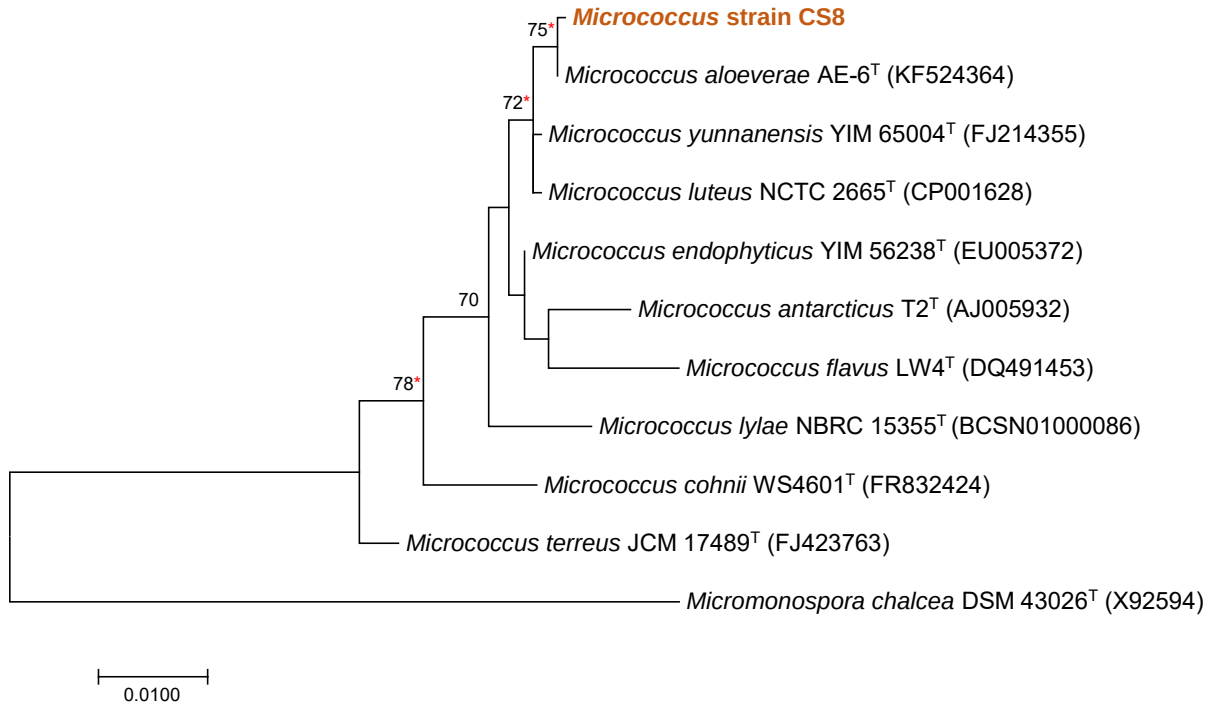

Figure 2.1. Maximum likelihood phylogenetic tree of *Micrococcus* strain CS8 and all nine type strains of the genus *Micrococcus* based on partial 16S rRNA gene sequences of 1358 nt. *Micromonospora chalcea* DSM 43026^T was used as the outgroup. The value at each node represents the percentage bootstrap support calculated from 1000 resampled datasets. Only bootstrap values of ≥ 70% are displayed. The scale bar indicates 1 nucleotide change per 100 base pairs. "*" Indicates matching topology with the neighbour joining and maximum parsimony trees.

2.3.3.2. Genus *Micromonospora*

There are currently over 80 species with validly published names in the genus *Micromonospora* (http://www.bacterio.net/micromonospora.html). The genus *Micromonospora* was first described by Ørskov in the year 1923 and the description was last emended by Gao *et al.* (2014). Species belonging to this genus are widespread in the environment and are known to be commonly isolated from water and soil, with most strains living in aquatic environments (Hernández *et al.*, 2000; Gutierrez-Lugo *et al.*, 2005) where they are known to play a vital role in the mineralization of organic matter (Ertaş *et al.*, 2013).

In this study, *Micromonospora* strain RS10 was isolated from the sediment in the shallow waters of a reservoir. Before 16S-rRNA identification confirmed that strain RS10 belonged to the genus *Micromonospora*, the isolate was selected as it displayed the colony morphology of

known micromonosporae, i.e. having wrinkled, folded colonies with deep orange pigmentation and a slimy, black spore mass (Zhao *et al.*, 2004; Kroppenstedt *et al.*, 2005).

A phylogenetic tree based on the 16S-rRNA gene sequences of 30 *Micromonospora* type strains was generated for *Micromonospora* strain RS10 in order to determine its phylogenetic position within the genus (Figure 2.2). *Micromonospora* strain RS10 can be seen clustered closely with *Micromonospora matsumotoense* DSM 44100^{T} (AF152109) in a separate clade. This clade displays moderate bootstrap support (76%) based on a 16S-rRNA gene sequence alignment of 1196 nucleotides. This association was also seen in the neighbour-joining and maximum parsimony trees. *M. matsumotoense* was also the top hit for *Micromonospora* strain RS10 with 99.51% 16S-rRNA gene sequence similarity over 1226 nucleotides (Table 2.3). Further *gyrB* gene analysis could be conducted to increase the robustness of the phylogenetic analysis.

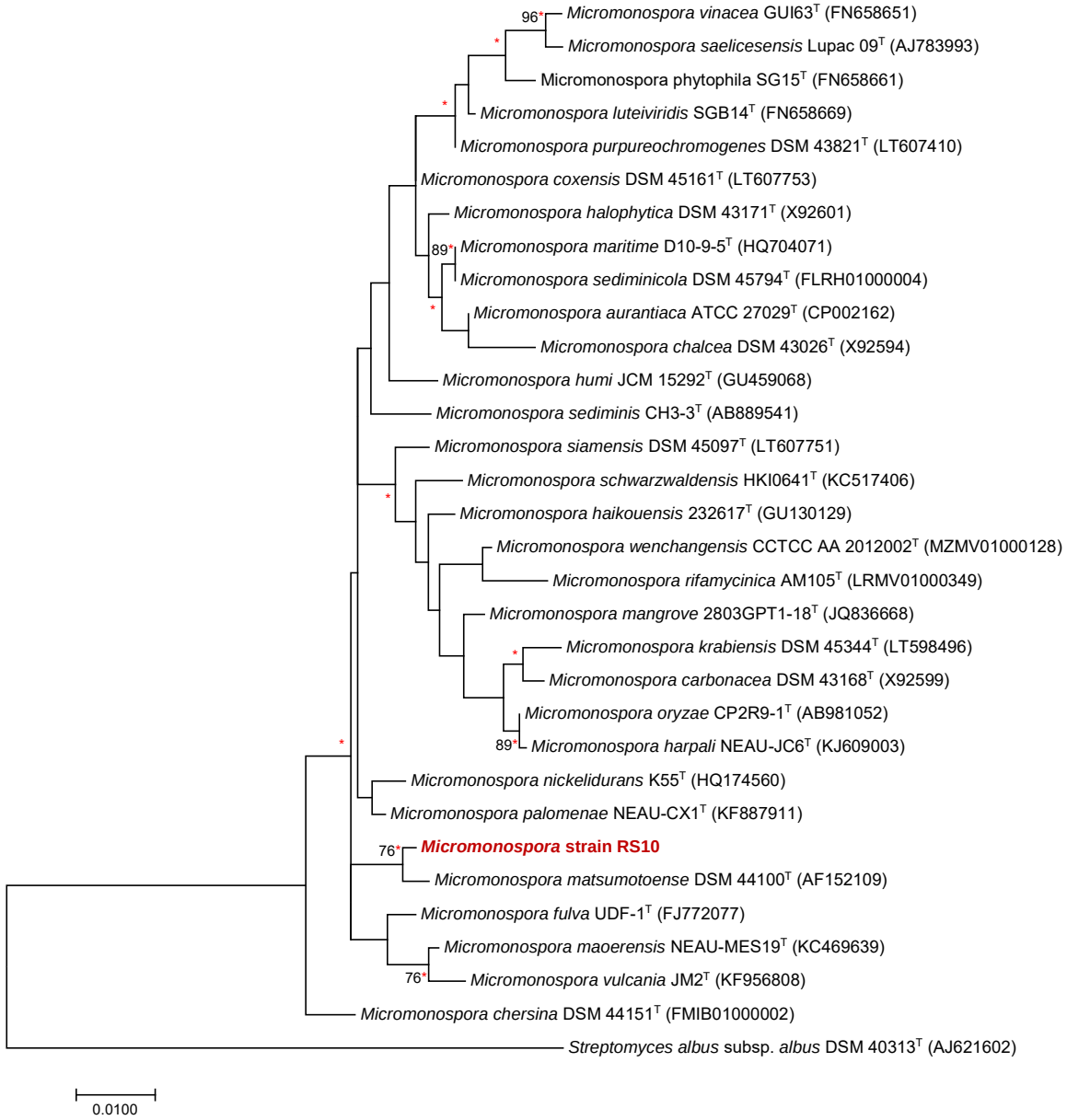

Figure 2.2. Maximum likelihood phylogenetic tree of *Micromonospora* strain RS10 and the top 30 *Micromonospora* hits by EzBioCloud analysis based on partial 16S rRNA gene sequences of 1196 nt. *Streptomyces albus* subsp. *albus* DSM 40313^T was used as the outgroup. The value at each node represents the percentage bootstrap support calculated from 1000 resampled datasets. Only bootstrap values of ≥ 70% are displayed. The scale bar indicates 1 nucleotide change per 100 base pairs. "*" Indicates matching topology with the neighbour joining and maximum parsimony trees.

2.3.3.3. Genus *Streptomyces*

The genus *Streptomyces* is the largest genus within the phylum *Actinobacteria*, currently comprised of 852 species with validly published names according to the LPSN (http://www.bacterio.net/streptomyces.html). The type species of this genus, *Streptomyces albus*, was first described as *Streptothrix alba* by Rossi Doria in the year 1891. Thereafter, it

was amended as *Actinomyces* by Krainsky until being established as the type species of the genus *Streptomyces* (Waksman and Henrici, 1943).

In this study, two separate phylogenies of the *Streptomyces* isolates were constructed based on the lengths of their partial 16S-rRNA gene sequences: 1) 16S-rRNA gene sequences longer than 1300-nt and 2) 16S-rRNA gene sequences shorter than 950-nt. In order to determine the phylogenetic position of the isolates within the genus *Streptomyces,* the type strains of the top five EzBioCloud hits of each isolate was used to generate the trees. The next top hit in line was selected in the case of overlapping top hits for different isolates (hence the inclusion of *Kitasatospora cinereorecta* NBRC 15395^T (AB184646) in Figure 2.3a). Due to the large number of selected *Streptomyces* top hits with validly published names, part of each phylogenetic tree was compressed to make the trees more compact.

2.3.3.3.1. *Streptomyces* phylogenetic analysis: extended 16S-rRNA gene sequences

Figure 2.3a and Figure 2.3b display the phylogeny of *Streptomyces* strains CS1, CS3, CS5, CS18, CS23 (isolated from cave soil), strains CW1, CW2, CW4 (isolated from a cave wall), and strains RS3, RS6, RS7, RS9, RS14, RS16 (isolated from sediment in the shallow waters of a reservoir). The trees show their phylogenetic positions based on a 16S-rRNA gene sequence alignment of 1325 nucleotides. In Figure 2.3a it is seen that *Streptomyces* strain CW2 groups closely with *Streptomyces luridiscabiei* NRRL B-24455^T (LIQV01000394), *Streptomyces fulvissimus* DSM 40593^T (CP005080), *Streptomyces fulvorobeus* NBRC 15897^T (AB184711) and *Streptomyces microflavus* NBRC 13062^T (AB184284). This is in accordance with EzBioCloud results where the aforementioned *Streptomyces* type strains were the top four hits of *Streptomyces* strain CW2 with 100% sequence similarity based on their 16S-rRNA gene lengths of approximately 1400-nt. This lack of sequence variation between the strains is also indicated by their branch lengths. However, the bootstrap support of the clade where *Streptomyces* strain CW2 clusters is weak with a value less than 70% (43%) and does not display matching topology with that of the neighbour-joining and maximum parsimony trees. Gyrase subunit B gene sequencing could help to further investigate whether *Streptomyces* strain CW2 belongs to the same species of one of its four top hits by analysing how similar their *gyrB* sequences are. However, whole-genome sequencing and analysis by ANI and dDDH would provide a definitive answer.

Streptomyces strains CS18 and CS23 grouped closely together in Figure 2.3a. Their identical 16S-rRNA gene sequences suggests that these two strains may be clones of a single strain. However, *gyrB* gene analysis would give us more insight into their relatedness (clones would have identical *gyrB* gene sequences). Furthermore, the clade in which they cluster is supported by a bootstrap value of 91% based on a 16S-rRNA sequence alignment of 1325-nt and displays matching topology with the neighbour-joining and maximum parsimony trees. *Streptomyces bacillaris* NBRC 13487^{T} (AB184439) branches off from *Streptomyces* strains CS18 and CS23 with weak bootstrap support (<70%). *S. bacillaris* NBRC 13487^{T} (AB184439) was not, however, the top hit of either *Streptomyces* strain CS18 or strain CS23, but rather the 20th hit of both strains with a sequence similarity of 99.4% over 1458-nt and 99.43% over 1417-nt, respectively, according to EzBioCloud analyses. The top hit for both *Streptomyces* strains CS18 and CS23 was *Streptomyces griseus* subsp. *griseus* (see Table 2.3). Genome sequencing followed by ANI and dDDH analyses would establish the relationship between *Streptomyces* strains CS18, CS23 and the type strain of *S. bacillaris* NBRC 13487^{T} (NZ_CP029378) as distinct genomic species or strains of a single species.

Figure 2.3a shows the position of *Streptomyces* strain RS16 very close to *Streptomyces pulveraceus* LMG 20322^{T} (AJ781377) (these strains share a 16S-rRNA gene sequence similarity of 98.46%). However, the clade displays weak bootstrap support with a value less than 70% over a sequence alignment of 1325-nt. Moreover, it does not match the topology of the neighbour-joining and maximum parsimony trees. The cluster does, however, match the information provided by the EzBioCloud database as *S. pulveraceus* LMG 20322^{T} (AJ781377) is the top hit of *Streptomyces* strain RS16 with a sequence similarity of 99.86% over 1452-nt (Table 2.3). A whole-genome comparison between the strains should be undertaken, although there is currently no genome sequence for the type strain of *S. pulveraceus* in the GenBank database.

Streptomyces strain RS6 clustered with *Streptomyces sanglieri* NBRC 100784^{T} (AB249945) in Figure 2.3a, which is in accordance with the EzBioCloud analysis, which showed that *S. sanglieri* was the top hit (Table 2.3). The 16S-rRNA gene sequence similarity between the strains was 99.52% over 1455-nt. The clade shows moderate bootstrap support with a value of 75% and displays topological conservation with the neighbour-joining and maximum parsimony trees.

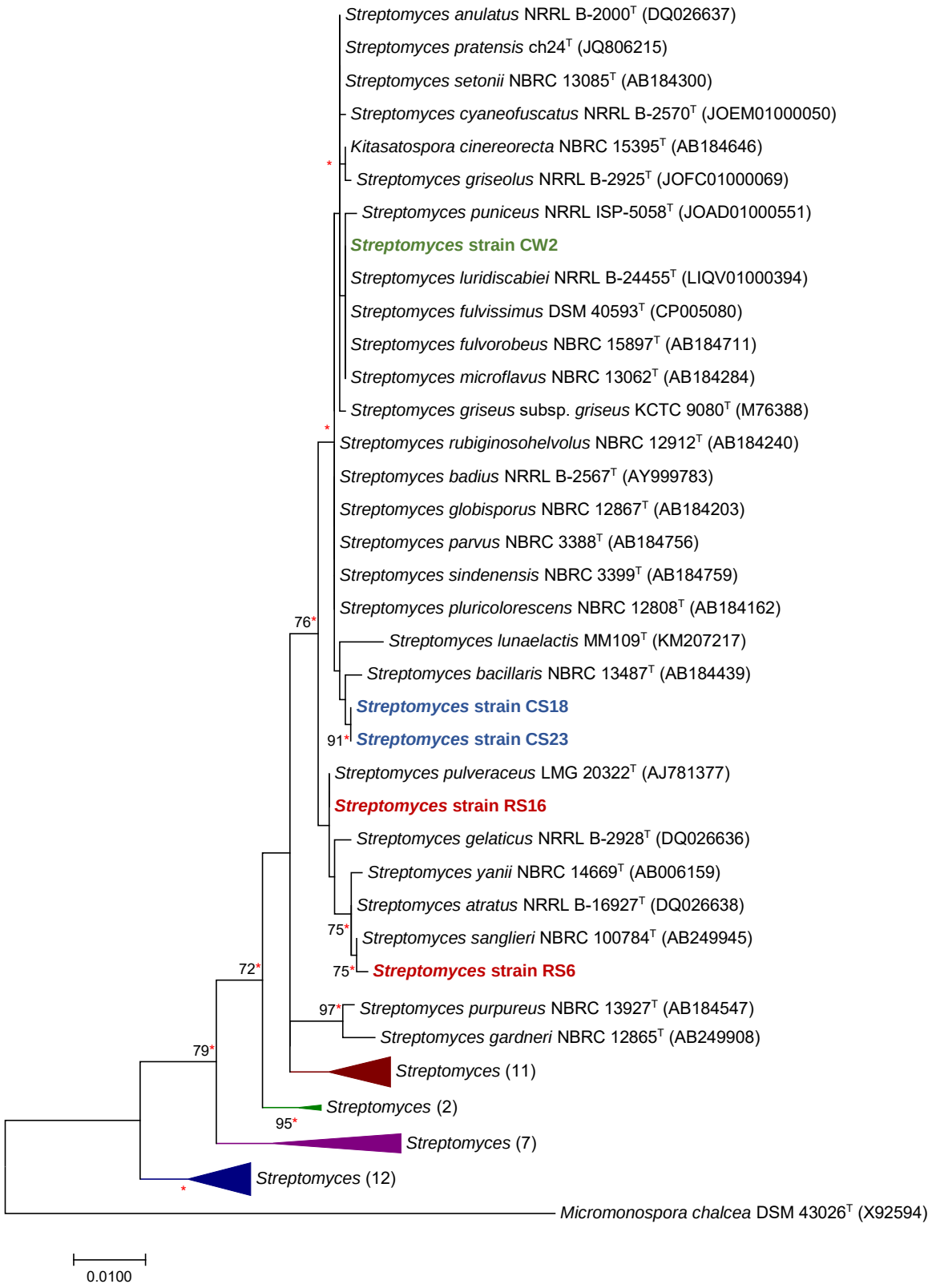

Figure 2.3a. Maximum likelihood phylogenetic tree of 14 *Streptomyces* strains compared to the top 50 hits based on 1325-nt 16S rRNA gene sequences (only five of the strains can be seen in this tree). *Micromonospora chalcea* DSM 43026^T was used as the outgroup. The lower half of the tree is compressed. The value at each node represents the percentage bootstrap support calculated from 1000 resampled datasets. Only bootstrap values of ≥ 70% are displayed. The scale bar indicates 1 nucleotide change per 100 base pairs. The values in parenthesis indicate the

number of strains in that compressed clade. "*" Indicates matching topology with the neighbour joining and maximum parsimony trees.

Streptomyces strains CS3 and CW4 clustered with *Streptomyces drozdowiczii* NBRC 101007^T (AB249957) (Figure 2.3b) with very strong bootstrap support of 98%. This topology was also seen in the neighbour joining and maximum parsimony trees. This agrees with Table 2.3 where *S. drozdowiczii* was the top hit of both *Streptomyces* strains CS3 and CW4 as determined in the EzBioCloud analysis (>99.5% 16S-rRNA gene sequence similarity over 1360-1446nt). The 16S-rRNA gene sequence similarity between strains CS3 and CW4 was 99.49% over 1361-nt. It can be noted that *Streptomyces* strain CS3 was isolated from cave soil and *Streptomyces* strain CW4 was isolated from a cave wall in the same cave, so it is possible that the two strains belong to the same species.

Streptomyces strain CS5 grouped closely with *Streptomyces brevispora* BK160^T (FR692104) in Figure 2.3b, which was also the top hit in an EzBioCloud analysis with 100% 16S-rRNA gene sequence similarity over 1452-nt (Table 2.3). The clade is supported by a strong bootstrap value of 86% and was also seen in the neighbour joining and maximum parsimony trees.

Streptomyces strain CS1, forms a branch between its top hit, *Streptomyces laculatispora* (99.72% 16S-rRNA gene sequence similarity over 1440nt; Table 2.3) and a cluster containing strain CW1 and the type strains of *Streptomyces beijiangensis* NBRC 100044^T (AB249973), *Streptomyces rectiviolaceus* NRRL B-16374^T (DQ026660) and *Streptomyces tauricus* JCM 4837^T (AB045879) (Figure 2.3b). The strain-CW1 clade displays 85% bootstrap support and has topological conservation with the neighbour joining and maximum parsimony trees. *Streptomyces* strain CW1 forms the deepest branch in the *S. beijiangensis* clade. Strain CW1 has highest 16S-rRNA gene sequence similarity to *S. beijiangensis* NBRC 100044^T (99.02% over 1429 nucleotides (Table 2.3).

Streptomyces strain RS9 clustered with *Streptomyces cyslabdanicus* K04-0144^T (AB915216), *Streptomyces olivaceoviridis* NBRC 13066^T (AB184288), *Streptomyces canarius* NBRC 13431^T (AB184396), *Streptomyces corchorusii* DSM 40340^T (KQ948396) and *Streptomyces galbus* DSM 40089^T (X79852) with strong bootstrap support (95%; Figure 2.3b). An EzBioCloud analysis revealed the type strain of *S. cyslabdanicus* as the top hit of *Streptomyces* strain RS9 with 99.51% 16S-rRNA gene sequence similarity over 1420 nt (Table 2.3). *Streptomyces* strain RS14 was loosely associated with the strain-RS9 clade (bootstrap value

43%; Figure 2.3b), having highest 16S-rRNA gene sequence similarity to the type strain of *Streptomyces galbus* (Table 2.3).

Streptomyces strain RS3 clustered with *Streptomyces rapamycinicus* NRRL B-5491^T (EF408733), *Streptomyces iranensis* HM 35^T (FJ472862), *Streptomyces yogyakartensis* NBRC 100779^T (AB249942), *Streptomyces javensis* NBRC 100777^T (AB249940) and *Streptomyces hygroscopicus* subsp. *hygroscopicus* NBRC 13472^T (BBOX01000593) with weak bootstrap support (51%; Figure 2.3b). The type strain of *S. rapamycinicus* was the top hit in an EzBiocloud analysis, with 98.94% 16S-rRNA gene sequence similarity over 1443-nt (Table 2.3).

Figure 2.3b depicts the phylogenetic position of *Streptomyces* strain RS7, which grouped with *Streptomyces geldanamycininus* NRRL B-3602^T (DQ334781), *Streptomyces melanosporofaciens* DSM 40318^T (FNST01000002), *Streptomyces castelarensis* DSM 40830^T (AY508511), *Streptomyces sporoclivatus* NBRC 100767^T (AB249934) and *Streptomyces antimycoticus* NBRC 12839^T (AB184185) with very strong bootstrap support (97%). The type strain of *S. geldanamycininus* was the top hit determined by EzBioCloud analysis, with 99.93% 16S-rRNA gene sequence similarity over 1423-nt (Table 2.3). The strain-RS7 clade was associated with the strain-RS3 clade with weak bootstrap support (47%), however, it displayed matching topology with that of the neighbour joining and maximum parsimony trees.

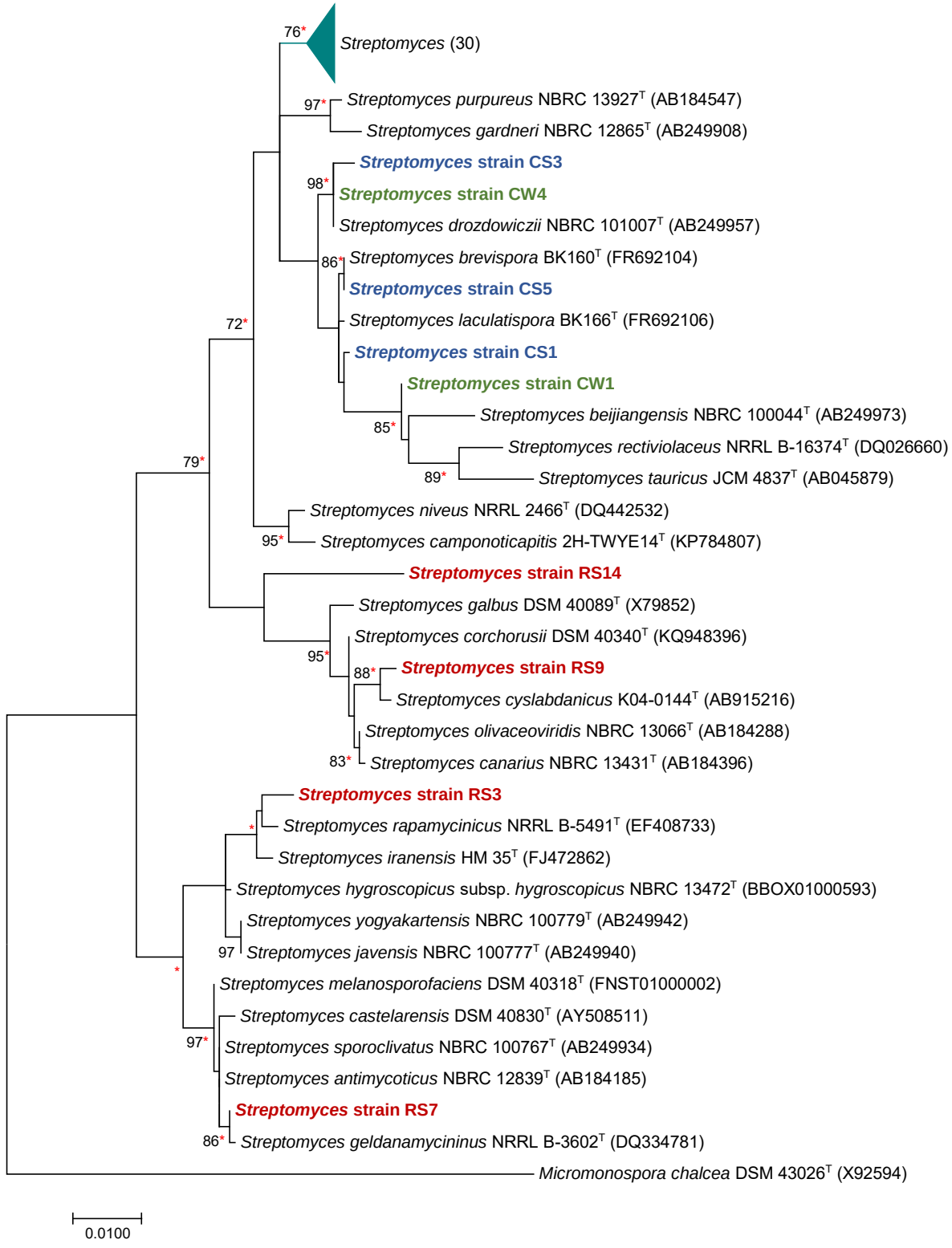

Figure 2.3b. Maximum likelihood phylogenetic tree of 14 strains identified as *Streptomyces* species compared to the top 50 hits based on 1325-nt 16S rRNA gene sequences (nine of the strains can be seen in this tree). *Micromonospora chalcea* DSM 43026T was used as the outgroup.The upper half of the tree is compressed. The value at each node represents the percentage bootstrap support calculated from 1000 resampled datasets. Only bootstrap values of ≥ 70% are displayed. The scale bar indicates 1 nucleotide change per 100 base pairs. The value in parenthesis indicates the number of strains in that compressed clade. "*" Indicates matching topology with the neighbour joining and maximum parsimony trees.

2.3.3.3.2. *Streptomyces* phylogenetic analysis: short 16S-rRNA gene sequences

Figures 2.4a and 2.4b display the phylogenetic analysis of *Streptomyces* strains CS12, CS14 (isolated from cave soil), CW3, CW5 (isolated from a cave wall), and RS13, RS15, RS17, RS18, RS19, RS20 and RS22 (isolated from sediment in the shallow waters of a reservoir). The phylogenetic position of each identified strain within the genus *Streptomyces* was determined by comparing their sequences against the top four hits for each strain, revealed by EzBioCloud analysis. The phylogenetic analysis was conducted based on a 16S-rRNA gene sequence alignment of 708 nt. The lower half (Figure 2.4a) and upper half (Figure 2.4b) of the tree were compressed for easier presentation.

In Figure 2.4a, *Streptomyces* strains CS14 and CW3 grouped with *Streptomyces fulvorobeus* NBRC 15897^T (AB184711), *Streptomyces griseus* subsp. *griseus* KCTC 9080^T (M76388), *Streptomyces microflavus* NBRC 13062^T (AB184284), *Streptomyces setonii* NRRL ISP-5322^T (MUNB01000146), *Streptomyces badius* NRRL B-2567^T (AY999783), *Streptomyces pluricolorescens* NBRC 12808^T (AB184162), *Streptomyces puniceus* NBRC 12811^T (AB184163), *Streptomyces sundarbansensis* MS1/7^T (AY550275) and *Streptomyces cyaneofuscatus* NRRL B-2570^T (JOEM01000050) with high bootstrap support (92%). This cluster was also seen in the neighbor joining and maximum parsimony trees. An EzBioCloud analysis, showed that *S. setonii* was the top hit of both *Streptomyces* strains CS14 and CW3 with 100% 16S-rRNA gene sequence similarity over approximately 936-937 nucleotides (Table 2.3). The sequence similarity between *Streptomyces* strains CS14 and CW3 was 99.68% over 938-nt. All the *Streptomyces* strains that grouped with strains CS14 and CW3 in the tree (Figure 2.4a) fell within the top ten hits in the EzBioCloud analysis. Longer 16S-rRNA gene sequences may help to distinguish strains CS14 and CW3 from their closest relatives, but whole genome sequencing would provide a definitive answer.

Streptomyces strain CS12 grouped in the same clade as *S. yanii* NBRC 14669^T (AB006159) and *S. brevispora* BK160^T (FR692104), with moderate bootstrap support (84%) and matching topology with the neighbor joining and maximum parsimony trees (Figure 2.4a). This is in accordance with the results provided by an EzBioCloud analysis, which showed that *S. yanii* was the top hit of *Streptomyces* strain CS12 with 99.89% 16S-rRNA gene sequence similarity over 941 nt (Table 2.3). Additionally, since *Streptomyces* strains RS16 and RS6 also grouped with the type strains of *S. yanii*, *S. atratus* and *S. sanglieri* (in addition to *S. pulveraceus* and *S.*

gelaticus) (Figure 2.3a), the sequence similarities between *Streptomyces* strains RS16, RS6 and CS12 were found to be 99.05% over 943 nt.

Streptomyces strain RS22 and *S. sanglieri* NBRC 100784^T (AB249945), *Streptomyces atratus* NRRL B-16927^T (DQ026638) and *S. laculatispora* BK166^T (FR692106) were loosely associated with the strain CS12 clade (bootstrap value 50%; Figure 2.4a). An EzBioCloud analysis showed that *S. atratus* was the top hit for *Streptomyces* strain RS22 with 100% sequence similarity over 936 nt (Table 2.3). For a more robust analysis of the phylogeny of RS22, a longer sequence of its 16S-rRNA gene could be used.

Figure 2.4a depicts the phylogenetic position of *Streptomyces* strain CW5, which grouped with *S. rectiviolaceus* NRRL B-16374^T (DQ026660) and *S. beijiangensis* NBRC 100044^T (AB249973) with very strong bootstrap support (99%), but this association was not seen in the neighbour joining and maximum parsimony trees. Nevertheless, *Streptomyces* strain CW5 clusters with its top hit, *S. beijiangensis,* as revealed by an EzBioCloud analysis with 99.79% 16S-rRNA gene sequence similarity over 953 nt. The sequence similarity between strain CW5 and CW1, for which the top hit was also the type strain of *S. beijiangensis*, was 99.56% over 911 nt.

Streptomyces strain RS20 grouped with *S. corchorusii* DSM 40340^T (KQ948396), *S. cyslabdanicus* K04-0144^T (AB915216), *S. olivaceoviridis* NBRC 13066^T (AB184288) and *S. canarius* NBRC 13431^T (AB184396) with strong bootstrap support (90%; Figure 2.4a). This clade was also seen in the neighbor joining and maximum parsimony trees. According to an EzBioCloud analysis, the aforementioned species clustering with *Streptomyces* strain RS20 were amongst the top five hits, with *S. cyslabdanicus* as the top hit. *Streptomyces* strain RS20 displayed 99.78% 16S-rRNA gene sequence similarity over 930-nt with its top hit (Table 2.3). The sequence similarity between strain RS20 and RS9, which had the same top hits (Figure 2.3b) is 99.56% over 909 nt.

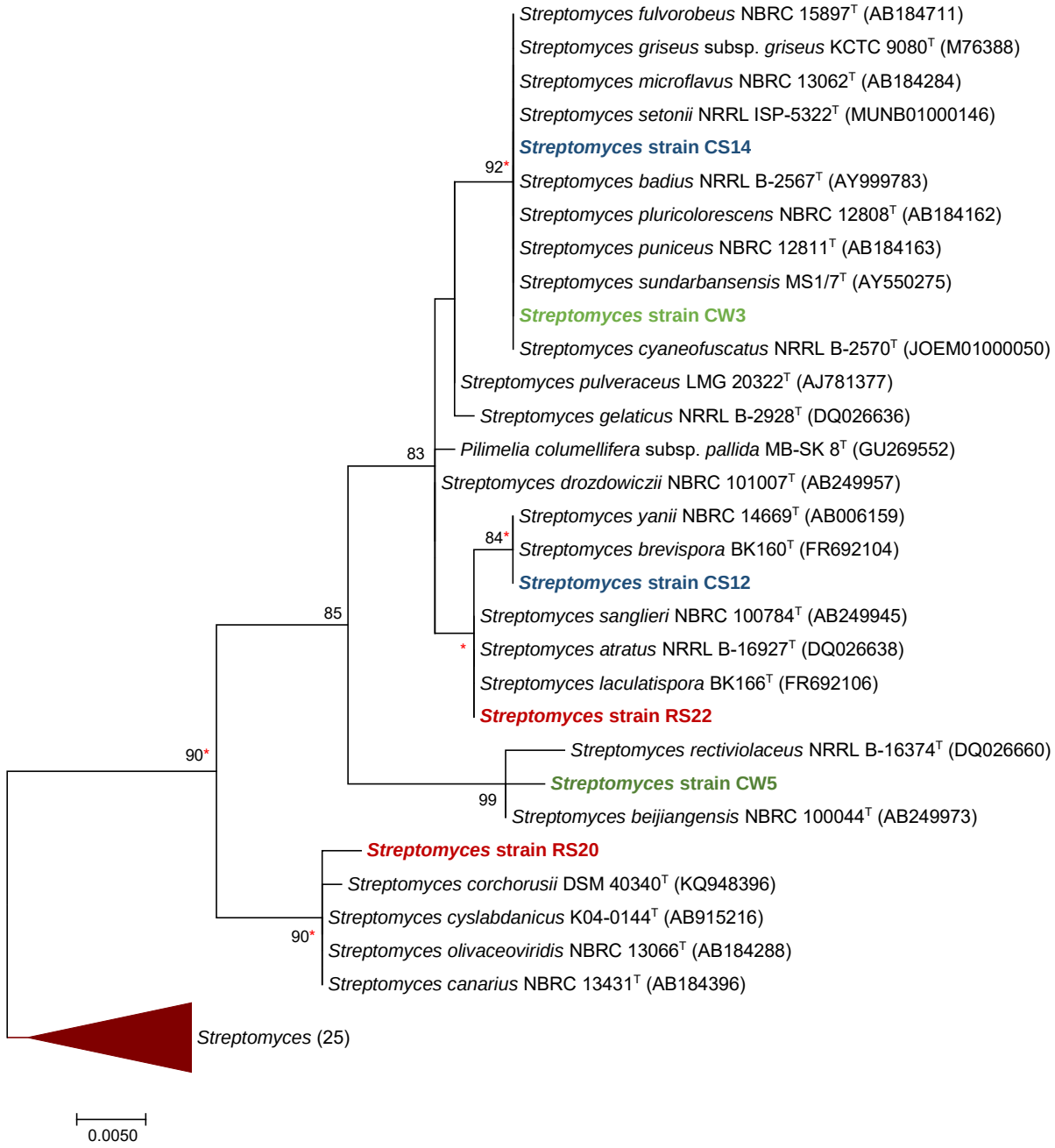

Figure 2.4a. Maximum likelihood phylogenetic tree of 11 *Streptomyces* strains compared to the top 44 hits in an EzBiocloud analysis based on partial 16S rRNA gene sequences of 708 nt. The lower half of the tree is compressed. The value at each node represents the percentage bootstrap support calculated from 1000 resampled datasets. Only bootstrap values of ≥ 70% are displayed. The scale bar indicates 5 nucleotide changes per 1000 base pairs. The value in parenthesis indicates the number of strains in that compressed clade. "*" Indicates matching topology with the neighbour joining and maximum parsimony trees.

In Figure 2.4b, *Streptomyces* strain RS18 is grouped with *Streptomyces hygroscopicus* subsp. *glebosus* NBRC 13786^T (AB184479), *Streptomyces caniferus* NBRC 15389^T (AB184640), *Streptomyces libani* subsp. *rufus* LMG 20087^T (AJ781351) and *Streptomyces decoyicus* NRRL 2666^T (LGUU01000106) with weak bootstrap support and no topological conservation with the neighbor joining and maximum parsimony trees. According to an EzBioCloud analysis, *S. decoyicus* was the top hit of *Streptomyces* strain RS18 with 100% 16S-rRNA gene sequence similarity over 946 nt (Table 2.3). Additionally, EzBioCloud revealed *S. hygroscopicus* subsp. *glebosus*, *S. caniferus* and *S. libani* subsp. *rufus* to be amongst the top four hits of *Streptomyces* strain RS18.

Streptomyces strain RS13 clustered with *Streptomyces xiamenensis* MCCC 1A01550^T (EF012099) with very strong bootstrap support (99%) and matching topology with the neighbor joining and maximum parsimony trees (Figure 2.4b). An EzBioCloud analysis revealed that *S. xiamenensis* was the top hit of *Streptomyces* strain RS13 with 100% 16S-rRNA gene sequence similarity over 729-nt (Table 2.3).

Figure 2.4b shows the clustering of *Streptomyces* strains RS15, RS17 and RS19 with *Streptomyces iranensis* HM 35^T (FJ472862) and *Streptomyces yatensis* NBRC 101000^T (AB249962) with weak bootstrap support. This grouping was not seen in the neighbour joining and maximum parsimony trees. EzBioCloud revealed *Streptomyces yatensis* as the top hit of *Streptomyces* strains RS15, RS17 and RS19 with 99.79% 16S-rRNA gene sequence similarity over 932 nt, 99.36% similarity over 935 nt and 99.36% similarity over 932 nt, respectively (Table 2.3).

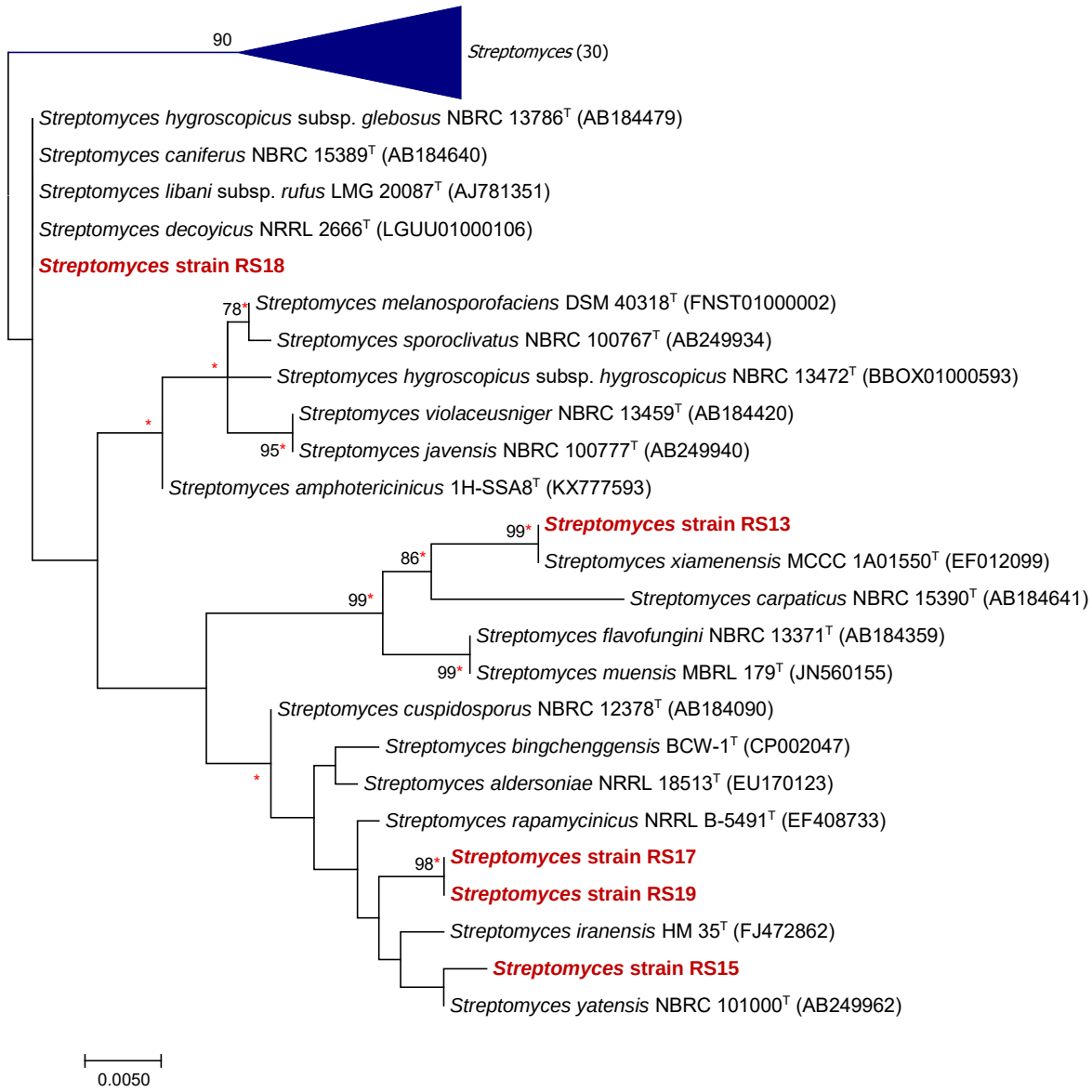

Figure 2.4b. Maximum likelihood phylogenetic tree of 11 *Streptomyces* strains compared to the top 44 *Streptomyces* taxon hits by EzBiocloud analysis based on partial 16S rRNA gene sequences of 708 nt. The upper half of the tree is compressed. The value at each node represents the percentage bootstrap support calculated from 1000 resampled datasets. Only bootstrap values of ≥ 70% are displayed. The scale bar indicates 5 nucleotide changes per 1000 base pairs. The value in parenthesis indicates the number of strains in that compressed clade. "*" Indicates matching topology with the neighbour joining and maximum parsimony trees.

Overall, the *Streptomyces* strains isolated from the three sites, namely, the cave soil, the cave wall and the sediment from the shallow waters of a reservoir, displayed great phylogenetic diversity within the genus *Streptomyces*, as revealed by how spread out they are in the phylogenetic trees.

2.3.3.3.3. Strain CS16: *Pilimelia* or *Streptomyces*?

The genus *Pilimelia* is a very small and rare genus with only three species and two subspecies with validly published names (http://www.bacterio.net/pilimelia.html). *Pilimelia terevasa* and *Pilimelia anulata* were the first two *Pilimelia* species to be discovered and were described by Kane in 1966. Later, in 1986, Vobis *et al.* described another species, namely, *Pilimelia columellifera* subsp. *columellifera* and *P. columellifera* subsp. *pallida.* Members of the genus *Pilimelia* are known to have tough, leathery textured colonies that are pale yellow, bright yellow, pale brown or sometimes colourless (Kane, 1966; Vobis *et al.*, 1986).

Before strain CS16 was identified, the isolate from cave soil was selected based on its interesting, wrinkly, circular-shaped, small colonies that appeared very pale yellow in colour. These features matched those of *P. columellifera* subsp. *pallida* described by Vobis *et al.* (1986). In order to determine its phylogenetic position within the genus, a phylogenetic tree was generated based on the 16S-rRNA gene sequences of all *Pilimelia* species with validly published names and *Micromonospora chalcea* DSM 43026^T (X92594) was used as the out-group (Figure 2.5a). Figure 2.5a shows strain CS16 clustering with *P. columellifera* subsp. *pallida* MB-SK 8^T (GU269552) with very strong bootstrap support (100%; the 16S-rRNA gene sequence alignment was over 1226 nucleotides). This clustering was also seen in the neighbour-joining and maximum parsimony trees. The EzBioCloud analysis based on the 16S-rRNA gene had shown *P. columellifera* subsp. *pallida* to be the top hit of strain CS16 with 99.35% sequence similarity over 1240 nucleotides. However, instead of clustering with the members of the genus *Pilimelia*, strain CS16 and *P. columellifera* subsp. *pallida* MB-SK 8^T formed the deepest branch in the tree, indicating that even *M. chalcea* DSM 43026^T (chosen as the outgroup) is more closely related to the members of the genus *Pilimelia* than strain CS16 and *P. columellifera* subsp. *pallida* MB-SK 8^T (GU269552) are.

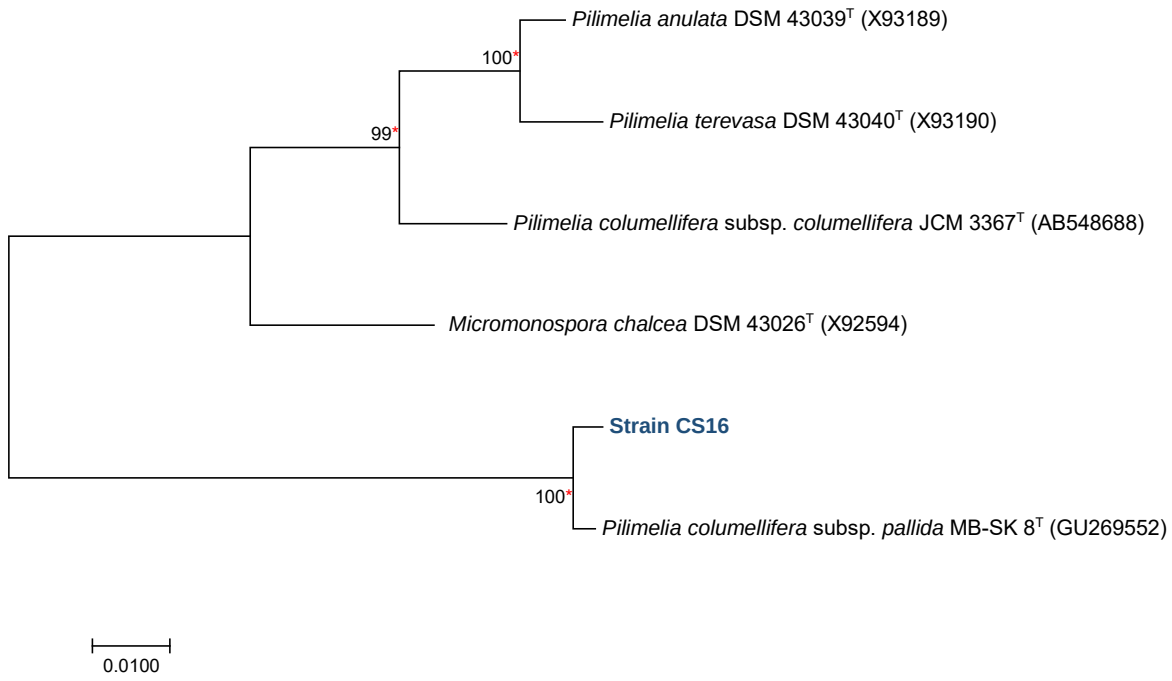

Figure 2.5a. Maximum likelihood phylogenetic tree of strain CS16 and the four type strains of the genus *Pilimelia* based on partial 16S rRNA gene sequences of 1226 nt. The 16S-rRNA gene sequence of *Micromonospora chalcea* DSM 43026^T was used as the outgroup. The value at each node represents the percentage bootstrap support calculated from 1000 resampled datasets. Only bootstrap values of ≥ 70% are displayed. The scale bar indicates 1 nucleotide change per 100 base pairs. "*" Indicates matching topology with the neighbour joining and maximum parsimony trees.

Further phylogenetic analysis was conducted using the 16S-rRNA gene sequences of the next five hits listed after *P. columellifera* subsp. *pallida* in the EzBioCloud analysis (all *Streptomyces* strains), resulting in the construction of an extended phylogenetic tree (Figure 2.5b). In Figure 2.5b it is seen that strain CS16 and "*P. columellifera* subsp. *pallida* MB-SK 8^T" (GU269552) clustered with the *Streptomyces* strains with very strong bootstrap support (100%; 16S-rRNA gene sequence alignment length was 1194 nucleotides). Strain CS16 clustered with "*P. columellifera* subsp. *pallida*" MB-SK 8^T (GU269552) with 83% bootstrap support. The *Pilimelia* and *Streptomyces* clades were also seen in the neighbour-joining and maximum parsimony trees. With this information, it is clear that GenBank sequence record GU269552 contains the 16S-rRNA gene sequence of a *Streptomyces* strain, not *P. columellifera* subsp. *pallida* MB-SK 8^T. According to a study by Yarza *et al.* (2013), the original *P. columellifera* subsp. *pallida* type strains (ATCC 43729^T and DSM 43799^T) were contaminated with a strain of *Streptomyces*. The GU269552 sequence was submitted to GenBank in 2009, prior to the discovery of the streptomycete contamination

(https://www.ncbi.nlm.nih.gov/nuccore/GU269552). Thus, strain CS16 belongs to the genus *Streptomyces* and is most closely related to *Streptomyces sanglieri* NBRC 100784^{T} (AB249945) showing 98.54% 16S-rRNA gene sequence similarity over 1240 nucleotides. Even though *S. sanglieri* NBRC 100784^{T} was the second hit of strain CS16 (after *P. columellifera* subsp. *pallida* MB-SK 8^{T}) according to the EzBioCloud database, strain CS16 clusters closely with *Streptomyces candidus* NRRL ISP-5141^{T}, which was the third hit according to the EzBioCloud database. The EzBioCloud database showed that strain CS16 also had 98.54% 16S-rRNA gene sequence similarity to *S. candidus* NRRL ISP-5141^{T} over 1240 nucleotides.

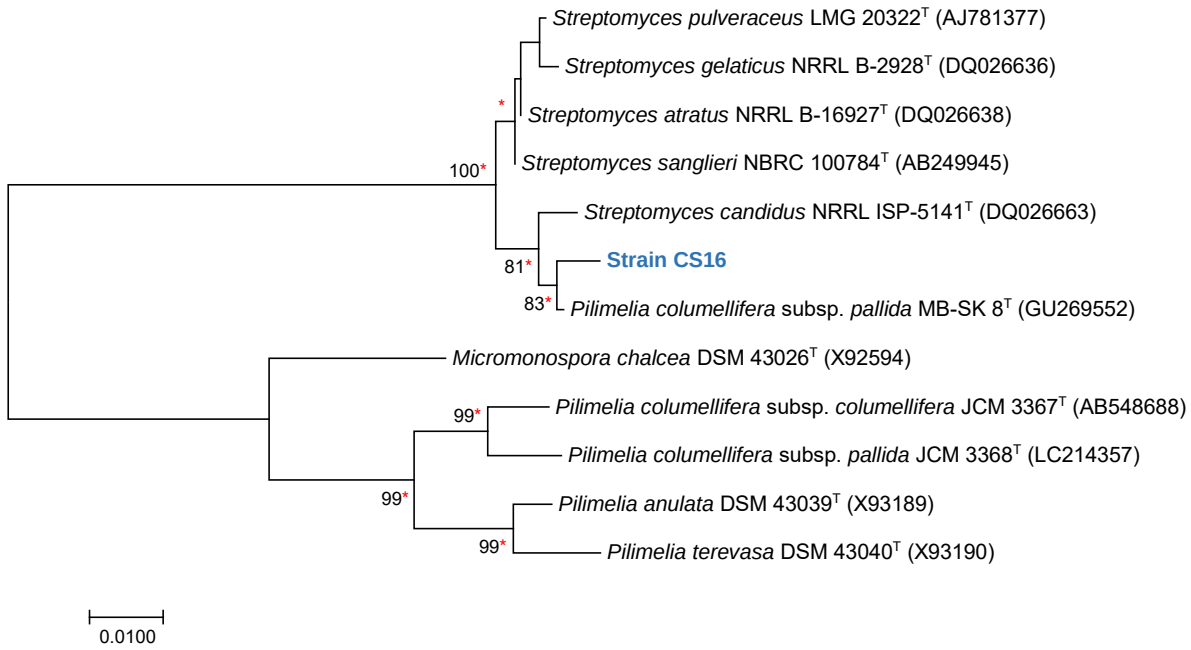

Figure 2.5b. Maximum likelihood phylogenetic tree of strain CS16 compared to strains of the genus *Pilimelia*, *Micromonospora chalcea* DSM 43026^{T} and the top five *Streptomyces* hits in an EzBioCloud analysis, based on partial 16S rRNA gene sequences of 1194 nt. The value at each node represents the percentage bootstrap support calculated from 1000 resampled datasets. Only bootstrap values of ≥ 70% are displayed. The scale bar indicates 1 nucleotide change per 100 base pairs. "*" Indicates matching topology with the neighbour joining and maximum parsimony trees.

2.3.3.4. Genus *Streptacidiphilus*

The genus *Streptacidiphilus* currently comprises of 13 species with validly published names (http://www.bacterio.net/streptacidiphilus.html). *Streptacidiphilus albus* is the type species (Kim *et al.*, 2003). Species belonging to the genus *Streptacidiphilus* are acidophilic and grow well over the pH range of 4.5 – 5.5. They can be found widely distributed in acidic environments, such as coniferous forest soil (Golinska *et al.*, 2013; Cho *et al.*, 2008).

2.3.3.4.1. *Streptacidiphilus* 16S-rRNA gene phylogenetic analysis

The phylogenetic position of *Streptacidiphilus* strain CS11 was determined by aligning its 16S-rRNA gene sequence with those of the 13 type strains of the genus *Streptacidiphilus* (Figure 2.6). *Micromonospora chalcea* DSM 43026^{T} (X92594*)* was used as the outgroup. *Streptacidiphilus* strain CS11 clustered with *Streptacidiphilus carbonis* DSM 41754^{T} (AF074412) with very strong bootstrap support (95%). This association was also seen in the neighbour joining and maximum parsimony trees. An EzBioCloud analysis revealed that the type strain of *S. carbonis* was the top hit of *Streptomyces* strain CS11 with 99.79% 16S-rRNA gene sequence similarity over 1452 nt. Furthermore, strain CS11 and the type strain of *S. carbonis* formed part of a bigger cluster containing the type strains of *Streptacidiphilus durhamensis*, *Streptacidiphilus albus*, *Streptacidiphilus hamsterleyensis*, *Streptacidiphilus neutrinimicus* and *Streptacidiphilus torunensis*, which had weak bootstrap support (68%). This clustering was also seen in the neighbour joining and maximum parsimony trees.

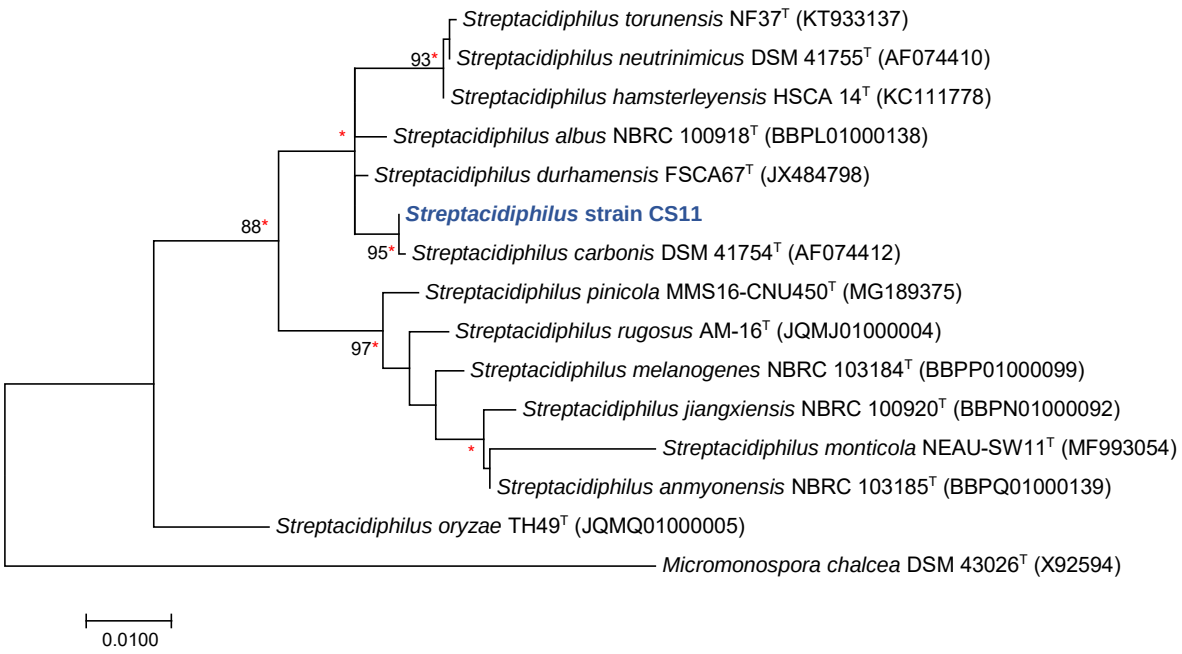

Figure 2.6. Maximum likelihood phylogenetic tree of *Streptacidiphilus* strain CS11 compared to all 13 type strains of the genus *Streptacidiphilus* based on partial 16S-rRNA gene sequences of 1364 nt. *Micromonospora chalcea* DSM 43026^T was used as the outgroup. The value at each node represents the percentage bootstrap support calculated from 1000 resampled datasets. Only bootstrap values of ≥ 70% are displayed. The scale bar indicates 1 nucleotide change per 100 base pairs. "*" Indicates matching topology with the neighbour joining and maximum parsimony trees.

2.3.3.4.2. *Streptacidiphilus gyrB* gene phylogenetic analysis

Since strain CS11 belongs to the rare genus *Streptacidiphilus*, it was decided to assess its phylogenetic relationship with the type strain of *S. carbonis* using another gene. The *gyrB* gene was chosen as there are *gyrB* gene sequences for most of the members of the genus *Streptacidiphilus* in the GenBank database. Furthermore, *gyrB* gene sequence analysis provides more information of the sequence similarity between species, as the gene is less conserved than the 16S-rRNA gene and therefore exhibits higher sequence variation. The strain CS11 *gyrB* gene sequence was aligned against the partial *gyrB* gene sequences of eight *Streptacidiphilus* type strains for which *gyrB* gene sequences are available. In Figure 2.7, it is seen that strain CS11 grouped with *S. carbonis* DSM 41754^T (JF424129) with very strong bootstrap support (99%). This association was also seen in the neighbour joining and maximum parsimony trees. This is in agreement with the *blastn* analysis, which revealed *S. carbonis* as the top hit of strain CS11 with 98.71% *gyrB* gene sequence similarity over 1133 nt (Table 2.3). The *gyrB* gene phylogenetic analysis provided support for the 16S-rRNA gene phylogenetic analysis, as strain CS11 and the type strain of *S. carbonis* formed part of a bigger, well supported cluster

(bootstrap value 100%) containing the same type strains as in the 16S-rRNA gene analysis (namely, *S. albus* and *S. neutrinimicus*) plus *S. oryzae* (the type strains of *S. durhamensis*, *S. hamsterleyensis* and *S. torunensis* were not included in the analysis, as there are no *gyrB* gene sequences for them in the GenBank database). This five-strain cluster was also seen in the neighbour joining and maximum parsimony trees.

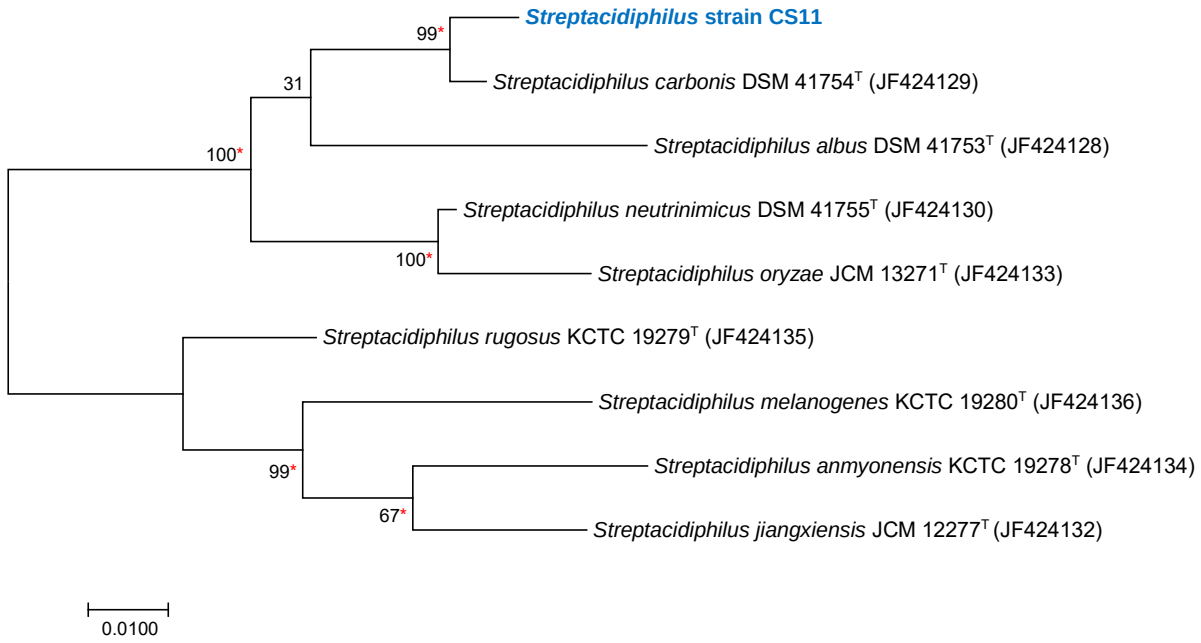

Figure 2.7. Maximum likelihood phylogenetic tree of *Streptacidiphilus* strain CS11 compared to eight strains of the genus *Streptacidiphilus* based on partial *gyrB* gene sequences of 852 nt. The value at each node represents the percentage bootstrap support calculated from 1000 resampled datasets. Only bootstrap values of ≥ 70% are displayed. The scale bar indicates 1 nucleotide change per 100 base pairs. "*" Indicates matching topology with the neighbour joining and maximum parsimony trees.

It can be noted that *Streptacidiphilus* strain CS11 clustered closely with the type strain of its top hit, *S. carbonis*, in both the 16S-rRNA and *gyrB* gene trees, indicating that strain CS11 is closely related to *S. carbonis*. As there is a genome sequence for *S. carbonis* NBRC 100919^T, it is recommended that the genome of strain CS11 be sequenced and that ANI and dDDH analyses be used to establish whether strain CS11 represents a new species in the genus *Streptacidiphilus*.

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CHAPTER 3

METAGENOMIC ANALYSIS: THE ACTINOBACTERIAL DIVERSITY OF SEDIMENT FROM THE SHALLOW WATERS OF A RESERVOIR USING CULTURE-INDEPENDENT METHODS

SUMMARY

Metagenomic analysis was used to analyse the actinobacterial diversity of a sediment sample from the shallow waters of the Silvermine reservoir in the Silvermine Nature Reserve, Cape Town. A total of 97 16S-rRNA gene clones was obtained from the reservoir sediment sample, RS1. The 16S-rRNA gene was amplified using actinobacterium-specific 16S-rRNA gene primers S-C-Act-0235-a-S-20-F and S-C-Act-0878-a-A-19-R, the PCR products were cloned and sequenced before identifying the species from which each PCR fragment was obtained using the EzBioCloud database. Rarefaction analysis based on unique phylotypes of the identified RS1 clone library revealed that the total actinobacterial diversity of the sample was not represented in the clone library. Therefore, further sampling and analysis of the sample site would uncover greater actinobacterial diversity. Pie charts were generated to analyse the total clonal diversity and the actinobacterial diversity of the reservoir sediment sample RS1 based on identifications to the order level (according to the EzBioCloud database). Additionally, a pie chart was generated to analyse the composition of cultured and uncultured clonal identifications. These pie charts revealed that 80% of the clone library were identified as actinobacterial species belonging to the orders *Acidimicrobiales, Streptomycetales, Streptosporangiales, Corynebacteriales*, *Sporichthyales* and the family *Jatrophihabitandaceae* (the remaining 20% was identified as non-actinobacterial species). The percentage compositions of the actinobacterial clonal diversity for each order were as follows: *Acidimicrobiales,* 56%; *Streptomycetales*, 29%; *Streptosporangiales,* 9%; *Corynebacteriales,* 4%; *Sporichthyales,* 1% and family *Jatrophihabitandaceae,* 1%.

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3.1. INTRODUCTION

The well-known and long-used culture-dependent approach to studying the bacteria in various environments has driven many advances in microbiology regardless of its limitations. These limitations are mainly related to the selectivity of the nutrient media and culturing conditions, which favour the growth of only a fraction of the bacterial community (Al-Awadhi *et al*, 2013). It is estimated that only 1% of bacteria in the natural environment can be cultured under laboratory conditions based on 16S rRNA gene sequence analysis (Vartoukian *et al.,* 2010). Over 88% of culturable isolates belong to one of four phyla: *Bacteroidetes, Firmicutes, Proteobacteria* and *Actinobacteria* (Nikolaki & Tsiamis, 2013). The main limitation of using culture-dependent techniques is that it underestimates the microbial diversity in samples that are under investigation (Vartoukian *et al.,* 2010). Species richness is the simplest way to describe microbial diversity. Quantifying species richness is important as it provides data addressing the total diversity of bacterial communities in various sites and also for comparison between sites under study (Cornell, 1999).

New methods, such as metagenomics, are necessary to overcome the limitations of culture-based methods. Metagenomics is a culture-independent approach of analysing large microbial communities irrespective of whether they can be cultivated on laboratory media or not. Instead, it identifies microbes by directly accessing their genetic material. This involves DNA extraction directly from an environmental source, PCR amplification of a region of the 16S rRNA gene, cloning the amplified DNA into an appropriate vector, and transforming it into bacteria that can be cultivated for DNA analysis (Handelsman, 2004). This allows access to the "concealed" DNA sequences of the 99% of microbes that cannot be cultured under laboratory conditions, in addition to those that can be cultured (Handelsman *et al.,* 1998). Metagenomics can therefore identify rare and new bacterial species and can also reveal DNA sequences encoding novel antibiotic compounds (Brady *et al.,* 2009).

It is important to note the limitations and advantages of both culture-dependent and culture-independent methods for the analysis of actinobacterial diversity (Al-Awadhi *et al.,* 2013). The major advantage of the traditional culture-dependent approach is that it offers the researcher the physical "material" that would be required for further study, whereas the more modern molecular techniques of the culture-independent approach do not. Metagenomic techniques are also subjected to bias problems, such as the preferential amplification of DNA from specific species, inadequate specificity of primers used in PCR, and the production of a single band

from several strains (Polz & Cavanaugh, 1998; Sipos *et al.*, 2007). Despite the limitations of these methods, the PCR based technique has proven to be one of the best tools for the analysis of species richness within complex bacterial environments (Xin *et al.*, 2008).

The aim of this part of the study was to analyse the actinobacterial diversity of sediment taken from the shallow waters of the reservoir in the Silvermine Nature Reserve, Cape Town, using culture-independent techniques. The metagenomic analysis of actinobacterial diversity served to complement the study of the actinobacterial diversity using culture-based techniques described in Chapter 2.

3.2. MATERIALS & METHODS

3.2.1. Sample collection

The sediment and soil samples used for the analysis of actinobacterial diversity would have preferentially been prepared simultaneously for the culture-dependent and culture-independent analyses, however, it was only decided at a later stage during the project to conduct a culture-independent analysis to further investigate the actinobacterial biodiversity in the Silvermine Nature Reserve. Fresh sediment from the shallow waters of the reservoir (RS) and soil from Elephant's Eye cave (CS) were collected in sterile Falcon® conical centrifuge tubes from the Silvermine Nature Reserve, Table Mountain National Park, Cape Town, during June 2018 (the samples for the culture-dependent analyses had been collected in February 2018). The fresh sediment and soil samples were stored at 4°C for no more than 24 hours before metagenomic DNA isolation for screening for the presence of actinobacteria. The pH of the sediment and soil samples were not measured at the time of metagenomic analysis, however, it was measured when acidic taxa were found amongst the clone hits.

3.2.2. DNA extraction and two step 16S-rRNA gene PCR amplification

Metagenomic DNA was extracted from 0.25g of each of the sediment and soil samples using the ZR Soil Microbe DNA miniprep Kit™ (Zymo Research, Irvine, California) according to the manufacturer's protocol.

Volumes of 50µL were used for each PCR set up, which contained 2mM $MgCl_2$, 0.5µM of each primer, 150µM of each dNTP, 1U Super-Therm *Taq* DNA polymerase (JMR Holdings, U.S.A.) and 100 – 500ng of metagenomic template DNA. The universal primers F1 (5'-AGAGTTTGATCITGGCTCAG-3') and R5 (5'-ACGGITACCTTGTTACGACTT-3') were used for the initial step of the two step 16S-rRNA gene PCR amplification and followed the method as described by Cook and Meyers (2003). The second step involved a "touchdown", nested PCR technique, previously described by Stach *et al.* in 2003, and was performed on the PCR product obtained from the first PCR step. The primers used in the "touchdown" PCR method involved specific actinobacterial primers, S-C-Act-0235-a-S-20-F (S20F) and S-C-Act-0878-a-A-19-R (A19R), designed to target and amplify a ~640bp fragment of actinobacterial 16S-rRNA genes (Stach *et al.,* 2003). These actinobacterium-specific primers were selected based on their ability to amplify 16S-rRNA gene sequences from most

actinobacterial species. The "touchdown" PCR conditions was the same as the first round of PCR in terms of reaction volume, $MgCl_2$ concentration, primer concentration and dNTP concentration. A volume of 2µL of the PCR product from the first round of PCR was used as the template DNA for the second round of PCR. The thermal-cycler set-up for the "touchdown" PCR was as follows: initial denaturation at 95°C for 4 min, 10 cycles of denaturation at 96°C for 30s, annealing at 72°C for 20s and extension at 72°C for 30s. The annealing temperature was decreased by 0.5°C per cycle throughout the first 10 cycles. This was followed by 15 cycles of 30s denaturation at 96°C, 20s annealing at 68°C, 30s extension at 72°C followed by a final 5 min extension at 72°C. All PCR reactions were carried out using a Techne TC-512 gradient thermal cycler.

The resulting PCR products were electrophoresed on 0.8% (w/v) agarose gels containing 0.8µg/mL EtBr at 90V in 1 X Tris-acetate-EDTA (TAE) buffer. *Pst*I-digested bacteriophage lambda DNA was used as a molecular-weight marker. The PCR products were visualized using an ultra-violet (UV) transilluminator at 365nm, excised from the gel and purified using the ISOLATE II PCR and Gel Kit (Bioline, www.bioline.com) following the manufacturer's instructions.

3.2.3. Cloning and plasmid purification

The purified PCR DNA was cloned using the pGEM®-T Easy Vector System (Promega, USA) and transformed into *Escherichia coli* cells following the manufacturer's protocols. The ligation and transformation steps were adjusted as follows: 1) the ligation reactions were set up using final volumes of 5µl instead of 10µl. 2) Transformation was performed according to the pGEM®-T Easy Vector System protocol using 15µl of *E. coli* DH5α competent cells (Bioline, London, United Kingdom) added to the 5µL DNA-ligation reactions. The transformed cells were mixed with 980µL of complete super optimal broth with catabolite repression (SOC) medium and incubated at 37 °C for 1 h. The transformed cells were then spread onto Luria-Bertani (LB) agar (10g tryptone, 5g yeast extract, 5g NaCl; dH_2O to 1L; pH 7) (Gerhardt *et al.*, 1994) plates that contained 80µL/mL X-galactosidase (X-gal), 0.5mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 100µg/mL ampicillin. Blue-white screening was used to identify putative recombinant clones, represented by white colonies, which were sub-cultured onto fresh LB agar plates containing only 100µg/mL ampicillin. Colony PCR was used to screen for inserts of the correct size (~640 bp) and was performed in 50µL reaction volumes

containing 2mM $MgCl_2$, 150µL of each dNTP, 0.5µM of each primer (S20F and A19R), 1U Super-Therm *Taq* polymerase (JMR Holdings, USA) and sterile dH_20 to make up to the final volume. Recombinant *E. coli* cells were transferred into the PCR tubes using sterile toothpicks. The resultant colony PCR products were electrophoresed on 0.8% (w/v) agarose gels containing 0.8µg/mL EtBr at 90V in 1 X TAE buffer and visualized using the ChemiDoc™ XRS+ Molecular Imager® (Bio-Rad) at 260nm.

Clones that were confirmed to contain inserts of the correct size were grown overnight (16 – 18 hours) at 37°C with shaking in 5ml volumes of liquid LB medium (Sambrook *et al.,* 1989) containing 100µg/mL ampicillin, before being subjected to plasmid DNA extraction. This was achieved with the use of a Zyppy™ Plasmid Miniprep Kit (Zymo Research, Irvine, USA) following the manufacturer's instructions. The concentration of the resulting purified plasmid DNA was measured using a Nanodrop™ spectrophotometer, model ND-2000. Samples of purified plasmid DNA, diluted to 100ng/mL in Zyppy™ Plasmid Miniprep Kit elution buffer, were prepared for sequence analysis.

3.2.4. Plasmid DNA sequencing, analysis and identification

All purified plasmid DNAs were sequenced with the S-C-Act-0205-a-S-20-F primer as a service by Macrogen Europe Inc., Amsterdam-Zuidoost, Netherlands. Sequences of ~640bp of the 16S-rRNA gene were edited using Chromas version 2.6.4, as described in section 2.2.1.6, to remove vector sequences. Edited sequences were uploaded to the EzBioCloud database to determine their closest related cultured or uncultured strain (Yoon *et al.,* 2017). All clones identified as actinobacteria were recorded together with their respective cultured or uncultured top hits.

3.2.5. Analysis of biodiversity

Clones that were suspected to be duplicates of the same 16S-rRNA gene sequence were identified by analysing the 16S-rRNA gene sequence similarity pairwise distance matrix for each order in DNAMAN version 4.13 (Lynnon Biosoft). Any clone with 100% sequence similarity to another clone in the clone library was removed from the analysis. Unique phylotypes were defined using ≤98.8% 16S-rRNA gene sequence similarity as the criterion for defining species. Actinobacterial rarefaction curves (Gotelli & Colwell, 2001) were manually

plotted in Microsoft Excel with the number of sequenced clones against the number of unique clones (defined as having ≤98.8% sequence similarity to their top hit in the EzBioCloud database).

Pie charts were generated using Microsoft Excel based on the number of unique clones identified as belonging to various actinobacterial orders, as well as the number of uncultured and cultured top hits according to the EzBioCloud database.

3.3. RESULTS & DISCUSSION

3.3.1. Clone sequencing & rarefaction analysis

A total of 97 clones containing inserts, identified by blue-white screening and colony PCR, was obtained from the reservoir sediment sample RS1. No positive clones were obtained for the cave soil sample (CS1) due to suspected poor DNA quality, and thus investigation of the CS1 sample was discontinued.

Appendix A provides a table displaying all the results for reservoir sediment sample RS1 collated from the EzBioCloud analysis of the clone library. It was noted that even though primers S20F and A19R, used for PCR amplification of the metagenomic DNA, were designed to target actinobacterial DNA, a few non-actinobacterial DNA sequences were also identified. Fifty five (55) of the 97 identified clones (56.7%) displayed sequence similarities equal to or less than 98.8% to all the sequences in the EzBioCloud database (therefore representing unique phylotypes). The 98.8% identity threshold was used to generate a rarefaction curve that is used to analyse the species richness of a sample. The slope of a rarefaction curve is an indication of the diversity of an environment and the rate at which new phylotypes would be discovered if more samples were to be analysed (Kuang *et al.,* 2018). The rarefaction curve indicates the possibility of finding additional new species if more clones were to be sequenced and analysed (Yang *et al.,* 2005). A flattening of the curve, in other words a curve reaching its asymptote, would mean that the total diversity of the sample had been sufficiently assessed and the number of species found would not increase by much if the sample size were to be increased.

The rarefaction curves were generated based on the number of sequenced clones that had percentage sequence similarities of ≤98.8% according to the EzBioCloud analysis (unique phylotypes) and are depicted in Figure 3.1. The rarefaction curve A (blue) was generated based on the entire clone library from the reservoir sediment sample RS1 (including non-actinobacteria), whereas the rarefaction curve B (red) was generated based only on the number of clones identified as actinobacteria. The slopes of both curves A and B do not reach their asymptotes and this indicates that the sampling of the clones for new phylotypes had not yet reached saturation point. This means that the clone library did not represent the full actinobacterial diversity of sample RS1. Therefore, further sampling of the reservoir sediment would be expected to identify new actinobacterial biodiversity in the sample.

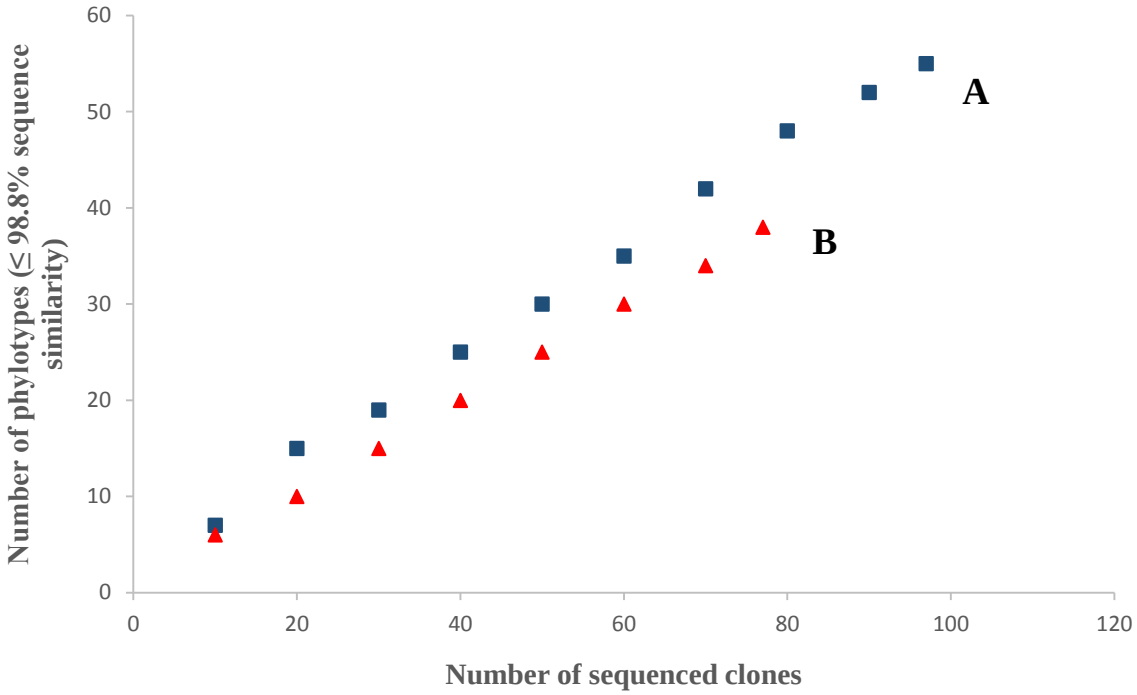

Figure 3.1. Rarefaction analysis of the clone library generated from the reservoir sediment sample RS1. The rarefaction curves depict the rate at which new phylotypes were discovered until the point where sampling ended based on the number of sequenced clones that had ≤98.8% sequence similarity to their top hit according to an EzBioCloud analysis. Curve A (■) includes all species identifications from the clone library, while curve B (▲) only includes actinobacterial identifications.

3.3.2. Actinobacterial composition of reservoir sediment

Pie charts were generated to represent the clone diversity found in the reservoir sediment sample RS1. Sequence analysis of the 16S-rRNA gene and sequence identification by EzBioCloud analysis revealed various actinobacterial species belonging to numerous genera, families and orders. In comparison to the results obtained from the culture-dependent analysis described in Chapter 2, it was noted that majority of the clone identifications obtained from the culture-independent analysis were those of acidotolerant or acidiphilic actinobacteria (Appendix A), whereas the culture dependent analysis did not identify any acidotolerant or acidiphilic actinobacteria. This can be attributed to the acidic pH of the reservoir sediment (pH 4.5 - 5) and the higher pHs (7.2 - 7.3) of the media used for isolating actinobacteria in the culture-dependent part of the study. Therefore, if the pH of the growth media had been adjusted

to match that of the reservoir sediment, acidotolerant and acidophilic actinobacteria may have been isolated.

Appendix A shows the detailed results of the RS1 clone library 16S-rRNA gene identifications to the lowest known taxonomic level available from EzBioCloud as, in some cases, the family or genus of many of the top hits is not currently known. Therefore, the pie charts were generated based on identifications at the order level. Figure 3.2 represents the actinobacterial composition of the reservoir sediment sample RS1 together with non-actinobacterial species, while Figure 3.3 shows only the actinobacterial composition of sample RS1. Eighty percent (80%; 78 clones) of the clone library was identified as actinobacterial species (Figure 3.2). Figure 3.4 shows that just 31% of the clones had top hits identified as culturable actinobacteria.

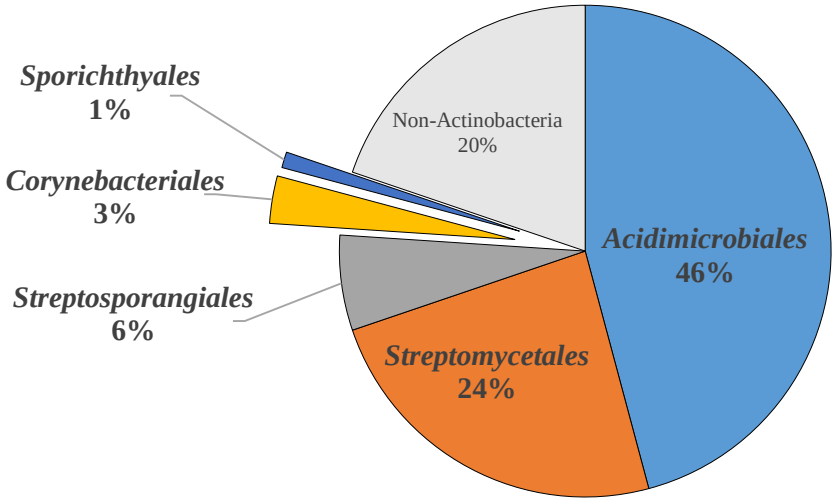

Figure 3.2. The percentage compositions of the total clone diversity (including non-actinobacteria) from the reservoir sediment sample RS1, based on identifications to the order level.

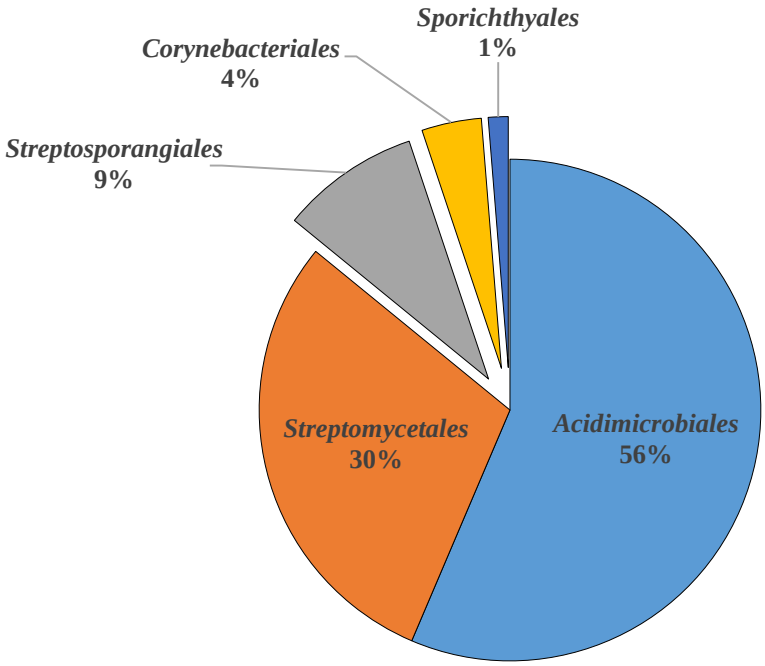

Figure 3.3. The percentage compositions of the total actinobacterial clone diversity from the reservoir sediment sample RS1, based on identifications to the order level.

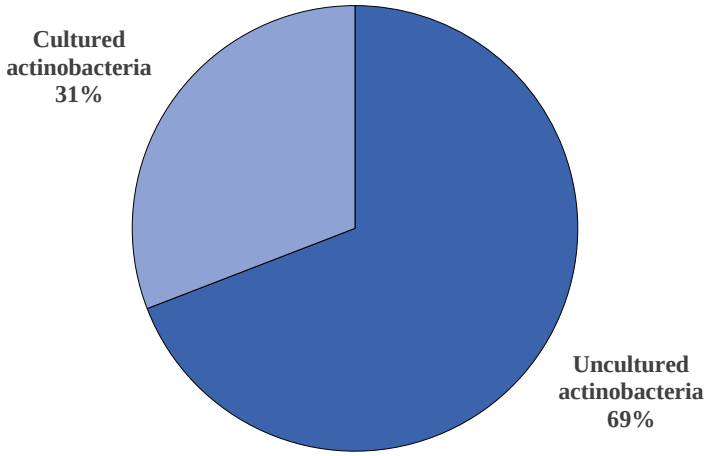

Figure 3.4. The percentage clonal compositions of cultured and uncultured actinobacteria in the reservoir sediment sample RS1.

3.3.2.1. Order *Acidimicrobiales*

In Figure 3.3, the order *Acidimicrobiales* is seen to represent the highest proportion of actinobacteria with 56% of the total identified actinobacterial diversity. The order *Acidimicrobiales* contains the families *Acidimicrobiaceae*, *Iamiaceae* and *Ilumatobacteraceae*. The family *Acidimicrobiaceae* is currently comprised of the genera *Acidimicrobium*, *Aciditerrimonas*, *Acidithiomicrobium*, *Acidithrix*, *Ferrimicrobium* and *Ferrithrix*. Different strains of the genus *Aciditerrimonas* were found, making up approximately 72.7% of the identified strains of the order *Acidimicrobiales* (32 of 44 clones), while the genera of the rest of the strains were unknown, as they had only been identified to the family level as members of the family *Acidimicrobiaceae*. Species belonging to the family *Acidimicrobiaceae* are known to be moderately thermophilic, acidophilic and are capable of iron oxidation and reduction (Clarke & Norris, 1996; Bridge & Johnson, 1998; Johnson *et al.*, 2009). The genus *Aciditerrimonas* currently contains one species with a validly published name (http://www.bacterio.net/aciditerrimonas.html) namely, *Aciditerrimonas ferrireducens*, which contains moderately thermoacidophilic actinobacteria. The first representative of this genus was isolated by Itoh *et al*. (2011) from a solfataric field. It is, therefore, fitting that species from the family *Acidimicrobiaceae* were identified from sample RS1 as the reservoir sediment is slightly acidic (pH 4.5 – 5). It would, therefore, be interesting to target and isolate novel acidophilic actinobacterial species from the Silvermine reservoir. All of the identified clones belonging to the order *Acidimicrobiales* form part of the 69% of uncultured actinobacteria (Figure 3.4), except for two clones (RS1-C9 and RS1-C113). However, the top hits of these clones currently do not have validly published names. Forty two (42) out of 44 *Acidimicrobiales* clones (95%) were found to be most closely related to uncultured strains. Therefore, even if the pH of the isolation plates discussed in Chapter 2 had been lowered to 4.5-5, it is likely that not much of the acidotolerant and acidiphilic actinobacterial biodiversity identified in the metagenomic analysis would have been isolated from the reservoir sediment.

3.3.2.2. Order *Streptomycetales*

The proportion of the identified actinobacteria belonging to the order *Streptomycetales* was 30% (Figure 3.3), representing the second largest group of actinobacteria identified in sample RS1. The order *Streptomycetales* contains a single family (*Streptomycetaceae*), consisting of six genera: *Allostreptomyces*, *Embleya*, *Kitasatospora*, *Streptacidiphilus*, *Streptomyces* and *Yinghuangia*. The 23 clones identified as belonging to the order *Streptomycetales* all belong to

the genus *Streptomyces*. Three of the clones identified as *Streptomyces* species formed part of the 69% of clones matching uncultured actinobacteria, while the rest formed the bulk of the cultured actinobacteria (31%) seen in Figure 3.4.

3.3.2.3. Order *Streptosporangiales*

The order *Streptosporangiales* made up 9% of the actinobacterial diversity identified in sample RS1 (Figure 3.3). This is a very low proportion compared to the two orders described above (sections 3.3.2.1 and 3.3.2.2). The order *Streptosporangiales* currently comprises the families *Nocardiopsaceae*, *Streptosporangiaceae* and *Thermomonosporaceae* (Bergey's Manual of Systematics of Archaea and Bacteria (BMSAB); Goodfellow *et al.*, 2012). All the clones identified as belonging to the order *Streptosporangiales* had top hits that were only identified to the order level with one exception, namely, clone RS1-C124, which was most closely related to *Actinocorallia longicatena*, previously known as *Actinomadura longicatena* (Zhang *et al.*, 2001). The genus *Actinocorallia* currently comprises of 9 species with validly published names (http://www.bacterio.net/actinocorallia.html) and belongs to the family *Thermomonosporaceae.* The family *Thermomonosporaceae* was first described by Stackebrandt *et al.* (1997) and emended by Zhang *et al.* (2001) and Zhi *et al.* (2009) and is known to encompass mesophilic and thermophilic actinobacteria (Zhang *et al.*, 2001). All the identified *Streptosporangiales* clones formed part of the 69% of uncultured actinobacteria (Figure 3.4), except for the clone most closely related to *A. longicatena*.

3.3.2.4. Order *Corynebacteriales*

The order *Corynebacteriales* represents 4% of the actinobacterial clonal diversity (Figure 3.3), forming the second smallest proportion of identified actinobacterial clones. The order includes many members that are found in various environmental habitats, such as marine and soil systems, and some strains are known to be pathogenic to humans and domesticated animals (Shahraki *et al.*, 2017; Toney *et al.*, 2010; Meena *et al.*, 2015). The family *Mycobacteriaceae* is one of eight families in the order *Corynebacteriales* (*Corynebacteriaceae*, *Dietziaceae*, *Gordoniaceae*, *Lawsonellaceae*, *Mycobacteriaceae*, *Nocardiaceae*, *Segniliparaceae* and *Tsukamurellaceae*; Nouioui *et al.*, 2018) and includes the genus *Mycobacterium*. The genus *Mycobacterium* contains 199 species with validly published names

(http://www.bacterio.net/mycobacterium.html). Only three clones were identified as belonging to the order *Corynebacteriales* and their top hits were *Mycobacterium conspicuum, Mycobacterium cookii* and *Mycobacterium asiaticum*. Further sampling of the reservoir sediment would allow for investigation of additional mycobacteria present at the site, and potential isolation of novel mycobacterial species. The *M. conspicuum* and *M. cookii* clones contributed to the 31% cultured actinobacterial composition while the clone (RS1-C54) most closely related to *Mycobacterium asiaticum* strain 1081914.2 contributed to the 69% of uncultured actinobacterial composition (Figure 3.4).

3.3.2.5. Order *Sporichthyales*

The proportion of actinobacterial clones belonging to the order *Sporichthyales* is 1% (Figure 3.3). The order *Sporichthyales* currently comprises the single family *Sporichthyaceae*, which contains the single genus, *Sporichthya* (Nouioui *et al*., 2018). The genus *Sporichthya* was first described by Lechevalier *et al.* in 1968 and is considered a rare taxon as only five strains were isolated in the Lechevalier laboratory. Other studies reported that strains belonging to the genus *Sporichthya* were isolated from soil samples, one being a sample from a greenhouse (Suziki *et al.,* 1999; Takeuchi *et al.,* 1996). However, *Sporichthya* strains are extremely difficult to isolate, as no other *Sporichthya* isolates have since been described. In this study, only one *Sporichthyales* clone (RS1-C66) was identified. *Sporichthyales* clone (RS1-C66) was found to be most closely related to *Sporichthyales* strain E1B-B6-114 (Appendix A) and was amongst the 69% of uncultured actinobacteria (Figure 3.4). One would have to use targeted isolation methods to attempt to isolate strains belonging to the genus *Sporichthya* (Suziki *et al.,* 1999; Takeuchi *et al.,* 1996).

3.3.2.6. Family *Jatrophihabitandaceae*

The number of clones belonging to the family *Jatrophihabitandaceae* contributed to 1% of the actinobacterial biodiversity found in the Silvermine reservoir sediment. The family *Jatrophihabitandaceae* is not currently assigned to an order (Nouioui *et al*., 2018) and was therefore not included in Figures 3.2 and 3.3. Clone RS1-C94 was identified as belonging to the genus *Jatrophihabitans.* The genus *Jatrophihabitans* was described by Madhaiyan *et al.* (2013) and currently comprises of five species with validly published names: *Jatrophihabitans*

endophyticus, *Jatrophihabitans fulvus*, *Jatrophihabitans huperziae*, *Jatrophihabitans soli* and *Jatrophihabitans telluris* (http://www.bacterio.net/jatrophihabitans.html). Members of the genus *Jatrophihabitans* have previously been isolated from grass soil and sediment from lava forest wetlands (Jin *et al.*, 2015; Lee *et al.*, 2018). *Jatrophihabitans* clone RS1-C94 contributed to the 69% of uncultured actinobacteria (Figure 3.4). Further sampling of the reservoir sediment sample site would provide more insight into the total actinobacterial diversity at the site, and targeted isolation might allow cultivation of strains belonging to the genus *Jatrophihabitans*.

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CHAPTER 4

ANTIMICROBIAL ANTIBIOTIC ANALYSIS OF ACTINOBACTERIAL ISOLATES

SUMMARY

Thirty seven (37) putative actinobacterial isolates out of the 56 that were isolated from reservoir sediment, a cave wall and the cave soil in the Silvermine Nature Reserve in Cape Town (Chapter 2) were screened for antimycobacterial activity and thereafter investigated for their antibiotic spectrum. The remaining isolates were disregarded, as they were deemed to be non-actinobacteria. A standard over-lay method was used for all antibiotic screening. The 37 putative actinobacterial isolates were screened for antimycobacterial activity against the non-pathogenic *Mycobacterium aurum* strain A+, which has a similar antibiotic susceptibility profile to that of *Mycobacterium tuberculosis*. A total of five identified actinobacterial strains (*Streptomyces* strains RS6, RS7, RS9, RS13 and RS15) and an unidentified actinobacterium, strain RS4, demonstrated very strong antimycobacterial activity (zone of growth inhibition of over 3000 mm^2). It was noted that most of the strong to very strong antimycobacterial activity was produced by *Streptomyces* strains isolated from the reservoir sediment, suggesting that it may be a good source for discovering novel antibiotic compounds. The antibiotic spectra of the 37 isolates were then investigated against *Staphylococcus aureus* strain ATCC 25923 and *Escherichia coli* strain ATCC 25922: 15 strains were active against *S. aureus* ATCC 25923 and only three were active against *E. coli* ATCC 25922. A total of 11 strains (*Streptomyces* strains CS1, CS3, CS12, CS18, CS19, CW5, RS3, RS6, RS9, RS13 and RS15), displaying varying strengths of antimycobacterial antimicrobial activity, were selected for antibiotic extraction. The resulting crude extracts were then subjected to spot bioautography to test for antibacterial activity. The organic compounds extracted from the cell mass of *Streptomyces* strain CS3 and the broth fraction of *Streptomyces* strain RS3 demonstrated strong activity against *M. aurum* strain A+. However, these fractions did not display strong activity against *S. aureus* strain ATCC 25923 (the test against *E. coli* strain ATCC 25922 was inconclusive). Lastly, the crude extracts of 15 actinobacterial strains (*Micromonospora* strain RS10 and *Streptomyces* strains CS1, CS3, CS12, CS18, CW2, CW5, RS3, RS6, RS7, RS9, RS13, RS15, RS18 and RS19) were additionally tested for antiplasmodial activity against *Plasmodium falciparum* strain NF54. There was a total of seven strains active against *Plasmodium* namely, *Streptomyces* strains

CW2, CW5, RS3, RS7, RS13, RS15 and RS19. *Streptomyces* strains CW2, CW5 and RS7 displayed the strongest activity against *P. falciparum* strain NF54 with IC_{50} values below the guideline threshold of 1 µg/mL (strain CW2 culture broth crude extract: IC_{50} 40 ng/mL, strain CW5 culture broth crude extract: IC_{50} 128 ng/mL and strain RS7 culture broth crude extract: IC_{50} 70 ng/mL).

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4.1. INTRODUCTION

The number of microbial diseases is increasing every year. This makes them a great threat to public health and welfare (Jones *et al.*, 2008; Morens *et al.*, 2004). Over 200 diseases are known to be caused by bacteria, fungi, protozoa and other microbes in humans (Diane *et al.*, 2010). Of these microbial pathogens, bacteria cause 10% to 30% of diseases and protozoa cause 10.7% of diseases, leading to numerous deaths per year (Taylor *et al*., 2001; Woolhouse & Gowtage-Sequeria, 2005; Cleaveland *et al*., 2001). Tuberculosis (TB), caused by the bacterium, *Mycobacterium tuberculosis*, is one of the top ten leading causes of death in Africa (https://www.who.int/tb/en/). *M. tuberculosis* is constantly evolving and becoming resistant to the first-line drugs for treating TB. It was estimated that multidrug-resistant TB (MDR-TB) was the cause of 82% of global TB cases in 2017 (https://www.who.int/tb/en/). This is a major problem faced by the medical and pharmaceutical sectors, thus, novel antimicrobial compounds with new structures and mechanisms of action are urgently required.

Actinobacteria are a natural resource from which potent antibiotics have already been isolated (Valan Arasu *et al.*, 2008; Ikeda *et al.*, 2003). Rare actinobacteria (non-*Streptomyces* species) are an ideal place to find novel compounds, as they are understudied. However, they are more difficult to isolate and culture under standard laboratory conditions. Another option is to look for actinobacteria in unexploited environments, such as aquatic habitats, caves and endophytes from plants. The study of rare actinobacterial species and *Streptomyces* species isolated from lesser studied environments could, therefore, allow for the discovery of compounds with unique structures. Members of the genus *Streptomyces* are widely studied for their antibiotic compounds and over 70% of described antibiotic compounds of microbial origin are produced by this genus (Berdy, 2005). Furthermore, a study by Watve *et al.* (2001) showed that only ~3 percent of all *Streptomyces* antimicrobial compounds have been discovered. Thus, *Streptomyces* strains represent a huge resource of novel antimicrobial compounds. Examples of *Streptomyces* antibiotics include streptomycin, produced by *Streptomyces griseus* (Waksman & Schatz, 1945), and streptothricin, produced by *Streptomyces lavendulae* (Kobayashi *et al*., 1986). Streptomycin was the first antibiotic used to treat TB and is a good-broad spectrum antibiotic, acting against multiple groups of bacteria, although it causes serious side effects. An example of TB drugs produced by non-*Streptomyces* actinobacteria is the rifamycins, produced by *Amycolatopsis rifamycinica* (Saxena *et al.*, 2014) and *Micromonospora rifamycinica*, which were isolated from an aquatic environment (mangrove

sediment; Huang *et al.*, 2008). Rifampicin, one of the front-line drugs used to treat TB, is a semi-synthetic derivative of a rifamycin produced by an actinobacterium.

This chapter presents the investigation of the antimycobacterial antibiotic potential of all presumptive actinobacterial isolates that were isolated from the understudied habitats (Silvermine reservoir sediment, soil from Elephant's Eye cave and the cave wall) and described in Chapter 2. The isolates were screened against *Mycobacterium aurum* strain A+, a non-pathogenic mycobacterium with a similar antibiotic susceptibility profile to that of *M. tuberculosis* (Chung *et al.*, 1995). Therefore, screening for anti-*M. aurum* strain A+ antibiotic activity could give us more insight into the potential of the actinobacterial isolates to produce antibiotics to treat TB. In addition, each actinobacterial isolate was subject to antimicrobial analysis to determine their antibiotic spectrum (against *Staphylococcus aureus* strain ATCC 25923 and *Escherichia coli* strain ATCC 25922). The organic compounds of the actinobacterial strains that demonstrated anti-*M. aurum* activity in agar overlays were extracted from culture broths and subjected to spot bioautography to confirm their activity against *M. aurum* strain A+, *S. aureus* strain ATCC 25923 and *E. coli* strain ATCC 25922. Furthermore, the crude extracts of selected strains with antibacterial activity were selected for antiplasmodial testing by a PhD student in the Division of Clinical Pharmacology, Faculty of Health Sciences, University of Cape Town (UCT).

4.2. MATERIALS AND METHODS

4.2.1. Screening for antimycobacterial activity

All presumptive actinobacterial colonies described in Chapter 2 using culture dependent methods were tested for antibiotic activity using a standard agar-overlay method (Curtis, 2015). Antimycobacterial antibiotic activity was tested by screening all isolates against *M. aurum* strain A+ as it has a similar susceptibility profile to that of *M. tuberculosis* (Chung *et al.*, 1995). Additional bacterial test strains, *E. coli* strain ATCC 25922 and *S. aureus* strain ATCC 25923, were used to determine the antibiotic spectrum of antibiotic producing isolates. Each isolate was stab inoculated, using sterile toothpicks, onto DSMZ #553, JCM61 and MB agar plates (agar media described in section 2.2.1.2) divided into quadrants, so that there were four isolates per plate. All stab inoculated plates were incubated at 30°C for 10 days before being overlaid with the bacterial test strains. In preparation for the overlays, 5mL cultures of each bacterial test strain were cultivated in 2YT medium [16g tryptone (Merck Biolab), 10g yeast extract (Merck Biolab), 5g NaCl (Saarchem/MERCK), distilled water to 1L, pH 7.0] overnight (14 – 18 hours) with shaking at 37°C on the 9th day of incubation. Each overnight bacterial culture was inspected for contamination by Gram staining and streaking for single colonies on 2YT agar plates (1.5% agar) before measuring the optical density at 600nm (OD_{600nm}) using a Beckman DU®-64 spectrophotometer. The optical density reading was then used to determine the appropriate volume of each bacterial test culture to be added to the stab inoculated plates using the empirical formulae of OD_{600nm} × culture volume (µL) = 160 for *M. aurum* strain A+ and *S. aureus* strain ATCC 25923 and OD_{600nm} × culture volume (µL) = 4 for *E. coli* strain ATCC 25922. This was done in order to ensure that a standardized amount of cells was used for the overlays in each test. The calculated volume of each bacterial culture was inoculated separately into 6 mL of 2YT sloppy agar (0.7% agar) and poured gently over the stab inoculated plates without washing any spores from the actinobacterial colonies. The overlaid plates were incubated at 37°C for 2 days (48 hours) for *M. aurum* strain A+, while plates were incubated overnight (14 – 18 hours) for *E. coli* strain ATCC 25922 and *S. aureus* strain ATCC 25923, before inspecting them for zones of growth inhibition.

The presence or absence of aerial mycelium on the actinobacterial colonies was recorded prior to the test bacterium overlays. Antibiotic activity was observed as zones of clearing surrounding the actinobacterial colonies, indicating inhibition of growth of the test bacterium. The zone of inhibition (ZOI) was calculated by subtracting the area of the colony (mm^2) from

the area of the clear zone (mm^2). The area of the zone of inhibition (ZOI) represents the strength of the activity of the antibiotic compounds produced, where ZOI < 1000 mm^2 indicates weak activity, 1000mm^2 < ZOI < 2000mm^2 indicates moderate activity, 2000mm^2 < ZOI < 3000mm^2 indicates strong activity and ZOI > 3000mm^2 indicates very strong activity.

4.2.2. Organic compound solvent extraction

Eleven isolates displaying antimycobacterial antibiotic activity across a range of weak, moderate and strong levels were selected for organic compound solvent extraction. Bacterial cultures were incubated in 20 mL volumes of either DSMZ #553 or JCM61 in 250-mL Erlenmeyer flasks, depending on the medium in which the isolate produced the best activity in the agar overlays. The cultures were incubated at 30°C, with shaking, for a period of 4 days or until sufficient cell mass was obtained to inoculate 100 mL of the same medium in a 1-litre Erlenmeyer flask. The entire volume of the 20-mL culture was used as the inoculum for the 100 mL of medium. The scaled-up culture was then incubated for a further 10 days at 30°C with shaking to obtain a higher cell mass and to allow sufficient time for antibiotic/s to be produced. Before extraction, the cultures were checked for purity by Gram stain and streaking for single colonies on DSMZ #553 and JCM61 agar plates.

Each pure culture was filtered through a pair of paper coffee filters (size 1 × 4, House of Coffees) to separate the filamentous cell mass from the liquid broth, giving two fractions: cell mass (CM) and broth. The cell mass was mixed with 4 mL 100% ethyl acetate (EtOAc) in a Schott bottle and topped up with enough 100% methanol (MeOH) to submerge the cells. This cell suspension was shaken at room temperature at 120 rpm for one hour to allow organic compounds to be extracted from the cells into the MeOH. After one hour, the extracted cells were removed from the MeOH suspension by filtering through a pair of size-102 paper coffee filters into a glass beaker. The MeOH in the cell mass extract was left to evaporate in a fumehood to obtain a dry crude cell mass extract. Antibiotic compound/s from the broth fraction were extracted by adding EtOAc to the broth fraction at one third of the culture volume (approximately 30 mL) in a separating funnel. The mixture of culture broth and EtOAc was shaken vigorously for 60s to extract the mid-polarity organic compounds from the culture broth into the EtOAc. This mixture was then put aside to stand at room temperature for one hour to allow the organic phase to separate from the aqueous phase. The lower, aqueous phase was slowly drained from the separating funnel and collected in a glass beaker. Thereafter, the

EtOAc layer was poured into a separate glass beaker. The extraction of the culture broth was repeated twice more with EtOAc to extract all the mid-polarity organic compounds. The EtOAc layers from the three extractions of each culture broth were pooled, while the aqueous phase was discarded. The pooled EtOAc fractions were then mixed vigorously by shaking in a separating funnel and left to stand overnight at room temperature to allow separation of any traces of aqueous phase. The final EtOAc extract was poured into a glass beaker and left in a fumehood to allow the EtOAc to evaporate, leaving the dry crude broth extract.

For both the dried organic crude cell mass and broth extracts, 2 mL of EtOAc was added to the glass beaker and swirled around. The EtOAc was removed to a weighed, labelled, 2-mL bench-top centrifuge tube. Then, 2 mL of MeOH was added to the same beaker to dissolve compounds that did not dissolve in the EtOAc. The MeOH was removed to another weighed, labelled, 2-mL bench-top centrifuge tube. These concentrated extracts were left to evaporate overnight (16 – 18 hours) at room temperature in a fumehood. The tubes were weighed again to determine the mass of each crude antibiotic extract and stored at -20°C until needed for antimicrobial activity analysis by spot bioautography (Betina, 1973) and antiplasmodial activity analysis.

4.2.3. Spot bioautography

Each of the dried, concentrated crude cell mass and culture broth fractions extracted from each selected actinobacterial strain were re-dissolved in appropriate amounts of MeOH and EtOAc to give a concentration of 20mg/mL. Volumes of 5µL of each cell mass and culture broth crude extract were spotted separately onto clean strips of silica gel 60F aluminium thin-layer chromatography (TLC) sheets (Merck 1.05554.0001). The solvents were left to evaporate from the TLC plates for 5 – 10 mins at room temperature before covering with a test bacterium culture.

The solvent-extracted organic compounds were tested for activity against *M. aurum* strain A+, *E. coli* strain ATCC 25922 and *S. aureus* strain ATCC 25923. These bacterial test strains were grown in 5mL volumes of 2YT broth overnight (14-18 hours), with shaking, at 37°C. The purity of the overnight cultures was confirmed by Gram stain and streaking for single colonies on 2YT agar plates. A spectrophotometer was used to measure the optical density (OD) of the cultures at 600nm before diluting to an OD of 0.5 with sterile 2YT medium, to ensure that the same amount of cells was used in each test. Non-absorbent, sterile cotton wool pads (Dove) were used to dab each of the bacterial test strain cultures (separately) over the surface of the

prepared TLC plates containing the dried organic compounds. The TLC plates were carefully placed on damp paper towel in a sealed plastic container and were incubated overnight for 14-16 hours at 37°C to allow any antibiotics to interact with the cells of the test bacterium. Thereafter, a solution of 0.25% MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma M2128) was dabbed over the culture-covered TLC plates with non-absorbent, sterile cotton wool pads. The MTT-treated TLC plates were then incubated at 37° C in a sealed container for one hour or until a colour change from yellow to blue/purple was observed. The colour change is due to MTT being reduced to an insoluble formazan derivative by the metabolism of living cells. The expected results were to observe zones of clearing (no colour change) around the spots of the organic compounds that had antibacterial activity against the bacterial test strains. Negative results was observed when the entire silica TLC plate changed colour from yellow to blue/purple, indicating no antibiotic activity. The resultant bioautographs were scanned to record the results, as the blue/purple colour fades over time.

4.2.4. Testing for antiplasmodial activity

The crude cell mass and culture broth extracts of interesting actinobacterial strains, displaying varying strengths of antimycobacterial activity, were tested for antiplasmodial activity against a drug sensitive strain of *Plasmodium falciparum*. *P. falciparum* strain NF54 was cultured continuously by Mrs Sumaya Salie and Mr Virgil Verhoog in the Division of Clinical Pharmacology's Tissue Culture Lab at the University of Cape Town, Faculty of Health Sciences, using the method of Trager and Jensen (1976). The *in vitro* efficacy of the actinobacterial crude cell mass and culture broth extracts was determined using the parasite lactate dehydrogenase assay (pLDH) by Mr Daniel Watson, a PhD student at UCT's Division of Clinical Pharmacology, using the method of Makler *et al.* (1993). Samples of each crude extract made up to 20 mg/mL in the same solvent they were re-dissolved in (i.e. ethyl acetate or methanol), were tested over a concentration range from 5 µg/mL to 9.8 ng/mL. The crude extracts were tested together with two well-known antimalarial drugs, artesunate and chloroquine, as positive controls. The presence of living *Plasmodium* cells was determined by reduction of nitro blue tetrazolium (NBT) to a formazan derivative. The amount of reduced NBT was measured at 600 nm using a Modulus Microplate Plate reader. Based on the absorbance readings, Graphpad PRISM 4 was used to calculate parasite survival, which was

expressed as the half maximal inhibitory concentration (IC_{50}) of each active crude actinobacterial extract.

4.3. RESULTS & DISCUSSION

4.3.1. Screening for antibiotic activity

All presumptive actinobacterial strains isolated from reservoir sediment in the shallow waters of the Silvermine reservoir, the soil of Elephant's Eye cave and sampling of the Elephant's Eye cave wall, described in Chapter 2, were screened for antimycobacterial activity against *M. aurum* strain A+. Antibiotic activity against two additional test bacteria, *S. aureus* strain ATCC 25923 (a Gram positive bacterium) and *E. coli* strain ATCC 25922 (a Gram negative bacterium), were also tested to determine the antibiotic spectrum of each presumptive actinobacterial isolate. The antibiotic activities of 29 confirmed actinobacterial isolates (by 16S-rRNA gene analysis) are summarised in Table 4.1. The antibiotic activities of eight unidentified presumptive actinobacterial strains are also included in Table 4.1. Antibacterial activity displayed against *M. aurum* strain A+ provides insight into the potential of an isolate to produce antimycobacterial antibiotics, which may include *M. tuberculosis.* It is important to note that the antibiotic activity against each test bacterium for each actinobacterial strain varied across the three agar media they were grown on. This is because the nutrient composition of the environment is known to influence antibiotic biosynthesis (Prescott *et al.*, 2008).

4.3.1.1. Antimycobacterial activity against *M. aurum* strain A+

From Table 4.1, 14 strains displayed no activity or weak activity (ZOI < 1000mm^2), 11 strains displayed moderate activity (1000mm^2 < ZOI < 2000 mm^2), six strains displayed strong activity (2000mm^2 < ZOI < 3000mm^2) and six strains displayed very strong activity (ZOI > 3000mm^2). Table 4.1 shows *Streptomyces* strains RS6, RS7, RS9, RS13, RS15 and presumptive actinobacterial strain RS4 (unidentified) displayed very strong activity against *M. aurum* strain A+. Furthermore, *Streptomyces* strains CW3, CW4, CS3, RS18 and presumptive actinobacterial strains CS21 and RS25 exhibited strong activity against *M. aurum* strain A+ (2000mm^2 < ZOI < 3000mm^2) (Table 4.1). These *Streptomyces* strains, therefore, show great potential to produce antimycobacterial antibiotics. It was noted that the DSMZ #553 and JCM61 media induced the strongest activity from the actinobacterial strains, ranging from strong to very strong activity. It is unusual that a nutrient rich medium, such as DSMZ #553, induces such strong activity, because antibiotic synthesis is normally activated under stressful conditions (such as the lower nutrient conditions of JCM61) (Tormo *et al.*, 2003). This could be due to the high growth rate of the actinobacteria in DSMZ #553 causing an increased amount

of activated cells producing antibiotics and thus a stronger antibiotic response. Additionally, more cells would be present in the medium leading to more rapid utilization of nutrients resulting in starvation conditions. This could then activate antibiotic synthesis during the 10 day growth period, leading to a strong antibiotic response. Weak activity observed from actinobacteria grown on Middlebrook 7H9 medium could possibly be a result of the presence of glucose, as glucose is known to inhibit the biosynthesis of several antibiotics (Martin & Demain, 1980). This problem can be solved by exchanging glucose for a different carbon source to induce antibiotic synthesis. Nevertheless, four inhibition zones of >1000mm^2 were recorded on Middlebrook 7H9 (for strains CW5, CS23, RS11 and RS25).

Overall, the strongest antimycobacterial activity was observed from the actinobacterial strains isolated from reservoir sediment. This suggests that the Silvermine reservoir may be a better source for finding *Streptomyces* strains with stronger antimycobacterial antibiotic activity than the Silvermine Elephant's Eye cave. According to past studies, many novel antibiotic compounds have been discovered from aquatic actinobacteria, such as rifamycin produced by *Micromonospora rifamycinica* (Huang *et al.*, 2008), marinomycins produced by a *Marinophilus* sp. (Jensen *et al.*, 2005), marinopyrroles produced by a *Streptomyces* sp. (Hughes *et al.*, 2008) and abyssomicin-C produced by a *Verrucosispora* sp. (Riedlinger *et al.*, 2004).

4.3.1.2. Determining antibiotic activity against *S. aureus* and *E. coli*

Antibiotic activity was also tested against another Gram positive bacterium (*S. aureus* strain ATCC 25923) and a Gram negative bacterium (*E. coli* strain ATCC 25922) to determine the antibiotic spectrum of each isolate (confirmed actinobacteria and unidentified presumptive actinobacterial isolates). Fifteen (15) isolates displayed antibiotic activity against *S. aureus* strain ATCC 25923, of which 13 were *Streptomyces* strains (Table 4.1). *Streptomyces* strains CW5, RS3, RS7 and RS15 displayed very strong activity against *S. aureus* (ZOI > 3000mm^2) on at least one of the three nutrient media (Table 4.1). This is interesting, as they also displayed moderate to very strong activity against *M. aurum* strain A+. This could mean that they have the ability to produce antibiotics against multiple Gram positive bacteria. It could be only one antibiotic compound producing the activity against both *M. aurum* strain A+ and *S. aureus* strain ATCC 25923, or it could be two compounds that are structurally different from each other and that inhibit the growth of different test bacteria. In order to determine whether the activity is produced by one or multiple antibiotic compounds, thin layer chromatography could

be used (discussed further in Chapter 5). Only three isolates (CW5, CS18 and RS4) displayed activity against *E. coli* strain ATCC 25922. This was expected as Gram negative bacteria, such as *E. coli*, have an outer membrane in addition to the cytoplasmic membrane, which makes them less susceptible to antibiotics (Salton & Kim, 1996). While the anti-*E. coli* activity of strains CS18 and RS4 was weak, strain CW5 exhibited moderate activity (ZOI = 1307 mm^2) (Table 4.1). *Micrococcus* strain CS8 and *Streptacidiphilus* strain CS11 showed no antibiotic activity against any of the test bacteria. *Micromonospora* strain RS10 showed activity against only *M. aurum* strain A+ (1218 mm^2 on JCM61; Table 4.1).

It is worth noting that the absence of antibiotic activity against all of the bacterial test strains for an isolate does not mean that that isolate is incapable of producing bioactive compounds. It is possible that antibiotic activity was not induced on the media tested and that the biosynthesis of antibiotics may be induced if the isolate was grown on other agar media. Furthermore, it is possible that an antibiotic *was* produced on one or more of the tested media, but that that antibiotic is only active against test bacteria not used in this study.

Table 4.1. The antibiotic activity of each confirmed and presumptive actinobacterial isolate against test bacteria *M. aurum* strain A+, *S. aureus* strain ATCC 25923 and *E. coli* strain ATCC 25922. Each isolate was grown on DSMZ #553, JCM61 and Middlebrook 7H9 (MB) media for 10 days at 30°C to allow antibiotic production before overlaying with the test bacterium. The agar overlay plates were examined after two days of incubation at 37°C. The zone of inhibition (ZOI) was calculated by subtracting the area of the colony from the area of the zone of growth inhibition. Measurement of the strength of antibiotic activity: - = no activity, ZOI < 1000 mm^2 = weak activity, 1000mm^2 < ZOI < 2000mm^2 = moderate activity, 2000mm^2 < ZOI < 3000mm^2 = strong activity and ZOI > 3000mm^2 = very strong activity. Blue shading = moderate activity, green shading = strong activity, orange shading = very strong activity.

	ZONE OF INHIBITION (mm^2) AGAINST								
Isolates	***M. aurum* strain A+**			***S. aureus* strain ATCC 25923**			***E. coli* strain ATCC 25922**		
	DSMZ #553	**JCM61**	**MB**	**DSMZ #553**	**JCM61**	**MB**	**DSMZ #553**	**JCM61**	**MB**
Streptomyces strain CW1	-	483	-	-	-	-	-	-	-
Streptomyces strain CW2	1437	188	-	-	-	-	-	-	-
Streptomyces strain CW3	1850	2222	-	-	-	-	-	-	-
Streptomyces strain CW4	1831	2189	-	-	-	-	-	-	-
Streptomyces strain CW5	-	1442	1103	5714	3753	267	594	1307	-
Streptomyces strain CS1	-	204	-	-	-	-	-	-	-
Presumptive actinobacterial strain CS2	-	-	-	-	-	-	-	-	-

Isolates	*M. aurum* strain A+			*S. aureus* strain ATCC 25923			*E. coli* strain ATCC 25922		
	DSMZ #553	**JCM61**	**MB**	**DSMZ #553**	**JCM61**	**MB**	**DSMZ #553**	**JCM61**	**MB**
Streptomyces strain CS3	2439	672	-	-	-	126	-	-	-
Streptomyces strain CS5	1850	-	-	-	-	-	-	-	-
Presumptive actinobacterial strain CS7	1218	1925	-	-	-	-	-	-	-
Micrococcus strain CS8	-	-	-	-	-	-	-	-	-
Streptacidiphilus strain CS11	-	-	-	-	-	-	-	-	-
Streptomyces strain CS12	-	924	-	-	-	-	-	-	-
Streptomyces strain CS14	1178	412	153	459	90	177	-	-	-
Streptomyces strain CS16	-	-	-	-	-	-	-	-	-
Streptomyces strain CS18	34	537	157	219	123	151	-	170	-
Presumptive actinobacterial strain CS21	2029	-	-	-	276	-	-	-	-
Streptomyces strain CS23	1822	276	1885	378	75	115	-	-	-

Isolates	*M. aurum* strain A+			*S. aureus* strain ATCC 25923			*E. coli* strain ATCC 25922		
	DSMZ #553	**JCM61**	**MB**	**DSMZ #553**	**JCM61**	**MB**	**DSMZ #553**	**JCM61**	**MB**
Presumptive actinobacterial strain RS2	-	-	-	-	-	-	-	-	-
Streptomyces strain RS3	1885	330	-	1913	4998	1913	-	-	-
Presumptive actinobacterial strain RS4	3695	2886	-	553	3716	1885	-	574	-
Presumptive actinobacterial strain RS5	841	-	-	-	-	-	-	-	-
Streptomyces strain RS6	2695	3164	-	-	-	-	-	-	-
Streptomyces strain RS7	4458	2011	1850	163	1885	4948	-	-	-
Streptomyces strain RS9	3672	1964	-	302	1144	1218	-	-	-
Micromonospora strain RS10	-	1218	-	-	-	-	-	-	-
Presumptive actinobacterial strain RS11	1056	-	1696	-	-	-	-	-	-
Streptomyces strain RS13	1787	3534	-	-	-	-	-	-	-

Isolates	*M. aurum* strain A+			*S. aureus* strain ATCC 25923			*E. coli* strain ATCC 25922		
	DSMZ #553	**JCM61**	**MB**	**DSMZ #553**	**JCM61**	**MB**	**DSMZ #553**	**JCM61**	**MB**
Streptomyces strain RS14	-	1529	-	-	-	-	-	-	-
Streptomyces strain RS15	3537	1099	962	123	2263	4665	-	-	-
Streptomyces strain RS16	742	-	-	-	-	-	-	-	-
Streptomyces strain RS17	-	544	979	16	21	-	-	-	-
Streptomyces strain RS18	2045	1025	726	75	57	-	-	-	-
Streptomyces strain RS19	-	-	-	1956	295	-	-	-	-
Streptomyces strain RS20	553	1746	-	-	1925	954	-	-	-
Streptomyces strain RS22	-	-	-	-	-	-	-	-	-
Presumptive actinobacterial strain RS25	2885	236	2529	-	-	-	-	-	-

4.3.2. Organic solvent extract activity

Eleven (11) actinobacterial strains with different strengths of antimycobacterial activity were selected from Table 4.1 for organic compound extraction from liquid culture. In terms of antibiotic production, actinobacteria may act differently in liquid broth compared to solid media (Charousová *et al.*, 2017) and therefore may produce different results from those seen in section 4.3.1, hence the selection of actinobacterial strains with antimycobacterial antibiotic activity ranging from very weak to very strong. Additionally, the strains were to be tested for antiplasmodial activity (discussed in section 4.3.3).

Four (4) *Streptomyces* strains (RS6, RS9, RS13 and RS15) that displayed very strong anti-*M. aurum* activity, three *Streptomyces* strains (CS3, CW5 and RS3) that displayed moderate to strong anti-*M. aurum* activity and four *Streptomyces* strains (CS1, CS12, CS18 and RS19) that displayed very weak or no anti-*M. aurum* activity were chosen for organic compound extraction. Solvent extraction was performed on the culture broth (EtOAc extract), as well as the cell mass (MeOH extract) for each strain. The extracts were then concentrated by re-dissolving in small volumes of EtOAc (for the culture broth extract) and MeOH (for the cell mass extract). The crude extracts were then tested for activity against the three test bacteria: *M. aurum* strain A+, *S. aureus* strain ATCC 25923 and *E. coli* strain ATCC 25922, by performing spot bioautography using silica TLC plates. Figure 4.1 shows examples of positive and negative results on the TLC plates. A positive result was observed by a zone of clearing, indicating the inhibition, by an active compound, of the ability of the test bacterium to reduce MTT. A negative result was observed by no zone of clearing, indicating that the test bacterium was not inhibited.

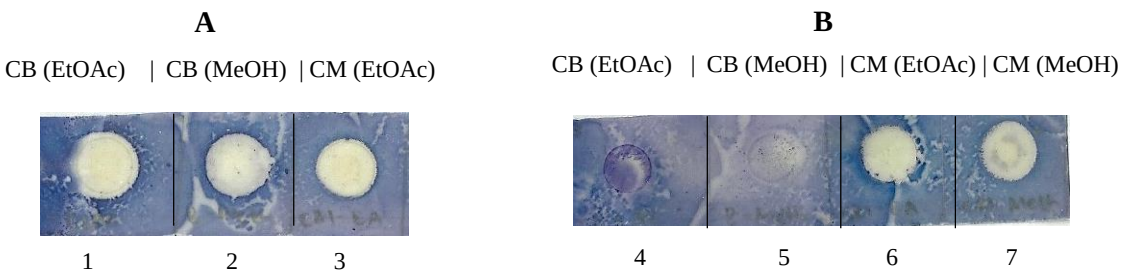

Figure 4.1. A) Spot bioautography of *Streptomyces* strain RS3 against *M. aurum* strain A+: 1– culture broth (CB) extract (dissolved in EtOAc), 2– CB extract (dissolved in MeOH), 3– cell mass (CM) extract (dissolved in EtOAc). B) Spot bioautography of *Streptomyces* strain CS3 against *M. aurum* strain A+: 4- CB extract (dissolved in EtOAc), 5– CB extract (dissolved in MeOH), 6– CM extract (dissolved in EtOAc), 7- CM extract (dissolved in MeOH).

Spots 1, 2 and 3 show the activity of *Streptomyces* strain RS3 and spots 4, 5, 6 and 7 show the activity of *Streptomyces* strain CS3 against *M. aurum* strain A+ (Figure 4.1). Spots 1 and 2 showed that there was activity in the culture broth (CB) extract implying that the active compound/s were excreted from the *Streptomyces* strain RS3 cells into the broth in high concentrations. However, the same was observed for the active compound/s of spot 3, implying that the active compound/s were also present in the cells. Spots 4 and 5 of *Streptomyces* strain CS3 showed that the active compound/s were not excreted into the broth, instead, they were present within the cell mass (CM) at high concentrations (indicated by spots 6 and 7; Figure 4.1B).

The spot bioautography results of each crude extract against test bacteria *M. aurum* strain A+ and *S. aureus* strain ATCC 25923 for 11 *Streptomyces* strains are summarised in Table 4.2. The crude extract activity against *E. coli* strain ATCC 25922 was not included due to inconclusive results. It was observed that the active organic compound/s extracted from *Streptomyces* strains CS3 (CM – EtOAc and CM - MeOH) and RS3 (CB – EtOAc, CB – MeOH and CM – EtOAc) showed the strongest activity against *M. aurum* strain A+. *Streptomyces* strain RS13 (CB – EtOAc) and RS15 (CM – MeOH) displayed strong activity against *S. aureus* strain ATCC 25923. Overall, six *Streptomyces* strains (CS3, RS3, RS6, RS13, RS15 and RS19) displayed either moderate or strong anti-*M. aurum* activity. The crude extracts of these strains could be studied further by determining the number of active compounds present in the extracts (discussed further in Chapter 5).

Table 4.2. Summarised bioautography results of the activity from cell broth (CB - EtOAc/ MeOH) and cell mass (CM - EtOAc/ MeOH) crude extracts of 11 *Streptomyces* strains against *M. aurum* strain A+ and *S. aureus* strain ATCC 25923. The *Streptomyces* strains were either grown in JCM61 or DSMZ #553 liquid media at 30°C with shaking before extracting their organic compounds. CB = cell broth, CM = cell mass. Strong activity: +++, moderate activity: ++, weak activity: +, no activity: -, NT: not tested.

***Streptomyces* strain**	**Growth medium**	**Test Bacterium**	**CB (EtOAc)**	**CB (MeOH)**	**CM (EtOAc)**	**CM (MeOH)**
CS1	JCM61	*M. aurum* strain A+	Inconclusive	-	-	-
		S. aureus strain ATCC 25923	++	+	NT	-
CS3	DSMZ #553	*M. aurum* strain A+	-	-	+++	+++
		S. aureus strain ATCC 25923	-	-	-	-
CS12	JCM61	*M. aurum* strain A+	-	-	-	-
		S. aureus strain ATCC 25923	+	-	-	-
CS18	JCM61	*M. aurum* strain A+	-	-	-	-
		S. aureus strain ATCC 25923	-	-	-	-
CW5	JCM61	*M. aurum* strain A+	+	+	-	-
		S. aureus strain ATCC 25923	++	NT	NT	-
RS3	DSMZ #553	*M. aurum* strain A+	+++	+++	+++	+
		S. aureus strain ATCC 25923	+	-	-	+
RS6	JCM61	*M. aurum* strain A+	+	++	++	-
		S. aureus strain ATCC 25923	+	NT	++	-
RS9	DSMZ #553	*M. aurum* strain A+	-	-	-	-
		S. aureus strain ATCC 25923	+	-	-	-
RS13	DSMZ #553	*M. aurum* strain A+	++	+	+	+
		S. aureus strain ATCC 25923	+++	-	-	-
RS15	DSMZ #553	*M. aurum* strain A+	-	-	-	++
		S. aureus strain ATCC 25923	-	-	-	+++
RS19	DSMZ #553	*M. aurum* strain A+	++	++	-	-
		S. aureus strain ATCC 25923	++	++	-	-

4.3.3 Antiplasmodial activity

Malaria is another leading cause of death and disease worldwide. Malaria is a mosquito-borne infectious disease caused by the *Plasmodium* parasite and resulted in 219 million cases and 435 000 deaths in 2017 (https://www.who.int/malaria/en/). According to the World Health Organisation (WHO), the most common antimalarial drugs used to date include artemisinin-based combination therapies (ACTs) and chloroquine phosphate. These front-line drugs work by killing the parasite. However, there is a constant struggle of evolving drug-resistant parasites (similar to the situation with bacterial pathogens, such as *M. tuberculosis*) and therefore novel antimalarial drugs need to be researched and developed. Since actinobacteria are not well studied for antimalarial compounds, Mr Daniel Watson, a PhD student in the Division of Clinical Pharmacology at UCT, is investigating actinobacteria as a source of natural products that could potentially be used to treat malaria.

Fifteen (15) identified actinobacterial strains (one *Micromonospora* strain and 14 *Streptomyces* strains) were selected from this study to be analysed for antiplasmodial activity by Mr Watson (see section 4.2.4). Antiplasmodial activity was assessed against the drug sensitive *P. falciparum* strain, NF54. Table 4.3 displays the antiplasmodial screening results of each actinobacterial strain where *Streptomyces* strains CW2, CW5, RS3, RS7, RS13, RS15 and RS19 displayed activity against *P. falciparum* strain NF54.

Table 4.3: *In vitro* activity of actinobacterial strains against *P. falciparum* strain NF54. +, activity; -, no activity. Blue shading = active antiplasmodial *Streptomyces* strains.

Strain name	Growth medium	*In vitro* antiplasmodial activity against *P. falciparum* strain NF54.
Micromonospora strain RS10	DSMZ #553	-
Streptomyces strain CS1	JCM61	-
Streptomyces strain CS12	JCM61	-
Streptomyces strain CS18	JCM61	-
Streptomyces strain CS3	DSMZ #553	-
Streptomyces strain CW2	DSMZ #553	+
Streptomyces strain CW5	JCM61	+
Streptomyces strain RS3	DSMZ #553	+
Streptomyces strain RS6	DSMZ #553	-
Streptomyces strain RS7	DSMZ #553	+
Streptomyces strain RS9	DSMZ #553	-
Streptomyces strain RS13	DSMZ #553	+
Streptomyces strain RS15	DSMZ #553	+
Streptomyces strain RS18	DSMZ #553	-
Streptomyces strain RS19	JCM61	+

Tables 4.4 and 4.5 show the antiplasmodial activity of the two positive controls (artesunate and chloroquine – to which the parasite is known to be sensitive) and the antiplasmodial activity of each active *Streptomyces* strain. The half-maximal inhibitory concentration (IC_{50}) (ng/mL) is the concentration of a molecule that is required to inhibit 50% of the target, in this case *P. falciparum* strain NF54. The lower the IC_{50} of the molecule, the less is needed to inhibit the parasite. Therefore, the molecule would be more potent. The activity of artesunate and chloroquine against *P. falciparum* strain NF54 were within acceptable ranges (Table 4.4). The acceptable range for activity is considered to be between 1 and 10 ng/mL for chloroquine between 0.5 and 8 ng/mL for artisunate based on lab standards. The IC_{50} (ng/mL) was determined for each crude extract, i.e. culture broth (EtOAc/ MeOH) and cell mass (EtOAc/

MeOH). *Streptomyces* strains CW2, CW5 and RS7 displayed the strongest activity against *P. falciparum* strain NF54 with IC_{50} values below 1 µg/mL for the culture broth and/or cell mass crude extracts (Table 4.5). According to Pink, *et al.* (2005), the crude extract should display *in vitro* activity of ≤ 1 µg/mL (≤ 1000 ng/mL) against protozoa for it to be worth considering for antiplasmodial drug discovery. Therefore, *Streptomyces* strains CW2, CW5 and RS7 would be worth prioritizing for further study. This could be done by scaling up the bacterial culture volume to extract a larger amount of material, purifying the active compounds, determining the structure of each active molecule to determine whether it is novel and then evaluating each novel molecule as a candidate new drug based on the methods of Pink *et al.*, (2005).

Table 4.4. *In vitro* antiplasmodial activity of positive controls against *P. falciparum* strain NF54. The IC_{50}'s were within the acceptable range for each drug.

Control	***P. falciparum* strain NF54: IC_{50} (ng/mL)**
Artesunate	5.5 ± 2
Chloroquine	4.1 ± 0.7

Table 4.5: *In vitro* antiplasmodial activity of each active actinobacterial crude extract. Gold shading = strongly active antiplasmodial *Streptomyces* strains (IC_{50} values ≤ 1000 ng/mL).

***Streptomyces* strain**	***In vitro* antiplasmodial activity against *P. falciparum* strain NF54: IC_{50} (ng/mL)**			
	Culture broth (EtOAc)	**Culture broth (MeOH)**	**Cell mass (EtOAc)**	**Cell mass (MeOH)**
CW2	40	< 125	311	> 5000
CW5	128	< 312	> 5000	> 5000
RS3	> 5000	3270	> 5000	> 5000
RS7	70	< 312	21	< 312
RS13	4493	> 5000	> 5000	> 5000
RS15	2293	> 5000	1631	2000
RS19	1933	2722	> 5000	> 5000

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CHAPTER 5

GENERAL DISCUSSION

Exploring overlooked environmental habitats for the isolation of novel, rare actinobacteria has become a major interest over recent years as the discovery of novel microbial natural products has become increasingly important (Lazzarini *et al.,* 2000). The study of aquatic habitats, especially freshwater habitats, has been neglected compared to terrestrial environments (Rifaat, 2003; Ningthoujam *et al.,* 2011). It has been proven that aquatic actinobacteria, as well as actinobacteria from unexplored terrestrial habitats, have great potential to produce novel compounds (Zothanpuia *et al.*, 2018; Das *et al.*, 2018). Taxonomy assesses the relationships between species based on their characteristics (metabolism, physiology and morphology), as well as their molecular traits (example, G+C content of DNA and gene sequence variation). The study of actinobacterial taxonomy enhances the search for antibiotics with novel structures as it allows one to identify new actinobacterial species capable of producing novel antibiotics, as biological diversity is known to underpin chemical diversity. Exploring the diversity of unique actinobacteria from understudied terrestrial environments and aquatic ecosystems would, therefore, improve the probability of finding novel antibiotic compounds. Standard culture-based methods used for bacterial isolation and biodiversity analysis can be limiting, as many environmental bacteria do not grow on laboratory media. However, culture-independent approaches, such as metagenomic analysis, circumvent the limitations of traditional culture-based approaches. This study made use of the traditional culture-based method in combination with a culture-independent approach (metagenomic analysis) to investigate the actinobacterial diversity in the Silvermine Nature Reserve, Cape Town, South Africa.

The aims of this study were to analyse the actinobacterial diversity of three locations in the Silvermine Nature Reserve: the sediment in the freshwater reservoir, soil from Elephant's Eye cave and a swab from the Elephant's Eye cave wall. This was accomplished using two methods. Firstly, a culture-dependent method was used to isolate actinobacteria, which were subsequently identified to the genus level by analysing their 16S-rRNA gene sequences. Phylogenetic analyses based on the 16S-rRNA gene sequences were conducted to determine the most closely related species for each isolate. Secondly, a metagenomic analysis was carried out by directly extracting gDNA from environmental samples for identification of the strains

in the samples by 16S-rRNA gene sequencing and analysis. The actinobacteria that were isolated in the culture-dependent part of the study were screened for antimycobacterial antibiotic activity using a standard overlay method, as well as by spot bioautography of crude organic solvent extracts of culture broths of selected strains.

Analysing actinobacterial biodiversity in the three different locations of the Silvermine Nature Reserve using the culture-dependent approach was successful. The culture-independent approach of using metagenomic analysis to analyse the actinobacterial biodiversity of the Silvermine reservoir sediment revealed biodiversity that was not seen in the culture-dependent part of the study.

The standard culture-dependent technique for isolating actinobacteria from the Silvermine Nature Reserve produced a total of 29 confirmed actinobacterial isolates out of 56 presumptive actinobacterial isolates. Twenty six (26) isolates were identified as *Streptomyces* strains and three isolates were identified as rare actinobacteria belonging to the genera *Micrococcus, Streptacidiphilus* and *Micromonospora. Micromonospora* species have been frequently isolated from various aquatic ecosystems (Rifaat, 2003; Eccleston *et al*., 2008) and, in this study, *Micromonospora* strain RS10 was isolated from reservoir sediment.. It was also noted that the Silvermine Nature Reserve is a good source for finding acidophilic actinobacteria, as *Streptacidiphilus* strain CS11 was isolated from Elephant's Eye cave soil. This observation is supported by the results obtained from the metagenomic analysis of the reservoir sediment, where 56% of the metagenomically identified actinobacterial species belonged to the order *Acidimicrobiales.* That only one actinobacterial strain representative of a genus associated with acidic environments was isolated can be attributed to the pH mismatch between the media used for isolation of actinobacteria (pH range 7.2 - 7.4) and the pH of the reservoir sediment (pH 4.5 - 5). In future, the pH of the sample should be recorded before sediment collection, so that the pH of the isolation media can be adjusted to match the pH of the environment from which the actinobacteria will be isolated. Adjusting the pH of the isolation media to match the pH of the sample will allow the isolation of acidotolerant and acidophilic actinobacteria. Some of these isolates may produce antibiotics with novel structures. However, it should be borne in mind that the majority of the metagenomic clones identified as belonging to the order *Acidimicrobiales* (95%) had top 16S-rRNA gene sequence hits that represent uncultured actinobacterial strains. Therefore, it may not have been possible to isolate these organisms, even if the pH of the isolation media had been in the pH 4.5-5 range. Nevertheless, a lower pH

in the isolation media would surely have allowed the isolation of different, acidotolerant and/or acidiphilic actinobacterial strains.

Streptomyces strain RS3, which had the lowest sequence similarity to its top hit amongst all of the identified actinobacteria (98.94% over a 16S-rRNA gene sequence length of 1429 nt), would be worth investigating further to determine whether it represents a new species. Additionally, *Streptomyces* strain CS16 displayed a low 16S-rRNA gene sequence similarity to its second and third hits after the top hit (*P. columellifera* subsp. *pallida* MB-SK 8^T) was disregarded, as the sequence that was submitted to GenBank (GU269552) was that of a contaminating *Streptomyces* strain. The second hit of *Streptomyces* strain CS16 was *Streptomyces sanglieri* NBRC 100784^T and the third hit was *Streptomyces candidus* NRRL ISP-5141^T and both hits displayed a sequence similarity of 98.54% over 1240 nt. To determine whether strains RS3 and CS16 represent new species, their genomes and those of their closest phylogenetic relatives (determined from 16S-rRNA gene sequence analysis) should be sequenced. The relatedness of the genome sequences would be analysed using Average Nucleotide Identity (ANI) and digital DNA-DNA hybridisation (dDDH) measurements. If the ANI values between the genomes of strain RS3 and strain CS16 and their respective closest relatives are below 96% (Ciufo *et al*., 2018) and the dDDH values are below 70% (Wayne *et al*., 1987), this would prove that strains RS3 and CS16 each belongs to a unique genomic species. Phenotypic characterisation to show that strains RS3 and CS16 are phenotypically distinct from their closest relatives would complete the polyphasic characterisation of strains RS3 and CS16 and would allow each of them to be formally described as a new species. In addition, the chemotaxonomic markers of strains RS3 and CS16 would need to be determined to show that these are consistent with membership of the genus *Streptomyces*. The chemotaxonomic markers that would need to be assessed are the phospholipids, fatty acids and menaquinones in the cell membrane and the isomer of diaminopimelic acid and the sugars in the peptidoglycan.

The metagenomic analysis of the Silvermine reservoir sediment revealed great actinobacterial biodiversity. Actinobacteria belonging to the orders *Acidimicrobiales, Streptomycetales, Streptosporangiales, Corynebacteriales* and *Sporichthyales* were identified using partial 16S-rRNA gene sequences. However, the complete actinobacterial diversity of the reservoir sediment was not revealed by the clone library prepared in this study. This means that there is even greater actinobacterial diversity yet to be discovered from this source. A full assessment of the actinobacterial biodiversity in the Silvermine reservoir sediment would require one to

do further sampling of the reservoir to generate a larger clone library for analysis. Furthermore, it would be interesting to use metagenomics to analyse the actinobacterial diversity of other locations in the Silvermine Nature Reserve, such as soil from Elephant's Eye cave, and compare the results between the different locations.

The screening of the actinobacterial isolates for antimycobacterial antibiotic activity showed that *Streptomyces* strains RS6, RS7, RS9, RS13 and RS15 displayed very strong activity (ZOIs > 3000mm^2) against *M. aurum* strain A+, suggesting that they produce strong antibiotic compounds. *Streptomyces* strain RS7 additionally displayed very strong activity against *S. aureus* strain ATCC 25923, another Gram positive bacterium. Although this suggests that the compound/s produced by *Streptomyces* strain RS7 act strongly against Gram positive bacteria, further testing against Gram-positive and Gram-negative bacteria should be carried out to establish the spectrum of the antibiotic(s). Thin layer chromatography, by 1D and 2D methods, could then be used to determine the number of antibiotic compounds that are produced by *Streptomyces* strains RS6, RS7, RS9, RS13 and RS15. These chromatography techniques separate compounds based on their polarity and their interactions between the mobile and stationary phases.

In order to determine whether any of the compounds is novel, the active molecules would have to be purified and subjected to structural analysis by nuclear magnetic resonance and mass spectrometry. In this study, *Streptomyces* strain RS7 was screened for antimalarial activity in the Division of Clinical Pharmacology at UCT where it displayed high potency against *P. falciparum* strain NF54, in addition to its very strong antimycobacterial antibiotic activity. The active molecules produced by *Streptomyces* strain RS7 would, therefore, be worth prioritizing for purification and structural elucidation in order to determine whether they are novel and might, therefore, be candidates for development as new drugs.

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Appendix A. EzBioCloud results for all metagenomic clones.

Strain	Cultured/ uncultured	Phylum	Order	Order/Family/Genus	% Similarity	Size (bp)	Accession number
RS1-C109	Uncultured	Actinobacteria	*Acidimicrobiales*	*Acidimicrobiaceae*	99.82	567	PAC000733
RS1-C6	Uncultured	Actinobacteria	*Acidimicrobiales*	*Acidimicrobiaceae*	93.25	548	PAC000520
RS1-C9	Cultured	Actinobacteria	*Acidimicrobiales*	*Acidimicrobiaceae*	98.73	551	PAC002136
RS1-C43	Uncultured	Actinobacteria	*Acidimicrobiales*	*Acidimicrobiaceae*	99.29	563	GU731322
RS1-C52	Uncultured	Actinobacteria	*Acidimicrobiales*	*Acidimicrobiaceae*	99.47	564	GQ402597
RS1-C60	Uncultured	Actinobacteria	*Acidimicrobiales*	*Acidimicrobiaceae*	98.94	568	PAC000068
RS1-C72	Uncultured	Actinobacteria	*Acidimicrobiales*	*Acidimicrobiaceae*	98.24	567	PAC000733
RS1-C81	Uncultured	Actinobacteria	*Acidimicrobiales*	*Acidimicrobiaceae*	96.76	570	AY326627
RS1-C92	Uncultured	Actinobacteria	*Acidimicrobiales*	*Acidimicrobiaceae*	98.76	566	PAC002288
RS1-C110	Uncultured	Actinobacteria	*Acidimicrobiales*	*Acidimicrobiaceae*	99.47	565	PAC000068
RS1-C113	Cultured	Actinobacteria	*Acidimicrobiales*	*Acidimicrobiaceae*	99.29	566	DQ906076
RS1-C12	Uncultured	Actinobacteria	*Acidimicrobiales*	*Acidimicrobiaceae*	97.37	538	PAC002288
RS1-C8	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	99.09	551	PAC002285
RS1-C19	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	99.47	569	PAC002285
RS1-C23	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	99.29	566	AB552456
RS1-C24	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	96.84	569	AY792235
RS1-C31	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	99.12	571	PAC002285
RS1-C32	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	98.13	590	PAC002285
RS1-C33	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	98.6	571	AY345538
RS1-C35	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	96.14	570	PAC002285
RS1-C41	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	99.47	571	PAC002285
RS1-C42	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	91.37	594	PAC000376
RS1-C46	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	98.6	571	PAC002285
RS1-C49	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	99.64	551	PAC002285
RS1-C51	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	99.65	571	PAC002285

RS1-C58	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	98.95	569	PAC002285
RS1-C62	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	96.91	551	AY345538
RS1-C63	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	98.06	569	PAC002285
RS1-C64	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	99.27	551	PAC002285
RS1-C67	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	97.19	569	AY792235
RS1-C69	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	99.47	565	PAC002285
RS1-C76	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	95.8	571	PAC000376
RS1-C79	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	98	571	AY345538
RS1-C91	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	99.29	566	PAC002285
RS1-C93	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	97.35	566	PAC000376
RS1-C95	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	99.65	565	PAC002285
RS1-C100	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	99.29	565	PAC002285
RS1-C104	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	99.27	551	PAC002285
RS1-C111	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	95.09	575	AB254795
RS1-C118	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	96.73	550	DQ450881
RS1-C133	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	99.65	568	PAC002285
RS1-C16	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	98.58	564	AY345538
RS1-C26	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	99.47	566	PAC002285
RS1-C1	Uncultured	Actinobacteria	*Acidimicrobiales*	*Aciditerrimonas*	97.29	590	AY792235
RS1-C3	Cultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	100	547	MUNB01000146
RS1-C25	Cultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	100	572	MUNB01000146
RS1-C28	Cultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	100	573	M76388
RS1-C30	Cultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	99.83	574	M76388
RS1-C5	Cultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	99.83	574	MUNB01000146
RS1-C20	Cultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	95.39	564	X79852
RS1-C39	Uncultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	97.74	574	JOAP01000131
RS1-C40	Cultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	99.48	574	M76388
RS1-C45	Cultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	98.92	554	LIQV01000394
RS1-C50	Uncultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	97.56	574	JQ806215

RS1-C65	Cultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	99.83	574	LIQV01000394
RS1-C70	Cultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	99.3	572	MJAH01000006
RS1-C71	Cultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	98.14	430	LIQV01000394
RS1-C73	Cultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	100	574	LIQV01000394
RS1-C74	Cultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	97.81	593	JOAP01000131
RS1-C75	Cultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	99.47	571	LIQV01000394
RS1-C82	Cultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	99.65	574	LIQV01000394
RS1-C84	Cultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	99.3	574	LIQV01000394
RS1-C87	Cultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	99.3	573	LIQV01000394
RS1-C97	Uncultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	98.07	569	M76388
RS1-C103	Cultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	99.65	572	LIQV01000394
RS1-C117	Cultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	99.65	574	LIQV01000394
RS1-C119	Cultured	Actinobacteria	*Streptomycetales*	*Streptomyces*	99.47	567	LIQV01000394
RS1-C44	Cultured	Actinobacteria	*Corynebacteriales*	*Mycobacterium*	100	568	LQOR01000030
RS1-C54	Uncultured	Actinobacteria	*Corynebacteriales*	*Mycobacterium*	99.83	586	LZKQ01000114
RS1-C96	Cultured	Actinobacteria	*Corynebacteriales*	*Mycobacterium*	95.23	566	AF480598
RS1-C94	Uncultured	Actinobacteria	Family without order	*Jatrophihabitans*	92.97	570	HQ674865
RS1-C66	Uncultured	Actinobacteria	*Sporichthyales*	*Sporichthyaceae*	88.61	570	EF016809
RS1-C2	Uncultured	Actinobacteria	*Streptosporangiales*	*Streptosporangiales*	98.82	592	EU861937
RS1-C36	Uncultured	Actinobacteria	*Streptosporangiales*	*Streptosporangiales*	98.78	573	EU861937
RS1-C56	Uncultured	Actinobacteria	*Streptosporangiales*	*Streptosporangiales*	94.7	568	JX504954
RS1-C78	Uncultured	Actinobacteria	*Streptosporangiales*	*Streptosporangiales*	95.99	584	JX504954
RS1-C123	Uncultured	Actinobacteria	*Streptosporangiales*	*Streptosporangiales*	94.76	572	JX504954
RS1-C124	Cultured	Actinobacteria	*Streptosporangiales*	*Actinocorallia*	92.49	565	AF163117
RS1-C55	Uncultured	Armatimonadetes	Non-Actinobacteria	AB630921_g (unknown)	97.28	551	AB630921
RS1-C10	Uncultured	Proteobacteria	Non-Actinobacteria	*Deltaproteobacteria*	88.66	573	HM187154
RS1-C22	Uncultured	Proteobacteria	Non-Actinobacteria	*Deltaproteobacteria*	98.09	576	PAC001922
RS1-C37	Uncultured	Firmicutes	Non-Actinobacteria	*Sporomusaceae*	88.64	591	AB603498
RS1-C29	Uncultured	Proteobacteria	Non-Actinobacteria	*Syntrophorhabdus*	98.13	595	FJ538126

RS1-C47	Uncultured	Proteobacteria	Non-Actinobacteria	*Syntrophorhabdus*	94.41	572	GQ402743
RS1-C4	Uncultured	Nitrospirae	Non-Actinobacteria	*Thermodesulfovibrio_f*	98.49	529	HM243789
RS1-C11	Uncultured	Proteobacteria	Non-Actinobacteria	*Desulfobacca_f*	98.43	547	AB426218
RS1-C13	Uncultured	Proteobacteria	Non-Actinobacteria	*Desulfobacca_f*	98.99	592	AB262720
RS1-C86	Uncultured	Proteobacteria	Non-Actinobacteria	*Desulfobacca_f*	91.72	593	AB426215
RS1-C59	Uncultured	Gemmatimonadetes	Non-Actinobacteria	*Gemmatimonadaceae*	99.11	565	AY913252
RS1-C90	Uncultured	Gemmatimonadetes	Non-Actinobacteria	*Gemmatimonadaceae*	92.63	570	GU731332
RS1-C17	Uncultured	Gemmatimonadetes	Non-Actinobacteria	*Gemmatimonadaceae*	98.26	574	AB426207
RS1-C21	Uncultured	Gemmatimonadetes	Non-Actinobacteria	*Gemmatimonadales*	96.68	573	AB426207
RS1-C89	Uncultured	Gemmatimonadetes	Non-Actinobacteria	*Gemmatimonadales*	95.09	591	AB426207
RS1-C14	Uncultured	Proteobacteria	Non-Actinobacteria	*Anaeromyxobacteraceae*	93.32	569	PAC000124
RS1-C15	Uncultured	Proteobacteria	Non-Actinobacteria	*Anaeromyxobacteraceae*	94.59	591	PAC000124
RS1-C34	Uncultured	Proteobacteria	Non-Actinobacteria	*Anaeromyxobacteraceae*	96.46	593	PAC000124
RS1-C80	Uncultured	Proteobacteria	Non-Actinobacteria	*Anaeromyxobacteraceae*	94.71	567	EF516099

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