Bioactive actinobacteria associated with two South African medicinal plants, *Aloe ferox* and *Sutherlandia frutescens*

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Keywords

Actinobacteria
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Abstract

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Actinobacteria, a Gram-positive phylum of bacteria found in both terrestrial and aquatic environments, are well-known producers of antibiotics and other bioactive compounds. The isolation of actinobacteria from unique environments has resulted in the discovery of new antibiotic compounds that can be used by the pharmaceutical industry. In this study, the fynbos biome was identified as one of these unique habitats due to its rich plant diversity that hosts over 8500 different plant species, including many medicinal plants. In this study two medicinal plants from the fynbos biome were identified as unique environments for the discovery of bioactive actinobacteria, *Aloe ferox* (Cape aloe) and *Sutherlandia frutescens* (cancer bush).

Actinobacteria from the genera *Streptomyces*, *Micromonaspora*, *Amycolatopsis* and *Alloactinosynnema* were isolated from these two medicinal plants and tested for antibiotic activity. Actinobacterial isolates from soil (248; 188), roots (0; 7), seeds (0; 10) and leaves (0; 6), from *A. ferox* and *S. frutescens,* respectively, were tested for activity against a range of Gram-negative and Gram-positive human pathogenic bacteria. These isolates were mass screened for antibiotic activity using a perpendicular streak method followed by screening using a standard overlay technique. Twenty isolates from each plant with high antibacterial activity were subjected to further antibiotic screening and revealed activity against *B. cereus* (80%), *S. aureus* (85%) and *P. aeruginosa* (55%).

Investigation into the genomes of two *Streptomyces* strains, strain A81 and strain S149, revealed that these isolates have the genetic potential to produce a wide range of natural products, including genes involved in the production of antibiotics, anti-tuberculosis, anti-HIV, and anti-tumour compounds. Interestingly, strain A81 additionally had genes that are involved in the production of a typical plant metabolite, naringenin, which is also produced by the strain's host plant, *A. ferox*. The genome of strain S149 contained genetic machinery for the production of auxin, another typical plant hormone. This endophyte was isolated from the leaves of *S. frutescens* and production of auxin may indicate its involvement with plant growth and development.

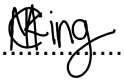
Findings from this study thus highlights that actinobacteria associated with medicinal plants, and unique environments in general, have great potential to produce a wide range of pharmaceutically important compounds. Furthermore, in addition to antibiotics the actinobacteria isolated in this study have the potential to produce anti-tuberculosis, anti-HIV and anti-tumour compounds.

August 2021

Declaration

I declare that "*Bioactive actinobacteria associated with two South African medicinal plants,* Aloe ferox *and* Sutherlandia frutescens*."* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name: Maria Catharina King Date: 2021-08-13

Signed: 

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Content

Chapter 1: Literature review

1.1 Natural product discovery

1.1.1 Introduction

Natural products (NPs) are chemical compounds that are produced by a diverse array of living organisms; including bacteria, fungi, plants, and some marine animals. Natural products include both primary and secondary metabolites, which possess a wide range of biological activity that have uses in human medicine, veterinary medicine and agriculture. The discovery of penicillin in 1929 by Dr Alexander Fleming (Fleming, 1929) was the most notable discovery of NPs produced by microorganisms. But it was not until the discovery of streptomycin from *Streptomyces griseus* by Dr Selman Waksman and colleagues (Schatz *et al*., 1944) in the early 1940s that sparked the global search for NPs from microorganisms. This marked the start of the 'Golden Age of Antibiotics'. The resultant large-scale systematic screening of predominantly terrestrial environments led to an increase in the discovery of natural products produced by microorganisms, with approximately 33 500 NPs that have been characterized to date (Bérdy, 2012). (Figure 1.1; data used from Bérdy, 2012).

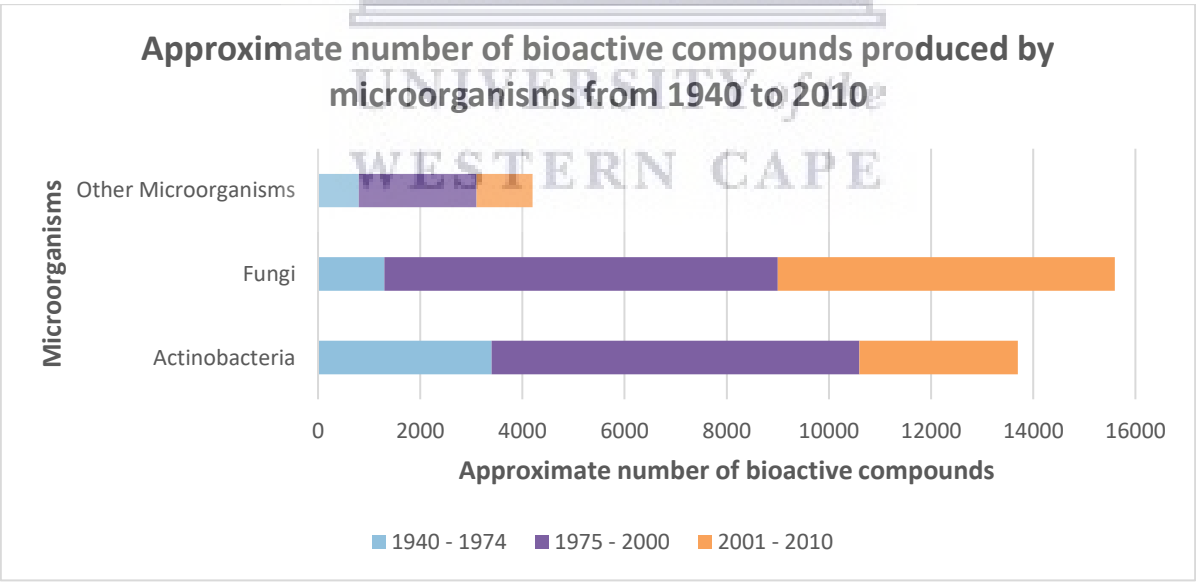


Figure 1.1: Number of bioactive compounds produced by fungi, actinobacteria and other microorganisms from 1940 - 2010. Data used from Bérdy, 2012.

1.1.2 A Brief History of NP's and drug discovery

New metabolites were discovered with relative ease during the early years (Golden Age; 1940 - 1974) of NP discovery from microorganisms. Streptomycin and many other compounds were detected by the following process: isolation of the microorganism, fermentation of the isolate, extraction of the compound of interest, and finally, testing of the fermentation broth and purified compound against a range of test pathogens (Schatz *et al*., 1944). This phenotypic screening approach formed the basis of pharmaceutical companies' assays in the discovery of NPs. With this approach, the activity of the organisms is evaluated using simple plate assays to observe activity without prior knowledge of the organism or the compound screened for. When cell inhibition or death is observed the compounds of interest can be extracted and identified using chromatography techniques. This was the most popular methodology during the Golden Age, with more than a thousand NPs identified to have antimicrobial activity. In the 1950s, early anti-cancer drugs were also discovered using this phenotypic approach, by cultivating cancer cell lines in animal models and later using *in vitro* methods (Bérdy, 2012).

The first step in the discovery of NPs from microorganisms is the acquisition and isolation of microorganisms from the environment. Scientists and employees at pharmaceutical companies such as Abbott Laboratories and Eli Lilly, were encouraged to collect soil samples from where ever they could; if they went on vacation overseas or even from their own backyards (Bérdy, 2012). From this, erythromycin (previously known as ilotycin), produced by *Saccharopolyspora erythraea* (formerly known as *Streptomyces erythreus*), was discovered when an employee of Eli Lilly and Company, sent a soil sample from his garden in the Philippines to Eli Lilly's research laboratory (McGuire *et al*., 1952; Lund, 1953; Bérdy, 2012). The indiscriminate collection of soil samples went unabated for decades until countries signed the Convention on Biological Diversity (CBD) Treaty in the 1990s that ensured that revenues made on discoveries from biological samples taken from the country of origin would be returned to the respective country (Secretariat of the Convention on Biological Diversity, 2005).

The NPs that were discovered during this era has been an invaluable source for new pharmaceutical products. Many of the antibiotic classes were discovered from a group of organisms called the actinobacteria during this era. These compounds are still used today, including the macrolides like tylosin, spiramycin, and erythromycin; aminoglycosides such as neomycin (Waksman *et al*., 1949), and gentamicin (Weinstein *et al*., 1963). The compound chlortetracycline, belonging to the class of broad-spectrum antibiotic called tetracyclines, was discovered in 1945 by Duggar and is since traded under the name Aureomycin (Jukes, 1985).

Other classes of antibiotics discovered during this era include polyenes, such as the potent antifungal nystatin (Hazen and Brown, 1950); chloramphenicol (Ehrlich *et al*., 1947); and glycopeptides like vancomycin.

During the mid-era of natural products discovery (1975 - 2000), the methods for drug discovery were mostly unchanged except for some advances in nuclear magnetic resonance (NMR) and mass spectrometry (MS) analysis (Katz and Baltz, 2016). The introduction of synthetic chemistry resulted in pharmaceutical companies shifting their focus from natural products to synthetic or semi-synthetic products. This, unfortunately, led to a decline in the discovery of natural products. Conversely, it led to an increase in semisynthetic production incorporating NPs or using NPs as a source of inspiration for the development of synthetic products. During this time frame, pharmaceutical companies shifted their focus to high throughput screening methods for the discovery of NPs, but soon after decided to focus on NP derivatives. For pharmaceutical companies, focusing on NP derivatives was a more cost-effective and faster technique, with greater scalability than searching for novel NPs.

Regardless of the introduction of synthetic chemistry, this era explored new techniques in NP discovery and screening. This period also marked the search for new sources of NPs and expanded the search of NP activities beyond antimicrobial activity - with a heavy focus on anti-cancer screenings. Marine environments were now being explored, including bacteria, algae and marine invertebrates. One such example is the discovery of the compound salinosporamide A that was isolated from the marine actinobacteria *Salinospora tropica* that has potential anticancer activity (Fenical *et al*., 2009).

Target-based approaches were implemented to complement phenotypic screenings, due to the inability of phenotype assays to confirm the mode of action of the tested compound. Target-based screening methods help to validate the understanding of the mode of action by having a quantifiable read-out when the compound of interest specifically interacts with the target, for example, *in vitro* enzyme assays.

Another big advancement made in the late 1970s was the development of genetic tools for *Streptomyces* species, including the development of plasmid vectors (Bibb *et al*, 1978). The implementation of genetic tools and sequencing helped to evaluate biochemical pathways of NPs. As more and more enzymatic genes were sequenced, the information was used to reconstruct the biochemical pathways predicted by the enzymes present. Scientists soon realised that the genes for the biosynthesis of the NPs were clustered. This facilitates the cloning of an entire NP pathway into vectors for the production of NPs. The advances made

during the 1970s to the 2000s, especially based on biochemistry and genetics, has helped to guide NP discovery into a genomic era that we use extensively today.

One of the noticeable genetic and biochemistry-based discoveries is that of two enzyme complexes, the non-ribosomal peptide synthases (NRPS) and polyketide synthases (PKSs). The NRPSs consist of modules made up of domains that are responsible for incorporating amino acids into a polypeptide chain. Penicillins, cephalosporins, vancomycin and daptomycin are examples of antibiotics that are produced by NRPSs. The PKSs synthesize polyketides through decarboxylative condensation of carboxylic acids (Yu *et al*., 2012). Polyketides include a diverse group of natural products, including anticancer (like kibdelones), anti-cholesterol (e.g. lovastatin) and antimicrobial compounds (including jadomycin B) (Ratnayake *et al*., 2007; Seenivasan *et al*., 2008; Jakeman *et al*., 2009). Polyketide synthases fall into three groups, type I, type II and type III, based on their sequences, primary structure and catalytic mechanisms. These enzyme complexes are further discussed in Chapter 4.

The first two *Streptomyces* genome sequences in the early 2000s revealed that these large genomes (around 8 megabases; Bentley *et al*., 2002) encode many more secondary metabolite gene clusters (SMGCs) than were previously thought. It is estimated that only around 10% of SMGCs are expressed in sufficient quantities to be detected under routine screening (Katz and Baltz, 2016; Ochi *et al*., 2014; Zhu *et al*., 2014). Whole genome sequencing of actinobacteria opened up a new door to the discovery of novel bioactive compounds and allows us to now peek into the biosynthetic gene clusters that produce a range of novel compounds.

1.1.3 Discovering NPs today

Today, the methods used for the discovery of natural products from microbial sources include a combination of techniques that includes phenotype and target-based screening, next-generation sequencing, bioinformatics, cloning, and chromatography. Using a combination of these methods will help in discovering and identification of novel bioactive compounds.

The advances in fields such as sequencing have dramatically decreased the cost of whole-genome sequencing. A better understanding of secondary metabolism and further improvements in analytical and bioinformatics methods will help to predict structures of new secondary metabolites, that was previously not possible.

Today, the discovery of natural products from actinobacteria uses a polyphasic approach, which combines genomic and phenotypic tools. This helps to identify products that are unique and would have possibly been missed if not screened for using a combination of techniques including, plate assays, solvent extraction, fractionation, preparative HPLC, and computational tools. These techniques were used to isolate and identify an antimicrobial compound, 2,3-Dihydroxy-9,10-Anthraquinone, from *Streptomyces galbus* (Balachandran *et al*., 2014). Similarly, compounds with antimicrobial activities (Kumar *et al*., 2014), cytotoxic potential (Law *et al*., 2017) and anti-inflammatory activity (Ma *et al*., 2017) have been identified from actinobacteria using similar techniques.

With the use of genome mining, various gene clusters can be identified, and products predicted using whole-genome sequencing technology and computational tools such as antiSMASH, MiBig and NaPDoS (further discussed in chapter 4). Iftime *et al*., (2016) used this approach to predict the gene clusters in the famous antibiotic producer, *Streptomyces collinus*. Various secondary metabolite gene clusters were predicted using these tools and included PKS, NRPS, hybrid PKS-NRPS, lanthipeptide, siderophores and terpenes. Predicted products of these gene products were subsequently identified using tools such as spectrometry and chromatography.

1.1.3.1 Phenotype screening

Phenotype screening can be performed to evaluate measurable outcomes, such as antimicrobial inhibition activity, and even antitumor activity. These tests can be performed using simple agar plate assays. Screening microbial isolates using plate assays was one of the first methods used to discover antibiotic compounds (Schatz *et al*., 1944). Even though this method was first used over 80 years ago, it is still a popular method to use today. This allows for an easy and cost-effective way to screen isolates for activity against a specific test criterion. With this method, microbes that do not display the desired activity can be eliminated. However, this has both positive and negative consequences. The positive side is that this method allows for the direct screening of the exact for the activity being investigated i.e. antimicrobial activity against a specific pathogen. Unfortunately, this also means that isolates producing compounds that are not screened for may be lost i.e. if it had antimicrobial activity against a pathogen that was not screened for. Isolates that produce small quantities of a compound that have the desired effect, might also be missed with this method. Nevertheless, this approach is still one of the easiest ways of screening for activity if the researcher knows exactly what the desired outcome needs to be. The attraction of this method is the ease of use, cost-effectiveness and (generally) does not require any specialized equipment.

1.1.3.2 Target-based screening

Phenotype screening cannot be used to evaluate and determine the mode of action of the compound that is produced. Using target-based screening, the mode of action can be determined which can be as simple as using enzymatic assays. With the target-based approach, the researcher knows which target the test compound should affect. Enzymatic screening is a popular approach, especially if the target enzyme activity can be coupled to a colourimetric substrate, which allows the activity to be measured efficiently. Using this method, conclusions can be made on the mode of action, i.e. the compound increases or inhibits the efficacy of the enzyme. An example of this is the inhibition of alpha-amylase enzyme which degrades sugar, such as starch and glycogen, that is important in the regulation of blood sugar levels. The inhibition of this degradation has potential value in the regulation of diabetes. In this assay, the enzyme, alpha-amylase, is used in combination with a dye, and a sugar source. The degradation can be evaluated spectrophotometrically to quantify the degradation (Nelson, 1944). The addition of the compound of interest will either inhibit, promote or have no effect on the enzyme and thus the mode of action of the compound can be determined. The combination of phenotype screening followed by target-based screening can help to identify possible compounds of interest. The potential to discover new compounds increase with the discovery of novel isolates, and isolates that have previously not been sequenced.

1.1.3.3 Next-generation sequencing

The low cost of next-generation sequencing has resulted in an increase of whole bacterial genome sequences. Genomes were sequenced at an exponential pace and today over 215 000 prokaryotic genomes are found in the GenBank database (Figure 1.2; https://www.ncbi.nlm.nih.gov/genome/browse/#!/prokaryotes/ accessed on 25/10/2019). Not only did the low cost of sequencing help move natural product discovery forward, but it also increased the quality of the data generated. The large amount of sequencing data that was generated contains an enormous amount of information that needs to be evaluated. The current problem is not the sequencing of the genomes but rather mining the genomes for data. Each genome holds information regarding everything to do with the organism, including the potential to produce bioactive compounds.

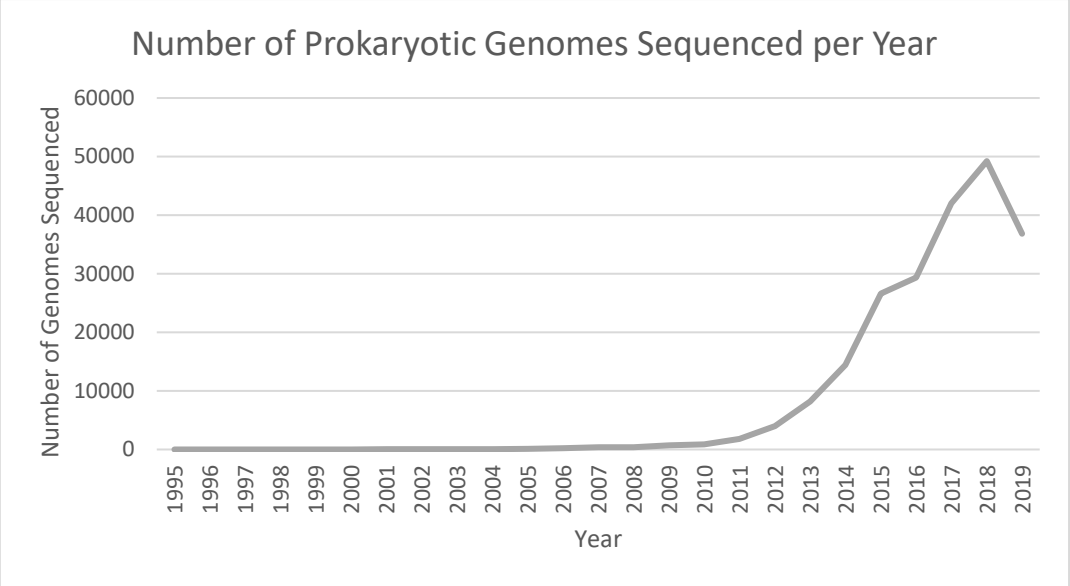


Figure 1.2: Number of prokaryotic genomes sequenced from 1995 to 2019. Data collected from the NCBI genome database. Accessed 25/10/2019.

The beginning of the 2000s has seen a return to natural product research due to advances in cultivation and activity screening, as well as the ability to harness the biosynthetic potential of actinobacteria captured in their DNA, but not expressed in culture (Harvey *et al*., 2015). Sequencing of microbial genomes, especially those of actinobacteria, revealed that the larger the genome the more SMGCs are found within the genome. Thus, actinobacteria have the potential to produce multiple bioactive compounds with varying activity. For example, the streptomycin-producer, *Streptomyces griseus*, was found to contain 34 biosynthetic gene clusters within its 8.5 Mb genome (Ohnishi *et al*., 2008). The model organism *Streptomyces coelicolor* (A3) also had a large genome of more than 8.6 Mb and contained more than 20 gene cluster that encodes for secondary metabolites (Bentley *et al*., 2002).

The genome sequence of a strain could be used to unlock its biosynthetic potential by using a strategy called genome mining. This strategy uses technologies such as next-generation sequencing (NGS) coupled with computing algorithms and databases for the annotation of metabolite gene clusters. These computing algorithms include, antiSMASH (Blin *et al*., 2019) and MIBiG (Medema *et al*., 2015), which identify gene clusters that drive the synthesis for both a known and an unknown compound. These clusters can be identified using multi-contig genome drafts and an automated pipeline for the annotation of natural products biosynthetic gene clusters (Blin *et al*., 2019).

Actinobacterial genomes have a high molecular percentage G+C content which poses difficulties for both the sequencing and the computation used for assembly. In addition, many

important actinobacteria contain a linear chromosome and plasmids with long terminal inverted repeats that can reach over one megabase. Polyketides and nonribosomal peptides represent the most relevant natural compounds. These compounds, at the nucleotide level, are made up by highly similar intragenic- and intergenic tandem repeats, often spanning over 700bp. These long repeats are normally longer than the read-length of NGS technologies, thus making it very difficult to correctly assemble these gene clusters. Illumina MiSeq offers a throughput of 2 x 300 nt, which does not resolve these highly repetitive and large gene clusters. Nonetheless, a complete and accurate genome is not a prerequisite for the application of the genome mining approach to natural product discovery.

1.1.3.4 Computational analysis

Whole genome sequencing in combination with bioinformatics tools, termed genome mining, is used to predict potential compounds produced by an organism. Various computer programs are on the market that will help to evaluate the genetic potential of a bacterial isolate to produce bioactive compounds such as AntiSMASH (Blin *et al*., 2019), NaPDoS (Ziemert *et al*., 2012) and RAST (Aziz *et al*., 2008). These applications predict possible biosynthetic gene clusters which are similar to known clusters. Using this method, potential novel bioactive compounds can be found. This valuable information helps to further investigate the compound of interest. These biosynthetic gene clusters can be evaluated in depth to determine the end product as well as its structure. Cloning of the biosynthetic gene clusters into a suitable expression host can help to determine the effect of the compound, as well as aid in the purification of the compound. Over 250 000 known natural compounds can be found in virtual NP libraries. These libraries can be used for virtual screening of products and help with computer-guided drug discovery. One such library, StreptomeDB, is a freely available online database focused on NPs produced by *Streptomyces* (Chen *et al*., 2017).

1.1.3.5 Cloning methods

Members of the order *Actinomycetia* are a major target for the discovery of natural products due to their ability to produce metabolites that are structurally novel, diverse and complex. Genome mining has further contributed to the discovery of biosynthetic gene clusters (BGCs) in these NP-producing actinobacteria. Numerous studies have shown that the majority of biosynthetic gene clusters are silent or cryptic. Additionally, even if they are active, they may be expressed poorly under laboratory conditions which makes it difficult to characterize the NP produced. Various strategies have been used to activate or stimulate these cryptic BGCs, some of which include ribosome engineering, co-culture fermentation, control of regulatory

genes, and adding elucidators (Chaudhary *et al*., 2013; Kurosawa *et al*., 2008; Ochi *et al*., 2004; Pettit, 2009; Wang *et al*., 2008). Heterologous expression of BGCs is one strategy used to express large clusters of up to 100 kb (Nah *et al*., 2017).

Traditionally, BGCs were partially digested and inserted into cosmid vectors to construct a library. However, the large size of BGCs (typically more than 20 kb and up to 100 kb) makes it difficult to clone into a cosmid vector system, making cloning and overexpression of the entire BGC challenging. Currently, manipulation of these large BGCs still remains challenging for heterologous expression, but systems have been developed which are suitable for large BGCs. The most popular systems used includes, the transformation-associated recombination (TAR) system, integrase-mediated site-specific recombination (SSR) system, and plasmid *Streptomyces* bacterial artificial chromosome (pSBAC) system (Baltz 2016; Du *et al*., 2015; Shizuya *et al*., 1992; Yamanaka *et al*., 2014; Figure 1.3).

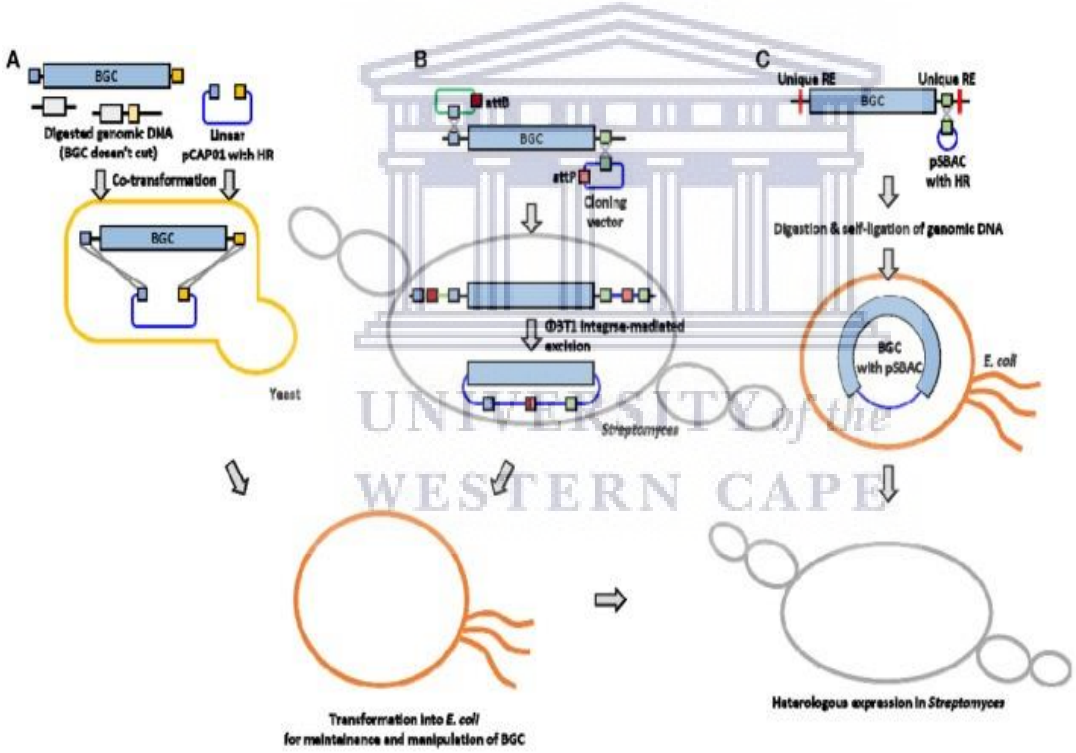


Figure 1.3: *Overview of biosynthetic gene cluster cloning system (A) TAR system (B) SSR system (C) pSBAC system. HR, Homologous region; RE restriction enzyme. Taken from Nah* et al*., 2017.*

The TAR system incorporates *Saccharomyces cerevisiae* natural capability to perform *in vivo* homologous recombination. The TAR cloning vector, pCAP01, was developed to contain three elements, one each from yeast, *E. coli*, and actinobacteria (Yamanaka *et al*., 2014). With the use of this vector, targets larger than 50 kb can be captured and manipulated in yeast. The

BGC can then be shuttled into *E. coli* and actinobacteria. Jordan and Moore (2016) identified the BGCs for ammosamides A-C, pyrroloquinoline alkaloids from *Streptomyces* sp. CNR-698. By utilising the TAR integrative cloning system, they validated ammosamide BGC and demonstrated that these molecules are derived from a complex set of biosynthetic genes.

The SSR system is based on the *Streptomyces* bacteriophages φBT1 and φC31, and is used to integrate exogenous DNA into the chromosomes of prokaryotes, eukaryotes and archaea. An integrase-mediated site-specific recombination in *Streptomyces* was developed using the φBT1 bacteriophage (Du *et al*., 2015). In this system, three plasmids are used, the pUC119-base suicide vector carrying a mutated *attP*, the pKC1139 vector carrying a mutated *attB*, and an integrative plasmid containing the φBT1 integrase gene. The pUC119 with mutated *attP* and a homologous region to the 5' end of the target BGC is introduced into the chromosome. Similarly, the pKC1139 vector containing the mutated *attB* and a homologous region to the 3' end of the target BGC is integrated into the chromosome by conjugation and single crossover through cultivation at a high temperature. The expression of the phage φBT1 integrase leads to the excision of the pKC1139 containing the target BGC. This plasmid is extracted from the original *Streptomyces* and transferred into *E. coli* for recovery and maintenance of the construct (Du *et al*., 2015). Using this method, the authors were successful in cloning the actinorhodin gene cluster from *Streptomyces coelicolor* M145, as well as the napsamycin and daptomycin gene clusters from *Streptomyces roseosporus* NRRL 15998 (Du *et al*., 2015).

Bacterial artificial chromosomes (BAC) are reported to carry inserts reaching 300 kb in length (Shizuya *et al*., 1992). Because of this, several *E. coli-Streptomyces* shuttle BAC vectors have been developed to carry large BGCs, such as pSBAC. Unique restriction enzyme recognition sites that flank both regions of the entire BGC are used to capture the BGC. These restriction enzymes (RE) sites can be naturally occurring or artificially inserted. The pSBAC vector is inserted within the unique RE sites by homologous recombination. The entire target BGC is then captured in the pSBAC through single restriction digestion and self-litigation (Nah *et al*., 2017). Using the pSBAC system with cluster tandem integration, a large 80-kb polyketide biosynthesis gene cluster of tautomycetin was homologously and heterlogously overexpressed. Overexpression of tautomycetin is of great importance because it is a protein phosphatase PP1/PP2A inhibitor and T cell-specific immunosuppressant, and resulted in a 14-fold enhancement of tautomycetin production (Nah et al., 2015).

The use of *Streptomyces* heterologous expression systems is an attractive strategy to awaken and express cryptic BGCs and can be applied to large BGCs from actinobacteria. The overexpression of BGCs using these techniques can furthermore help to improve production

of known BGCs. Despite there being several systems available, the precise cloning and expression of an entire BGC still remains challenging due to difficulty in manipulating large gene clusters.

1.1.3.6 Chromatography and Spectrometry

Chromatography techniques can be used to separate compounds and, when coupled with mass spectrometry, can be used to identify compounds based on their mass, solubility, and elution rate (time). Different chromatography techniques can be used to identify compounds at different purity levels and compound characteristics. For example, a bioactive microbe, *Streptomyces sannanensis* strain SU118, was isolated from phoomdi soil in Loktak lake in India and a potent antimicrobial agent active against Gram-positive bacteria was extracted with ethyl acetate, and purified using the silica gel TLC technique (Singh *et al*., 2014). Screening and bioassay-fractionation led to the isolation of the potential compound with a minimum inhibitory concentration of 0.5 µg/L against *Staphylococcus aureus* and 3 µg/L against *Mycobacterium smegmatis* and *Bacillus circulans*. In another study from India, three bioactive compounds were extracted, purified and structurally elucidated from the liquid culture broth of the strain *Streptomyces coelicoflavus* BC 01 isolated from mangrove soil. The compounds were named as BC 01_C1, BC 01_C2 and BC 01_C3. The bioactive compounds were extracted with ethyl acetate and purified by column chromatography. The purity of the compounds was evaluated via reverse-phase high-performance liquid chromatography (RP-HPLC). Structural elucidation was performed by evaluating the solubility pattern, melting points, ultraviolet (UV) spectrum, and the infrared spectra of the purified compounds. Furthermore, the mass spectrum was evaluated using the GC-MS system. The nuclear magnetic resonance (NMR) spectra were also recorded. The structure of compound BC 01_C1 was identified as 5-amino-2-(6-(2-hydroxyethyl)-3-oxononyl) cyclohex-2-enone, compound BC 01_C2 identified as 8-(aminomethyl)-7-hydroxy-1-(1-hydroxy-4-(hydroxylmethoxy)-2,3-dimethyl-butyl)-2-methyl dodecahydro phenanthren-9(1H)-one and compound BC 01_C3 was established as 1-((E)-2-ethylhex-1-en-1-yl)2-((E)-2-ethylidenehexyl)cyclohexane-1,2-dicarboxylate (Rao *et al*., 2017).

Doroghazi and colleagues (2015) developed a systematic bioinformatics framework to study natural product biosynthetic gene clusters. They combined several metrics to develop a system for the global classification of the gene clusters into families (GCFs). The GCF network consisted of 11 422 gene clusters grouped into 4 122 GCFs. Mass spectrometry data were used to verify GCFs and demonstrate the *de novo* correlation of natural products and biosynthetic genes to enable the discovery of novel natural products from large data sets

without the use of bioassays. This technique combines genomics, bioinformatics, and metabolomics to get a complete picture of the bacterial isolate and their bioactive potential.

1.1.4 NP discovery and the future

The future for bioactive compound discovery is very promising. Firstly, only a small portion of the earth's environments, including soil, marine and extreme habitats, have been sampled. Expanding the sampling sites will vastly expand the discovery of new microbes and ultimately lead to the discovery of new bioactive products. This will lead to future discovery and identification of novel SMGCs that will further drive metabolite discovery from microbes. These clusters can be overexpressed by genetic or physiological manipulation in the organism or expressed heterologously in a host. Furthermore, structural biology and bioinformatics advances will help to predict products from identified gene clusters and can be used to manipulate NRPSs and PKSs clusters to synthesize compounds that were not previously possible.

Recently, a new cloning system has been developed based on the RNA-guided Cas9 nuclease. This cloning method, known as CATCH (Cas9-assisted targeting of the chromosome), employs the use of Cas9 nuclease to cleave the target DNA from the bacterial chromosome *in vitro*. These chromosomes are embedded in agarose plugs and can be subsequently ligated with a cloning vector via Gibson assembly. This method holds promise for the future cloning and overexpression of BGCs, due to its ability to clone large pieces of DNA (Jiang *et al*., 2015).

As discussed previously, the discovery of natural products from bacteria follows a multipronged approach, and no one approach, or combination of approaches is correct or incorrect. The combination of approaches used is dependent on the availability of equipment, finances, expertise, and time. Nonetheless, the future of NP discovery from actinobacteria is promising and NP discovery is on the brink of a new era thanks to advances in genomics, metabolomics and computational analysis.

1.2 Actinobacteria

Actinobacteria are widely distributed and found in both terrestrial and aquatic environments. In the environment they play an important role in the decomposition of organic materials. These Gram-positive bacteria have large genomes and a high mol% G+C content in their DNA (Bentley *et al*., 2002; Ohnishi *et al*., 2008). This phylum is also one of the largest phyla of Bacteria and is well known for their ability to produce bioactive compounds and contains the well-known antibiotic-producing genus of *Streptomyces*. Actinobacteria are exploited for their potential to produce a variety of secondary metabolites. The vast majority of species produce a variety of bioactive metabolites, making them biotechnologically valuable for the development of pharmaceuticals and other important compounds (Baltz, 2016; Choi *et al*., 2015; Du *et al*., 2000).

The classification of actinobacteria requires the use of a polyphasic approach. With this approach, a variety of factors are investigated to identify and classify actinobacteria into the correct taxa. This approach is followed to ensure that the phenotypic and genotypic characteristics relate to the phylogenetic classification of the organisms (Nouioui *et al*., 2018; Shirling and Gottlieb, 1966).

1.2.1 Lifecycle

The majority of the actinobacterial species are saprophytic, soil-dwelling organisms that spend most of their life cycle as semi-dormant spores. This is especially true when nutrient levels are limiting. When conditions are favourable, the spores germinate into a vegetative state and form substrate mycelium. After this stage, the bacteria form reproductive aerial mycelium at which point they are also able to produce antibiotics. The aerial mycelium produces spores that mature and are released from the aerial hyphae. If the spores settle on a suitable substrate, they can result in a new actinobacterial colony (Figure 1.4).

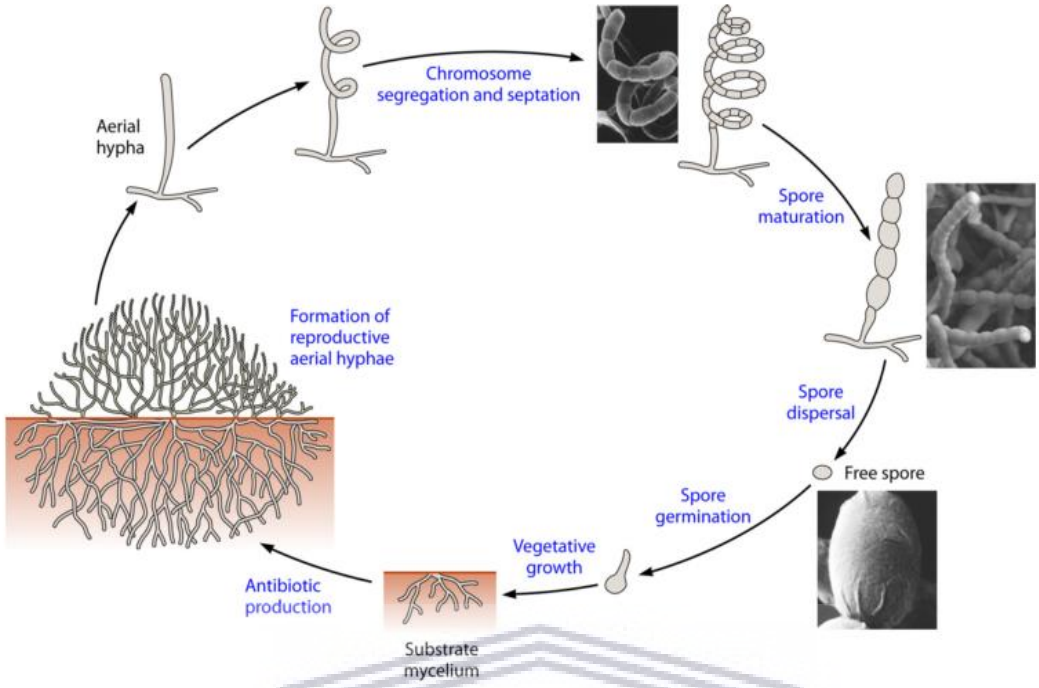


Figure 1.4: *The life cycle of sporulating actinobacteria. Taken from Barka et al., 2016*

1.2.2 Actinobacterial Taxonomy

A recent update in *Bergey's Manual of Systematic Bacteriology* (Ludwig et al., 2012) simplified the classification of the phylum Actinobacteria. With this update, previous subclasses were made into classes, as well as previous suborders were made into orders. This simplified the previous six classifications into only four which is now more consistent with other prokaryotic phyla. This phylum represents one of the largest taxonomic groups of Bacteria which include six classes (*Actinobacteria*, *Acidimicrobidiia*, *Coriobacteriia*, *Nitriliruptoria*, *Rubrobacteria*, and *Thermoleophilia*), and 23 orders (Ludwig *et al*., 2012) (Figure 1.5).

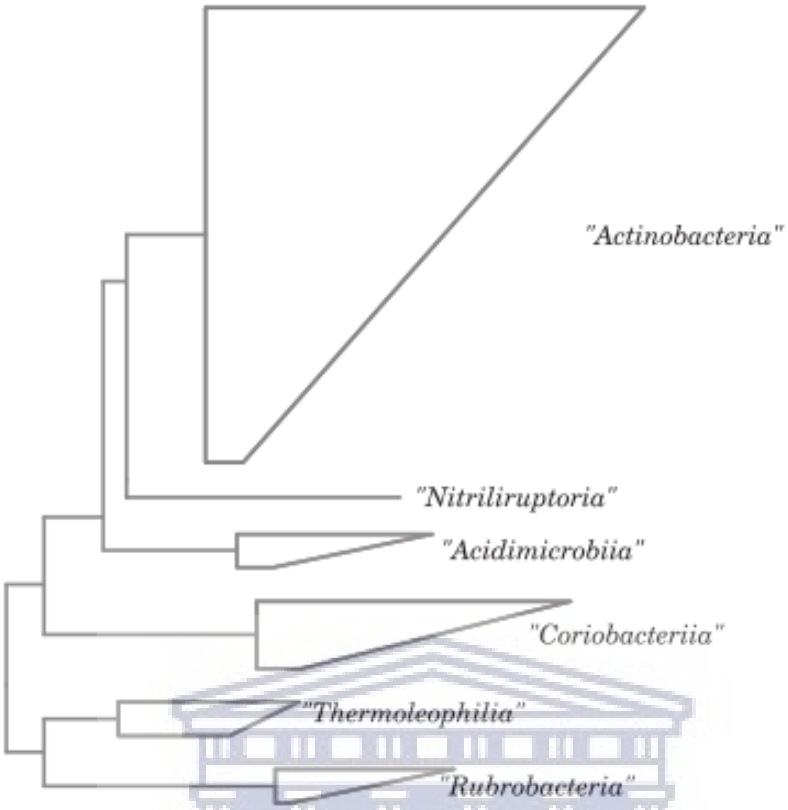


Figure 1.5: *Classes within the phyla* Actinobacteria*. Taken from Ludwig et al., 2012*

1.2.3 Genomic classification

Advances in the areas of sequencing and improved molecular techniques have made genomic taxonomy the preferred method for the classification of bacteria. Two approaches are routinely used in genomic taxonomy, namely whole genome and gene-specific methods. Actinobacteria are mainly identified using gene-specific methods, but with the ever-decreasing cost of genome sequencing, more and more actinobacteria are classified using genome sequencing methods (Nouioui *et al*., 2018).

Three types of genes are used to taxonomically classify bacteria. First, informational genes that encode for essential cellular functions and have a slow evolution rate, for example, the 16S rRNA gene (Ochman and Wilson, 1987). Housekeeping genes are also used because they are ubiquitous within a population and encode for proteins which are essential for metabolism. These genes have a moderate evolution rate and can be used to distinguish phylogenetic relationships at the species level. Examples of housekeeping genes include *gyrB, rpoB* and *recA* genes (Kasai *et al*., 1998). Lastly, hypervariable genes can be used. These genes are not ubiquitous within populations and code for virulence factors and surface

proteins. They have a fast evolution rate and allow for differentiation at the subspecies level (Jarraud *et al*., 2002).

16S rRNA gene sequences have been extensively used for the classification of organisms, but due to its highly conserved nature, it is found that related organisms share an almost identical 16S rRNA gene sequence (Stackebrandt and Goebel, 1994). Classification with the use of other genes, such as housekeeping genes, allows organisms to be classified at the species level with more certainty.

1.2.4 Physiological and Biochemical Characteristics

Actinobacteria have a range of physiological characteristics and grow under different conditions. Testing these characteristics help to classify these bacteria. Actinobacteria are tested for their ability to grow at different temperatures, pH and salt concentrations. The metabolic ability is tested by growing the bacteria on a range of carbon and nitrogen sources. The enzyme activity of the actinobacteria is tested by growing the bacteria in the presence of compounds such as casein, starch and xylan. Furthermore, the bacteria are also evaluated for their ability to grow in the presence of inhibitory compounds such as antimicrobial agents, dyes, and potassium cyanide (Li *et al*., 2016). Comparison of the physiological and biochemical characteristics is an indirect comparison of the organism's genome, because the proteins and enzymes that are produced are a result of gene expression, and thus can be used to make inferences about an organism's genome.

1.2.5 Morphological Characteristics

Actinobacteria comprise of unicellular organisms that are mostly filamentous. Most of the genera form aerobic substrate mycelia with aerial mycelia (Figure 1.3). Morphologically, actinobacteria form compact colonies that are often leathery or brittle and have chalky dust-like aerial mycelium when grown on solid agar. These bacteria come in a range of colours, from white to black and every colour in between. Morphology is an important characteristic and was used in the first descriptions of the genus of *Streptomyces.* The Golden Age of Antibiotic discovery saw an explosion in the number of "novel" *Streptomyces* species, which resulted in the same species being given several names. The International *Streptomyces* Project (ISP) was established which outlined a standardized protocol for classifying species (Shirling and Gottlieb, 1966).

Cultural characteristics of actinobacteria are determined using the strict guidelines of the ISP, where pure cultures are grown on 4 to 6 different media at 28°C for several days. Growth is evaluated according to: 1) the production and colour of substrate pigment, 2) the colour of the substrate mycelium, 3) the production and colour of the aerial mycelium, and 4) the amount of growth observed. These guidelines help to classify actinobacteria into their respective taxa.

Morphological traits include both cellular and colonial characteristics of the organism. Cellular characteristics include the shape and size of cells, spores, sporangia, sporangiospores, and the location of the spores on the sporangia. The shape, size, colour, form, and dimensions of the colonies are evaluated to further identify the morphology (Figure 1.6).

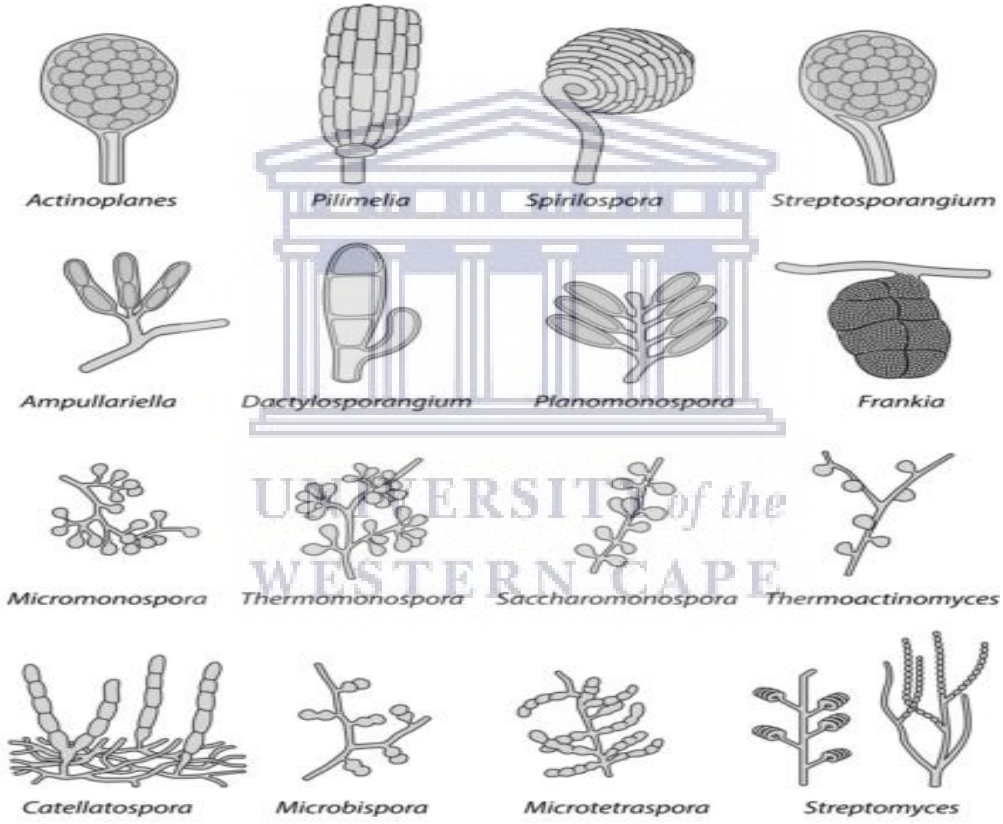


Figure 1.6: *The different types of spore chains produced by actinobacteria. Taken from Barka et al., 2016*

1.2.6 Chemotaxonomy of Actinobacteria

Chemotaxonomy of actinobacteria is the biological classification of organisms by the analysis of the chemical makeup of the cell. The use of chemotaxonomy helps in the classification of bacteria and forms part of the polyphasic approach of actinobacterial classification. This classification relies on the identification of cell wall diaminopimelic acid (DAP) isomer and

whole cell sugars, phospholipids, isoprene quinones, mycolic acids, and fatty acids. The cell structure is evaluated using different chemical techniques including extraction, fractionation, and purification of the cell compounds. The resolution of the target compounds is evaluated via chromatography techniques, including thin layer chromatography (TLC), high-pressure liquid chromatography (HPLC), and gas chromatography (GC).

The cell wall type can be used with morphology to classify actinobacteria into broad generic groups. The composition of the cell wall DAP isomers and whole cell sugars are widely used as taxonomic markers for this broad classification. Actinobacteria have a relatively thick peptidoglycan cell wall of around 20 - 80 nm thickness. The peptidoglycan represents around 40-80% of the cell wall with the remainder being other macromolecules such as lipids, proteins, and polysaccharides. The peptidoglycan type can be described by the amino acid sequences in the peptide chains, the diamino acids present, and the mode of cross-links between the chains. Actinobacteria cell walls are classified according to the diamino acids at the third position on the peptide chain. The diamino acids found at this position include *meso*- and LL-DAP, L-ornithine, L-lysine, and L-diaminobutyric acid (Table 1.1; Barka *et al*., 2016).

Table1.1: *Different types of cell wall components of Actinomycetia. Taken from Barka et al., 2016.*

Cell wall type	Major parietal constituent(s)	Genera
I	LL-DAP, glycine, no sugar	*Arachnia, Nocardioides, Pimelobacter, Streptomyces*
II	*meso*-DAP, glycine, arabinose, xylose	*Actinomyces, Actinoplanes, Ampulariella, Catellatosporia, Dactylosporangium, Glycomyces, Micromonospora, Pilimelia*
III	*meso*-DAP, madurose (3-*O*-methyl-D-galactose)	*Actinocorallia, Actinomadura, Dermatophylus, Frankia, Geodermatophilus, Kitasatospora, Maduromycetes, Microbispora, Microtetraspora, Nonomuraea, Planobispora, Planomonospora, Planotetraspora, some Frankia* spp*., Spirillosporia, Streptosporangium, Thermoactinomyces, Thermomonospora*
IV	*meso*-DAP, arabinose, galactose	*Micropolyspora, Nocardioforms*
V	Deprived of DAP; possesses lysine and ornithine	*Actinomyces*
VI	Deprived of DAP; variable presence of aspartic acid, galactose	*Arcanobacterium, Actinomyces, Microbacterium, Oerskovia, Promicromonospora*
VII	Deprived of DAP; diaminobutyric acid, glycine, with lysine variable	*Agromyces, Clavibacter*
VII	Deprived of DAP; ornithine	*Aureobacterium, Curtobacterium, Cellulomonas*

Phospholipids are the most common polar lipids and located in the plasma membrane of the cell. These lipids consist of hydrophilic head groups that are linked to two hydrophobic fatty acid chains. Polar lipids affect the permeability of the cell membrane and play a role in the movement of compounds across the membrane. Five phospholipid types (PI - PV) are used to describe and differentiate actinobacterial phospholipids. Type PI is characterised by having no nitrogenous phospholipids, whereas type PII has only one nitrogenous phospholipid phosphatidylethanolamine. Type PIII contains phosphatidylcholine and a characteristic phospholipid, glucosamine-containing phospholipids are classified as type PIV and lastly, type PV has phosphatidylglycerol and glucosamine-containing phospholipids (Lechevalier *et al*., 1977). The phospholipid types can be used to classify actinobacteria genera. For example, type PII is found in the genera of *Actinoplanes*, *Micromonospora*, *Nocardia* and *Streptomyces*, whereas type PIII is found in the genera of *Kribella* and *Nocardiopsis*. The phospholipids can be identified by comparing fingerprints generated by two-dimensional (2D) TLC.

Isoprenoid quinones are components of the bacterial cytoplasmic membrane, as well as the mitochondrial membrane. Here they play an important role in the electron transport chain. In actinobacteria, menaquinones are most commonly found in the cell envelope and the only type of respiratory isoprenoid quinones found in actinobacteria. The variations in the number of isoprene units and hydrogenated double bonds characterise this phylum. Mass spectrometry (MS) or HPLC can be used to determine the menaquinone present in the bacteria (Barka *et al*., 2016; Wang and Jiang, 2016).

Mycolic acids are long alkyl chain 2-alkyl-3-hydroxy fatty acids with a high molecular weight. These acids are found in bacteria with a cell wall type IV and are in the cell wall where it is thought to play a role in the architecture and impermeability of the cell envelope (Marrakchi *et al*., 2014). Mycolic acid is a characteristic feature of several actinobacterial genera, including *Gordonia, Mycobacterium*, *Nocardia*, and *Rhodococcus*.

Fatty acids are commonly found in the cell membrane as components of glycolipids and polar lipids. Unlike other cell wall components, the fatty acid composition is dependent on the growth conditions. Care needs to be taken when cultivating bacteria for biomass by ensuring that standardised growth conditions are implemented to ensure accurate analysis of fatty acids. Due to this lack of reproducibility, fatty acid analysis has limited use in taxonomy. Nonetheless, fatty-acid methyl ester (FAME) profiles have been used to resolve intrageneric relationships within the *Streptomyces* genus.

1.3 Genetic and Metabolic potential of Actinobacteria

Bioactive metabolites are primary and secondary metabolites that display biological activity. Primary metabolites have an essential role in the life cycle of the cell. Whereas, secondary metabolites have diverse and unusual structures, do not play a role in the cell life cycle, and are typically produced in late stages of growth (Solecka *et al*., 2012).

Whole genome sequencing of actinobacterial isolates has revealed that these organisms have a greater metabolic potential than previously thought. For example, *Streptomyces coelicolor* was one of the first actinobacteria whole genome sequenced, and was at the time was only known to produce four bioactive compounds. A study of the whole genome sequence revealed that the organism contained 20 cryptic bioactive pathways (Bentley *et al*., 2002). This new and exciting insight has prompted the search for new metabolites through the investigation of whole genome sequences of actinobacterial species.

Actinobacteria possess many gene clusters that are responsible for their bioactivity. With the help of sequencing, we can identify gene clusters that may produce the bioactive compounds. These technologies enable us now to unlock the genetic potential of bacteria. Previously, bacteria needed to be exposed to different conditions to produce a certain compound or to have an activity, known as the one strain many active compounds (OSMAC) approach (Pan *et al*., 2019). It is now possible to peek at the DNA of the organism to determine which compounds can be produced by the bacteria.

Next-generation sequencing has opened the doors into the genetic potential of bacteria. This allows us to identify previously unknown compounds and enable us to manipulate compounds by changing their biosynthetic pathways. Genome drafts can be used to identify the gene clusters that encode for a known compound, as well as gene clusters responsible for the production of an unknown but interesting products. Strategies to identify the unknown metabolic products from the biosynthetic gene clusters have been developed. The three main strategies that are used are; the activation of the expression of the gene cluster in the original organism, cloning and expression of the gene cluster in a heterologous host, or mutation of the gene cluster followed by metabolite profiling (Gomez-Escribano *et al*., 2016). Using these strategies, various novel compounds have been discovered. A 51-membered glycosylated macrolide, named stambomycins A-D, was discovered after the stimulation of a cryptic type I PKS gene cluster via constitutive expression of a gene in the cluster. The gene that was upregulated, encodes for a putative pathway specific activator that triggers the expression of

the PKS genes. This stimulation provided the key to the identification of the stambomycins, that could not be expressed under ordinary laboratory conditions (Laureti *et al*., 2011).

Cloning and heterologous expression, as well as the mutational analysis, was used to partially identify the chaxamycin biosynthetic gene cluster from *Streptomyces leeuwenhoekii*. Heterologous expression of the chaxamycin biosynthetic gene cluster in *Streptomyces coelicolor* A3(2) strain M1152, was used to confirm that the identified cluster was responsible for the synthesis of chaxamycin. Next, mutational analysis was performed by deleting the 3-amino-5-hydroxybenzoic acid (AHBA) producing gene (*cxmK*) and replacing it with the kanamycin-resistance gene, *neo*, by double-crossover homologous recombination. The mutation revealed that chaxamycin production can be restored in the non-producing Δ*cxmK* mutant when grown on medium supplemented with AHBA and further confirms the role of the *cxmK* gene in chaxamycin production. The authors further imply that supplementation of ABHA analogues can be a viable approach to generate chaxamycin variants (Castro *et al*., 2015).

Using genetic and high-throughput screening, approximately 22 500 bioactive metabolites have been discovered from microorganisms. Of this, 3 800 are from unicellular bacteria (predominantly *Bacillus* and *Pseudomonas* species), 10 100 are from actinomycetia species, and 8 600 are of fungal origin. Whole genome sequencing of actinobacterial isolates has revealed the ability of these organisms to produce a wide range of metabolic products, including antibiotics, antifungals, anticancer agents and immunosuppressants. Thousands of compounds are produced by actinobacteria, and from the *Streptomyces* online database (StreptomeDB), more than 4000 compounds from over 2500 organisms have been found (Klementz *et al*., 2016). The genetic and metabolic potential of undiscovered bioactive compounds from actinobacteria, make this class very attractive for academic- and industrial research.

Many secondary metabolites display activities such as antibacterial, antifungal, antiprotozoal, antiviral, anti-tumour, anti-inflammatory and other activities that are used in medicine, veterinary medicine, agriculture and industry. Antimicrobial activity is the main activity of the secondary metabolites that have been isolated from bacteria, with about 14 000 compounds exhibiting antibacterial, antifungal, and/or antiprotozoal activity. The majority of bacterial antibiotics and other industrially-important compounds are produced by the *Streptomyces* species, which are responsible for producing 7 600 metabolites (Bérdy, 2005).

1.3.1 Antibiotics

Antibiotics are a type of drug that inhibits the growth of specific bacteria and are used to treat bacterial infections. Originally, antibiotics were isolated from natural organisms, such as bacteria, fungi, and plants. Today, antibiotic compounds can also be produced synthetically or semi-synthetically. The discovery of the antibiotics, actinomycin and streptomycin from *Streptomyces antibioticus* (Waksman and Woodruff, 1941) and *Streptomyces griseus* (Schatz *et al*., 1944) respectively, during the early 1940s, established the commercial value of the genus *Streptomyces*. Since this discovery, thousands of compounds were discovered from this genus and utilized for the treatment of infections. Actinomycetia produce more than half of all known microbial antibiotic compounds (Bérdy, 2005).

Antibiotics are biosynthesized through complex biosynthetic pathways that use primary metabolites as the building blocks. These pathways are activated through the upregulation of biosynthetic genes, with these genes normally grouping together in the chromosome, or sometimes on a plasmid, and are known as biosynthetic gene clusters. These clusters contain all the genes that encode for the biosynthetic enzymes, and frequently contain the regulatory genes that control biosynthesis as well as genes involved in antibiotic transport and self-resistance (Bibb and Hesketh, 2009). Antibiotic production is regulated by external factors, such as the availability of carbon, nitrogen, and phosphate. In addition, the production of antibiotics can also be regulated by small diffusible extracellular signalling molecules, such as gamma-butyrolactone (Rokem *et al*., 2007, Bibb and Hesketh, 2009; Figure 1.7).

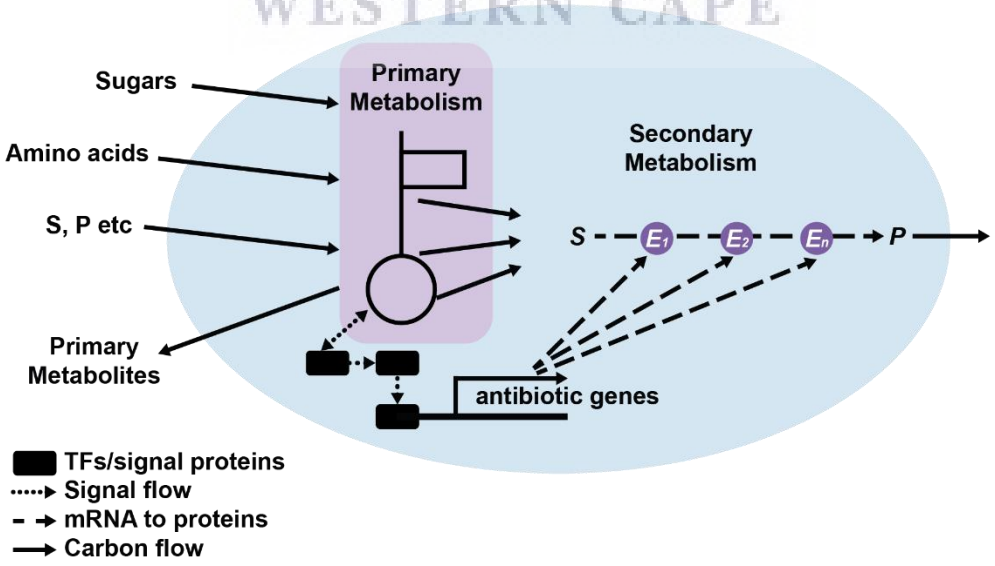


Figure 1.7: *A schematic overview of the regulatory systems that may be involved in the control of antibiotic production. Adapted from Rokem et al., 2007.*

Antibiotics target different components of a bacterial cell, and their mode of action is dependent on their class. The principal targets include the synthesis of the cell wall and cell membrane, DNA and RNA synthesis, folate metabolism, and protein synthesis (Figure 1.8). Actinobacteria produce a wide range of different antibiotic types. The main antibiotic types produced by actinobacteria include macrolides, tetracyclines, chloramphenicol, rifamycins, lipopeptides, aminoglycosides, glycopeptides, streptogramins, platensimycin, lincosamides, cephalosporin, penicillins, and quinolones (Coates *et al*., 2011).

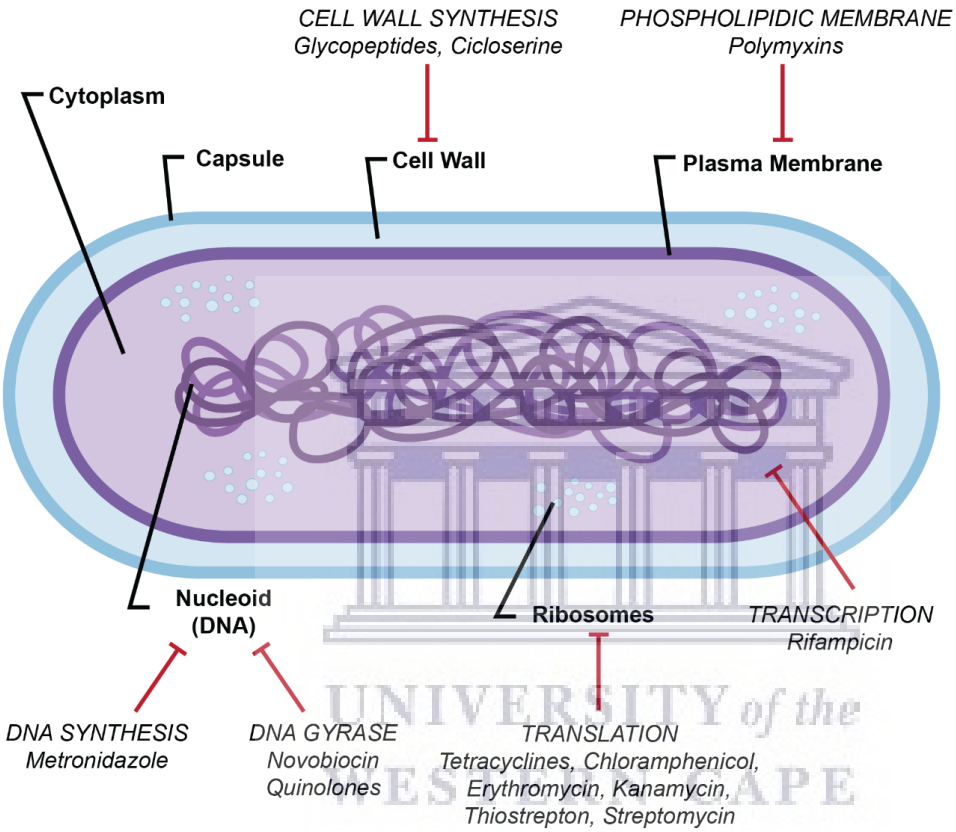


Figure 1.8: *Principal targets of antibiotics. Adapted from Grasso et al., 2016.*

1.3.2 Anti-tumour compounds

A substantial number of anticancer or antitumour compounds have been isolated from actinomycetia, with Actinomycin D being one of the first natural products to be used in tumour treatment. Actinomycin D was isolated from *Streptomyces antibioticus* and acts by binding DNA at the transcription initiation complex and prevents the elongation of the transcript by RNA polymerase. Due to its many side-effects, use has been limited but it is still used for the treatment of Wilms tumours in children (Waksman and Woodruff, 1941; Demain and Sanchez, 2009). Other antitumour compounds from actinobacteria include anthracyclines, bleomycin, mitomycin, and calicheamicin.

Bleomycin acts as an antitumour drug by inhibiting DNA synthesis (Blum *et al.,* 1972). Advances in gene sequencing have helped immensely with the identification of antitumour gene clusters from actinobacteria. The bleomycin gene cluster from *Streptomyces verticillus* was identified as a hybrid NRPS-PKS system (Du *et al*., 2000). With this information, the genes could be manipulated to synthesise different bleomycin analogues. Another biosynthetic gene cluster for an antibiotic antitumor compound that has been characterised is that of mitomycin C produced by *Streptomyces lavendulae*. The manipulation of a putative pathway regulator stimulated and led to an increase in mitomycin C production (Mao *et al*., 1999). Two new antitumor anthraquinones, lupinacidins A and B, were isolated from an endophytic actinobacterium, *Micromonospora lupini*. These lupinacidins had significant inhibitory effects against murine colon 26-L5 carcinoma cells without the inhibition of cell growth (Igarashi *et al*., 2007).

1.3.3 Antifungals

Amphotericin B is a potent antifungal that is produced by *Streptomyces nodosus*. This compound also has activity against some viruses, protozoa and prions. Due to the severe side effects of this antifungal compound, clinical use has been limited. In an attempt to lower toxicity and evaluate new analogues, the biosynthetic genes involved in the synthesis of amphotericin was identified and investigated. The PKS gene cluster was identified to contain six large PKS genes, two cytrochrome P450 enzymes, two ABC transporter proteins, and genes involved in biosynthesis and attachment of mycosamine. The genes involved were disrupted with phage KC515 that confirmed the gene cluster's involvement in the production of amphotericin. It is hoped that gene disruption and replacement could lead to the production and discovery of amphotericin derivatives with improved biological activity and reduced toxicity (Caffrey *et al*., 2001).

Polyoxins and nikkomycins are peptidyl nucleoside antibiotics that inhibit fungal chitin biosynthesis. Polyoxins are produced by *Streptomyces cacaoi* var. *asoensis* and *Streptomyces aureochromogenes* and are used extensively as an environmentally-friendly agricultural fungicide (Chen *et al*., 2009). Nikkomycins are synthesised by *Streptomyces tendae* Tü901 and *Streptomyces ansochromogenes,* and are used for the treatment of the human pathogenic fungal infection, Valley fever (Chen *et al*., 2016). Another group of fungicidal compounds produced by *Streptomyces* are the oligomycins which are produced by *Streptomyces diastatochromogenes*, *Streptomyces libani*, *Stretptomyces avermitilis* and *Streptomyces diastaticus* (Yang *et al*., 2010).

1.3.4 Antivirals

Natural products from actinobacteria have been studied extensively for the treatment of bacterial and fungal infections, and only recently, the antiviral properties have been investigated. Actinobacteria show promise for the production and isolation of antiviral compounds. Actinohivin is an anti-HIV protein that was isolated from the rare actinobacteria *Longispora albida*. This protein acts as a monomeric carbohydrate-binding agent with three carbohydrate binding sites. Actinohivin was found to prevent cell-free virus infection, cell-to-cell virus transfer, virus capture, and thus the subsequent transmission of HIV and has the potential to be developed as a microbicide drug (Hoorelbeke *et al*., 2010). Another rare species of actinobacteria, *Kibdelosporangium albatum*, is a producer of the antiviral compounds, cycloviracins, which have antiviral activity against herpes simplex virus type 1 (Tsunakawa *et al*., 1992; Tomita *et al*., 1993). Antimycin A1a, a secondary metabolite that displays potent activity against Western equine encephalitis virus, and was obtained from the marine streptomycete, *Streptomyces kaviengensis*. Antimycin A furthermore has a broad range of activity against RNA viruses, with inhibition activity against members from the *Togaviridae*, *Flaviviridae*, *Bunyaviridae*, *Picornaviridae*, and *Paramyxoviridae* families (Raveh *et al*., 2013).

1.3.5 Other bioactive compounds and activities

Other reported bioactivities from actinobacteria include antiparasitic, immunosuppressant, insecticidal, anti-inflammatory, nematocidal, neuroprotectant, and many more. Some compounds have more than one activity, such as valinomycin which is a cyclic depsipeptide that is produced by several *Streptomyces* species. This natural product has a wide range of activities including insecticidal, nematocidal, antibacterial, antiviral and apoptosis-inducing activities (Matter *et al*., 2009).

Sirolimus (rapamycin) and tacrolimus are immunosuppressive agents produced by actinobacteria (Barreiro *et al*., 2012). In 1999, the FDA approved sirolimus as a prophylaxis for acute rejection in renal transplant recipients. A clinical trial demonstrated that when used in combination with cyclosporine and steroids, it decreased the incidence of acute rejection episodes in the early post-transplant period, compared to either azathioprine or placebo (Goral and Helderman, 2010). Tacrolimus (a.k.a. FK-506; Prograf), produced by *Streptomyces tsukubaensis*, was approved by the FDA in 1994 for use in liver transplant recipients and in 1997 for use in kidney transplant recipients as the primary immunosuppressive agent or as

rescue therapy in steroid-resistant rejection due to its ability to block T-cell activation genes by a mechanism similar to that of cyclosporine (Vincenti *et al*., 1996, Varghese *et al*., 2014).

Streptomyces violaceoruber, isolated from zebra feces, produces anti-inflammatory compounds that inhibit nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated macrophage cells (Ma *et al*., 2017). Similarly, the endophytic *Streptomyces* sp. SUC1 produced anti-inflammatory compounds, lansais A - D, that also inhibited NO production in LPS-stimulated murine macrophage cells (Taechowisan *et al*., 2009). Neuroprotective activity of actinobacteria isolated from mangrove forest soils was tested using three different models, hypoxia, oxidative stress, and dementia, using SHSY5Y neuronal cells (Azman *et al*., 2017). Nocapyrones H–J, 3,6-disubstituted α-pyrones was isolated from the marine actinomycete *Nocardiopsis* sp. KMF-001 that exhibited both anti-inflammatory and neuroprotective activities. The neuroprotective effect of nocapyrone H was observed on inflammation-related brain damage induced by microglial cell activation (Kim *et al*., 2013). Actinobacteria produce secondary metabolites that are not only valuable as pharmaceuticals but also valuable as agricultural products. In agriculture, actinobacteria are used to combat insects, bacterial and fungal infections, and help to promote healthy plant growth and development (Palaniyandi *et al*., 2013).

1.4 Plant-microbe interactions

Plant-associated microorganisms are an extensive source of natural products that remains largely untapped. These organisms form associations with the plant within the phylosphere and rhizosphere where they are found on or in the plant itself. The endosphere of the plant includes the inner surface of the plant (including leaves, roots, flowers etc.) and harbours endophytic bacteria and fungi. The phylosphere includes the top outer surface of the plant (including the leaves, stem, flowers etc.) and hosts epiphytic bacteria and fungi. The rhizosphere of the plant includes the surrounding area of the plant roots and harbours rhizobacteria, mycorrhiza, nematodes and oocytes (Figure 1.9). These microorganisms produce biologically active metabolites that have applications as antibiotics, anticancer agents, antiparasitics, antifungals, antivirals, agrochemicals, among others (Ferdous *et al*., 2017).

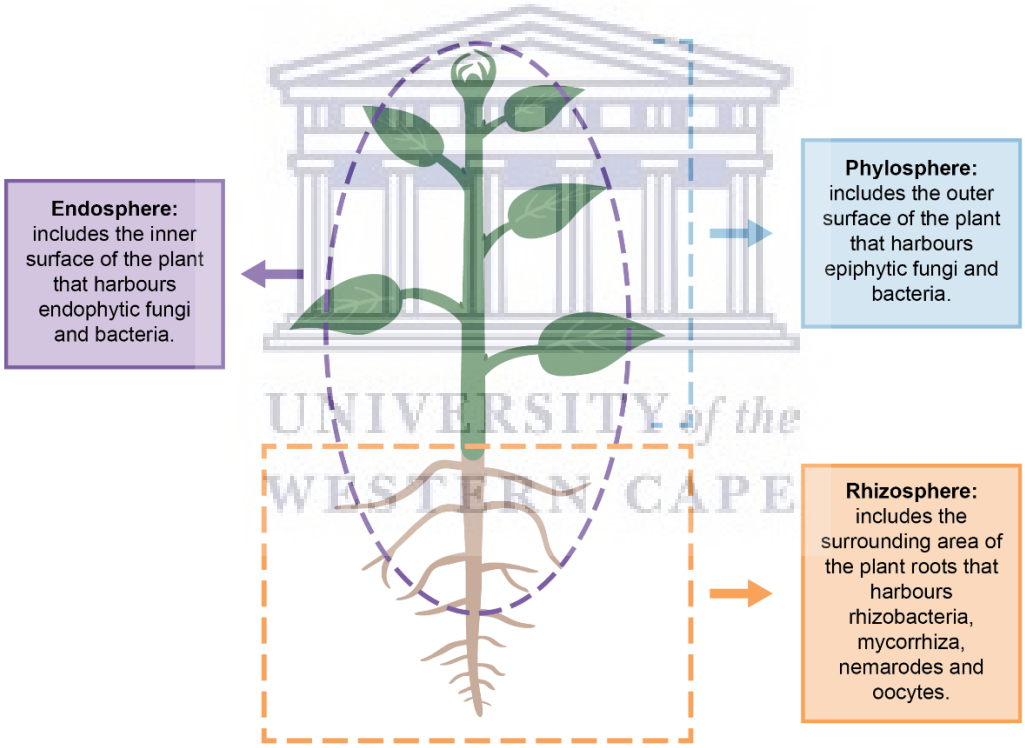


Figure 1.9: *Schematic representation of plant-associated microorganisms. Adapted from Ferdous et al., 2017.*

Certain types of pesticides and fertilizers have been banned or are under evaluation in some countries due to their negative effects on the environment and health concerns (Ntalli and Menkissoglu-Spiroudi, 2011). Microbial inoculants are a viable environmentally-friendly alternative for the control of plant disease and enhanced plant growth. Actinobacteria play a role in plant disease suppression by producing antimicrobials that inhibit the growth of plant

pathogens. Production of cell wall degrading enzymes by actinobacteria furthermore help with the biocontrol of plant pathogenic fungi and bacteria. Actinobacteria promote plant growth through the production of plant growth regulators, such as indole acetic acid (IAA) and cytokinins. Plant growth is further promoted by actinobacteria by enhancing iron availability through siderophore production, nitrogen fixation, and phosphate solubilisation (Palaniyandi *et al*., 2013).

Soil microbes influence plant productivity. A wide range of microorganisms forms symbiotic relationships with plants, with the relationship between plant and nitrogen-fixing microbes being the most extensively studied. These organisms convert atmospheric nitrogen into ammonium, a bioavailable form of nitrogen which is taken up by the plant. These N-fixing bacteria are normally found in close association with leguminous plants and contribute approximately 20% of all plant nitrogen that is required by vegetation annually (Cleveland *et al*., 1999, Van der Heijden *et al*., 2008). Microorganisms can also help improve plant productivity by inhibiting the growth of phytopathogens, decomposition of organic material into nutrients that the plant can use, and through phosphate solubilisation (Van der Heijden *et al*., 2008).

Some microorganisms have been found to be specific to certain plant species. Fynbos plant species were found to host plant specific bacterial communities. In a study by Miyambo *et al.* (2016) they focused on the bacterial communities associated with three Fynbos plant species, namely, *Erepsia anceps*, *Phaenocoma prolifera* and *Leucadendron laureolum*. Sequencing of the 16S rRNA gene revealed a high degree of variability within the different plant species tested (specialists), but also found bacterial communities that were present in all the plant species (generalists). The endophytic bacteria furthermore harboured plant-growth promoting activities that can influence plant growth and development. Plant associated bacteria protect the host plant against disease (Van der Heijden *et al*., 2008). Bacteria found within the soil can help to eliminate the growth of certain fungal species thus helping to protect the plant from fungal infections. While other bacteria can protect the plant from bacterial and fungal infections.

1.4.1 Rhizobacteria

Plants form symbiotic relationships with bacteria to help them adapt to their environment, to accumulate valuable nutrients and minerals, and to sustain growth. Interactions between legume root nodules and nitrogen-fixing bacteria have been studied extensively. This root nodule symbiosis involves host-specific recognition in order for nitrogen-fixing bacteria to form a mutualistic relationship with the plant. The plant provides a form of shelter to the rhizobia by

producing a root nodule, whereas the bacteria provide valuable nitrogen for the plant to use for growth. Leguminous plants initiate relationships with rhizobacteria by excreting flavonoids and aldonic acids via their roots which the bacteria senses to form interactions with the plant root. A sophisticated study by Haichar *et al*., (2008) investigated the ability of barrel clover, maize, rape, and wheat, grown separately in the same soil, to shape the microbial rhizosphere community. The investigators concluded that plant root exudates influence the soil microbial community. They also found that different plant species can promote the growth of specific bacteria, thus having a selective ability.

1.4.2 Epiphytes and Endophytes

Bacteria can also form symbiotic relationships with the entire plant by living on or inside it. Bacteria that live on plants are known as epiphytes and are found in the phyllosphere of the plant. These bacteria live near the plant without causing it any harm. Epiphytes have been found to alter floral scent emissions (Helletsgruber *et al*., 2017), effect leaf surface permeability (Schreiber *et al*., 2005), produce antifreeze proteins to protect Antarctic moss plants (Davies, 2016; Raymond, 2016), and protect plants from pathogens (Innerebner *et al*., 2011; Berg *et al*., 2005). Epiphytes have also been found to affect the main carbohydrate levels in plants. A recent study revealed that when *Sphingomonas*, *Pseudomonas* and *Methylobacterium* species were inoculated onto *Arabidopsis thaliana* leaves the composition of glucose, sucrose and fructose in the plants were affected in a species-specific manner (Ryffel *et al*., 2016).

Endophytes can be bacteria or fungi that are found within plant tissue, known as the endosphere, and includes the leaves and roots. Endophytes colonize plants internally without having a negative effect on the plant's structure and without displaying any disease symptoms (Reinhold-Hurek and Hurek, 2011). Endophytes are classified into two types, namely facultative and obligate. Facultative endophytes are microorganisms that can live inside plants or in other environments such as soil or water. Obligate endophytes are microorganisms that are strictly bound to life inside a plant. These organisms do not have any of its life stages outside the plant, except for plant-to-plant and plant-to-insect-to-plant transmission (Hardoim *et al*., 2008).

Studies have shown that endophytes have both a direct and indirect positive effect on plant growth and development. They affect plant growth directly by facilitating the acquisition of essential nutrients or by modulating the hormone levels of the plant. Endophytes have been reported to produce plant growth promoting regulators such as auxins, cytokinins and gibberellic acids. The production of ACC deaminase also suppresses the plant-stress

hormone, ethylene. The reduction of ethylene levels in plants under stress conditions can help to alleviate environmental stressors resulting from flooding, drought, salinity, metal and organic contamination, fungal and bacterial pathogens, nematode damage, and low temperature (Glick, 2004, 2012).

Plants often produce secondary metabolites as a response to environmental stress. In the laboratory, these stressors can be mimicked to investigate the plant's response. For example, the addition of a stress elicitor such as jasmonic acid to the plant's environment increases the amount of caffeic acid produced. Similarly, O_2 depletion, which is an abiotic stress factor, also increases the amount of caffeic acid produced (Nitzsche *et al*., 2004). Microorganisms living in close associations with plants can also produce environmental stressors which in turn induces the plant's secondary metabolism. For example, the production of taxol could be induced by the bacterial toxin, coronatine, which mimics the jasmonic acid-isoleucine conjugate (Katsir *et al*., 2008).

While both plant pathogens and plant endophytes can induce the plant's secondary metabolism, the long-term interaction of pathogen and plant is not desirable for the sustainable production of the compounds of interest due to the effect the pathogen has on the host. This one-sided interaction eventually leads to the decay of the host plant. Conversely, endophytes, do not harm the plant in any way. Furthermore, endophytes have been found to produce secondary metabolites with interesting bioactive properties. In recent years, research scientists have recognised the potential of these endophytes to produce secondary metabolites with possible applications in biotechnology (Casella *et al*., 2013). In many cases, endophytes produce secondary metabolites to compete with other microorganisms and thus these compounds have antimicrobial activities. The protection of plants from pests can sometimes be attributed to the secondary metabolites produced by the endophytes (Poling *et al*., 2008). Isolated endophytes that were identified as novel organisms with high antimicrobial potential due to their ability to produce new compounds (Casella *et al.,* 2013). There are several mechanisms by which plants and endophytes can interact. The plant can induce the endophyte's secondary metabolism and vice versa. The endophyte and plant can work together, were both are responsible for the partial production of a metabolite. Or lastly, the host plant can metabolize a produced compound partially (Ludwig-Müller, 2015).

It is of interest to investigate endophytes for bioprospecting because they have value not only in crop protection (Schulz *et al*., 2002) but also against human pathogens (Alvin *et al*., 2014), and can be used as a source or starting material for novel drug discovery (Suryanarayanan *et al*., 2009). Furthermore, microorganisms can produce potent cyclic peptides with high

antimicrobial potential via nonribosomal peptide synthesis, not found in plants. Compared to plant metabolites, microorganisms are favourable in commercial settings due to their short generation times, high biomass production, fast growth rates, and high scalability in bioreactors (Ludwig-Müller, 2015). However, when planning to isolate novel compounds from endophytes, the habitat of the host plant also needs to be taken into consideration to ensure successful production of the desired compound as the production media used should mimic that of the host plant (Strobel and Daisy, 2003; Strobel *et al*., 2004). To achieve this, additives like soil and plant extract can be used.

Actinobacteria are naturally found in soil and can easily interact with plant roots. Here they can form symbiotic associations with the plant. They can also enter the plant via the roots and become endophytic. From here the bacteria can stay in the roots or move to other parts of the plants. Bacteria can also become endophytic via integration into the plant through the stomata located on the leaves. Studies have found that the greatest endophytic actinobacterial diversity is found within the roots, then the stems and least in the leaves (Qin *et al*., 2009; Gangwar *et al*., 2014).

1.4.3 Actinobacterial interactions with Medicinal Plants

Medicinal plants are rich sources of bioactive metabolites. The long-term association of endophytes with these plants may have caused endophytes to participate in metabolic pathways within the plant, enhance its own bioactivity through the association or may have gained some genetic information to produce a specific active compound similar to that of the host plant (Stierle *et al*., 1993; Castillo *et al*., 2002; Li *et al*., 2008; Qin *et al*., 2009). Thus, endophytes isolated from medicinal plants can be of great biotechnological value. To isolate rare actinobacteria from medicinal plants, Machavariani and co-workers (2014) pre-treated leaves of medicinal plants, collected from the Moscow region in Russia, with heteroauxin and zircon. A total of 179 actinobacteria was isolated from 20 medicinal plant species. The endophytes isolated using this method included *Streptomyces* (65%), *Micromonospora* (27.5%) and *Nocardiopsis* (7.5%) species.

There is a great diversity of endophytic actinobacteria inside medicinal plants. Du and colleagues isolated 940 endophytes from 37 medicinal plant tissue samples which included more than 600 actinobacteria belonging to 34 genera and 7 unknown taxa. Furthermore, the physiological characteristics of the actinobacterial isolates related to the geographical distribution of their host. It was also noted that differences among strains from the same host were greater than from different plants grown in the same area (Du *et al*., 2013). In five

different mangrove plants, 101 endophytic actinobacteria were isolated that also showed high diversity. These actinobacteria were distributed within 7 orders, 15 families, and 28 genera (Jiang *et al*., 2018). Actinobacterial diversity was also observed to differ depending on the locations within the plant (Nimnoi *et al*., 2010).

Given the great diversity of actinobacteria in medicinal plants one can predict that they have the potential to host a variety of rare actinobacterial endophytes as well. Rare genera have been isolated, including *Actinocorallia*, *Blastococcus*, *Dactylosporangium*, *Dietzia*, *Jiangella, Oerskovia*, *Promicromonospora*, and *Saccharopolyspora* species from medicinal plants growing in rainforests in China (Qin *et al*., 2009). Similarly, rare actinobacteria genera such as *Microtetraspora* and *Intrasporangium* were isolated as endophytes from *Compositae* plant species from South Sinai in Egypt (El-Shatoury *et al*., 2013). Endophytic actinobacteria isolated from medicinal plants from a rainforest in China displayed antitumour and antimicrobial activities. Genome analysis revealed that the isolates harboured a plethora of PKS-I, PKS-II and NRPS biosynthetic genes. These genes indicated the genetic potential of the isolates to produce bioactive compounds, that may or may not have been expressed (Li *et al*., 2008). Similarly, actinobacteria were isolated from the rhizosphere soils of 16 medicinal plants from the Lamphun Province in Thailand. Some of the isolates had activity against phytopathogenic fungi with 36 of the isolates showing the ability to produce indole-3-acetic acid (IAA), a plant auxin which helps to stimulate plant growth and development (Khamna *et al*., 2009).

Bioactive compounds produced by endophytes isolated from medicinal plants have been investigated with much success. Munumbicins, are wide spectrum antibiotics, that was isolated from an endophytic streptomycete strain found in the medicinal snake vine plant (*Kennedia nigriscans*) native to Australia. The different munumbicins had differing biological activities. For example, munumbicin B had inhibitory activity against methicillin-resistant *Staphylococcus aureus*, whereas munumbicin A did not have any activity against this strain. The group of munumbicins generally had strong activities against Gram-positive bacteria such as *Bacillus anthracis* and *Mycobacterium tuberculosis*. Interestingly, munumbicin D, had inhibition activity against the malarial parasite *Plasmodium falciparum*. This important group of bioactive compounds thus has great potential in medicine (Castillo *et al*., 2002).

Extensive research has revealed that a wide diversity of actinobacteria are associated with medicinal plants, most of which has yet to be discovered. These bacteria have shown potential to produce similar bioactive compounds as their host plant. These bacteria also produce unique metabolites that have the potential to be developed into pharmaceutical products.

1.5 Medicinal plants

Medicinal plants or medicinal herbs are plants that have some sort of medicinal property and are used to treat ailments and diseases. The use of medicinal plants to treat illnesses is as old as mankind and has been documented all around the world. Medicinal plant usage has been documented from Traditional Chinese Medicine, Kampo medicine, Ayurvedic medicine, European medicine, and traditional medicines of Africa, Australia and the Americas (Wink, 2015).

Medicinal plants have been used throughout human history and are recorded in scriptures like the Bible, and the Ebers Papyrus which contains information on herbal medicine. The earliest recorded use dates back over 5000 years and is written on a Sumerian clay slab from Nagpur. This slab contained information on 250 plants species that are used in 12 recipes to make herbal concoctions. China's herbal medicine usage dates to around 2500 BC when the Chinese emperor Sheng Nung wrote the "Pen TS'ao", which contained information on 365 drugs made from dried medicinal plants. Some of these plants, such as ginseng, camphor, and cinnamon bark, are still used today (Hassan, 2015).

Medicinal plant usage has been documented in various cultures from around the world, from Africa to Greece to China. Often these medicinal properties were discovered by experimentation and anecdotes. These treatments were then passed down from generation to generation. Today we can verify the medicinal characteristics of these plants and are able to identify the compounds that are linked to the plant's healing properties.

Medicinal plants serve as a valuable resource for the discovery of new antibiotics, anticancer agents and other drugs. These plants have evolved to produce secondary metabolites for a variety of reasons, which includes for the protection against herbivores, other plants, microbes, but also as signalling compounds. Plants evolved to produce these secondary metabolites for an obvious reason, in that they are unable to move away from undesirable conditions or move towards optimal conditions. Thus, the production of secondary metabolites is the plant's mechanism to cope with environmental conditions (Wink, 2015).

Traditional usage of medicinal plants has guided scientists to explore certain properties of plants. For example, Sumerian cultures used poppies (known to them as the joy-plant) for narcotics (Theis and Lardau, 2003). In the 1970s it was elucidated that the *Papaver somniferum*, or opium poppies, produce high amounts of codeine and morphine which are now established pain medications (Miller *et al*., 1973; Ziegler *et al*., 2009). Opium poppies

became so important due to these alkaloids that they produced, that they were the first plant to be cultivated for reasons other than nutritional value (Theis and Lardau, 2003).

The well-known anti-cancer compound taxol, was isolated from the bark of the medicinal plant *Taxus brevifolia*, the Pacific yew tree, which is now used clinically to treat breast, ovarian, lung, bladder, prostate, melanoma, esophageal, as well as other types of solid tumor cancers (Wani *et al*., 1971). Another widely used group of anti-cancer metabolites produced by plants are the Catharanthus alkaloids which comprise of a group of 130 terpenoid indole alkaloids. These alkaloids are produced by *Catharanthus roseus*, the Madagascar periwinkle. The pharmaceutical product made from these alkaloids, Vinblastine, has now been used for over 40 years as an anti-cancer drug (Van der Heijden *et al*., 2004).

African medicinal plants have been shown to have antibacterial, antifungal, antischistosomal, anti-amoebic, antimalarial, anti-inflammatory, and antioxidant activities. Some plants have also been found to help treat mental illness such as anxiety, epilepsy, hysteria and even depression (Fennell *et al*., 2004). Coupling traditional knowledge with science can help us to unlock the potential of medicinal plants.

1.5.1 Medicinal plants of South Africa

South Africa has a very diverse climate and is divided into nine major terrestrial biomes, namely the Nama Karoo, succulent Karoo, fynbos, forest, Albany thicket, savanna, grassland, Indian Ocean coastal belt, and desert biome (Figure 1.10). Having so many unique biomes found in a relatively small geographical area (1 219 912 km^2) makes the Southern Africa's landscape an ideal host for a diverse number of plants, harbouring an estimated 22 750 flowering plant species (Van Wyk, 2011).

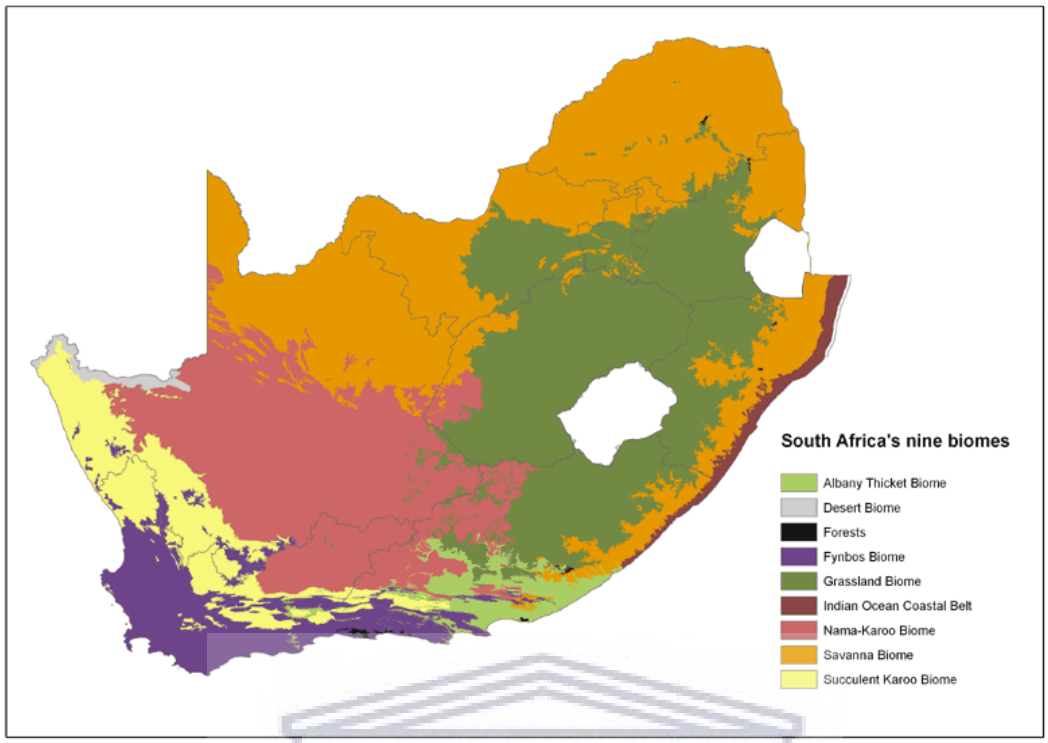


Figure 1.10: *The distribution of the nine biomes in South Africa. Taken from Manning and Goldblatt, 2012.*

The Fynbos biome is defined by climate, life-form patterns and major natural disturbances and comprises three different, naturally fragmented vegetation types, namely fynbos, renosterveld and strandveld. These vegetations are dominated by small-leaved, evergreen shrubs whose regeneration is dependent on natural fires. The Fynbos biome occurs in the winter- and summer-rainfall areas and is one of two (with Albany Thicket) biomes that are endemic to South Africa (Rebelo *et al*., 2006). The Fynbos region forms part of the Cape Floral Kingdom, which is the smallest of the six floristic kingdoms but is the most diverse per unit of area. Impressively, this rich biome only covers about 90 000 km^2 of South Africa yet contains around 9000 species of which over 80% are endemic to the area (Manning and Goldblatt, 2012).

An estimated 3000 species in southern Africa are administered to humans for their medicinal properties. Some of these medicinal plants have been used for decades and have been passed on from one generation traditional healers to the next. A staggering 72% of people in South Africa use traditional medicine with the traditional medicinal plant trade valued at about R2.9 billion. As a result of the high number of medicinal plants used, medicinal plants of high commercial importance are threatened by overharvesting and have in some cases resulted in local extinction.

Street and Prinsloo (2013) reviewed the top ten medicinal plants with commercial importance which are: *Agathosma betulina* (buchu), *Aloe ferox* (Cape Aloe), *Aspalathus linearis* (rooibos), *Harpagophytum procumbens* (devil's claw), *Hypoxis hemerocallidea* (African potato), *Merwilla natalensis* (blue squill), *Pelargonium sidoides* (kalwerbossie), *Sclerocarya birrea* (Marula), *Siphonochilus aethiopicus* (African ginger) and *Sutherlandia frutescens* (cancer bush). This study will only focus on two of these medicinal plants namely, *Aloe ferox* and *Sutherlandia frutescens*.

1.5.2 *Aloe ferox*

Aloe ferox Mill (commonly known as bitter aloe, Cape aloe, or bitter aalwyn) is indigenous to the Cape coastal region of South Africa and is found abundantly from Swellendam to the Eastern Cape, and even extends into KwaZulu-Natal (Chen *et al*., 2012). This plant grows well in a variety of environments and is generally found within the Fynbos, Albany Thicket, Grassland and Indian Ocean Coastal Belt biomes (Raimondo *et al.,* 2018). *Aloe ferox* is a single-stemmed, hardy arborescent species that grows up to 10 meters tall and is identifiable by its characteristic fleshy curved leaves with red-brownish thorns which refers to the *ferox* in the botanical name meaning fierce or ferocious in Latin. During the winter, *A. ferox* produces single-stemmed racemes with either bright red, orange, yellow or rarely white flowers (Figure 1.11).

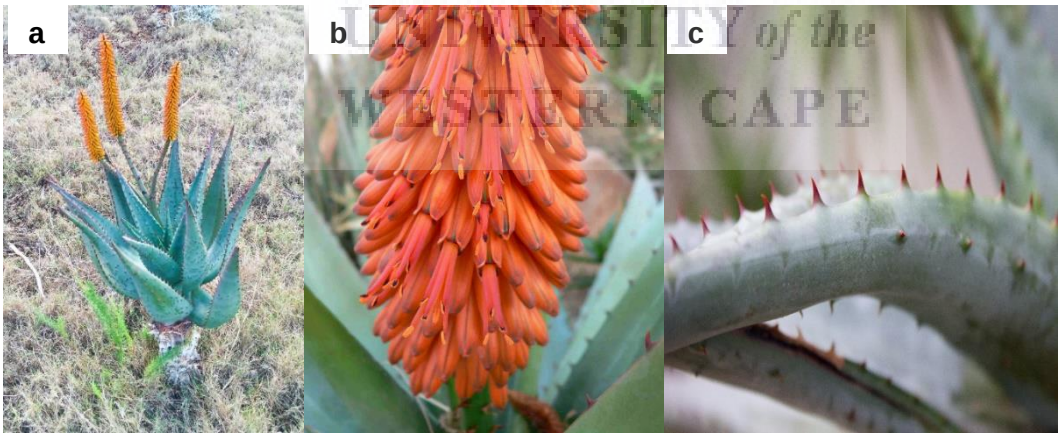


Figure 1.11: *(a)* Aloe ferox plant *with its (b) bright orange flowers and (c) fleshy thorns. Images by Katrien King (a and c) and Stan Shebs (b).*

Traditionally, *A. ferox* has been used as a laxative, to treat skin ailments, and for the treatment of cancers. In recent years, aloe has been incorporated in food and cosmetic products. The use is these industries increased the popularity of aloe-based products (Chen *et al*., 2012).

Aloe ferox is also one of a few plants to be depicted in San rock paintings (Van Wyk, 2013). The bitter gel is used in Africa and Europe as a laxative and has been proven to provide relief for constipation in Wistar rats (Wintola *et al*., 2010). Furthermore, leaf extracts have high antioxidant activity that can add value to cosmetics, medicine and food industries (Wintola and Afolayan, 2011). The value of the *Aloe ferox* industry to small-scale rural farmers was estimated in 2006 to be worth between R12 and R15 million, and the value of the total industry is estimated to be closer to R150 million per year due to mark up through retail (Shackleton and Gambiza, 2007). Due to growth in the industry over the last decade and the shift to using more naturally based products, it can be assumed that the value of the *A. ferox* industry has also increased significantly.

Aloe ferox produces a broad range of metabolites including chromones, anthraquinones, and phenolic compounds. The aloe leaf can be divided into two parts, the outer green rind and the fleshy inner pulp (Figure 1.12a). The aloe bitters are found within the inner pulp in the alloin cells (parenchyma cells) (Figure 1.12b). The aloe bitters contain mainly aloeresin A, aloesin and aloin in a ratio of approximately 4:3:2, respectively (O'Brien *et al*., 2011). The inner pulp contains a clear gel that is used for its emollient and moisturising properties. Upon further investigation of this freeze-dried gel, an 1H NMR spectrum recorded signals for glucose, fructose, malic acid and quinic acid (Andersen, 2007; Chen *et al*., 2012). *Aloe ferox* has a broad range of activities such as a laxative, wound healing, skin protectant, antioxidant, anti-inflammatory, anti-cancer, antimalarial, and anthelmintic. A study showed that spraying cattle with an aqueous leaf extract aided in controlling ticks on cattle (Moyo and Masika, 2009).

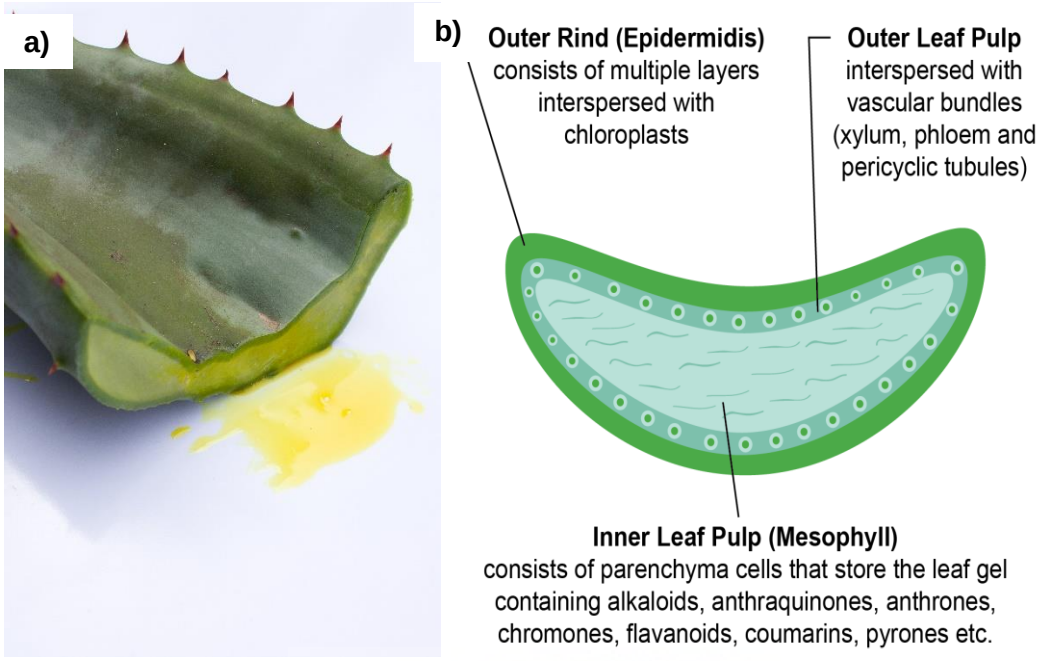


Figure 1.12: (a) The fleshy leaves of *A. ferox* consist of a green outer rind and a fleshy inner pulp. (b) Schematic cross-section of the leaf showing the outer rind, outer leaf pulp and inner leaf pulp (Adapted from Cock, 2015).

Aloe bitters are well known for its purgative effect. Anthrone-C-glycosides is considered typical in aloe bitters and occurs as aloin A and B. These compounds are responsible for the bitter and laxative properties (Dagne *et al*., 2000). It is hypothesised that the compounds are stable in the stomach and that the sugar moiety prevents them from being absorbed into the upper gastrointestinal tract and later the detoxification in the liver. Thus, they do not get broken down in the intestine before reaching the site of action in the colon and rectum. In the large intestine, the aloins break up through bacterial digestion and release the aglycones that have the laxative effect (Chen *et al*., 2012). The effect of aloe bitters on constipation was confirmed when loperamide-constipated Wistar rats were fed an aqueous leaf extract for 7 days. The aloe extract performed at the same level of senokot, a standard laxative drug (Wintola *et al*., 2010).

Aloe ferox has been extensively studied for its effect on the skin. The leaf gel had an anti-erythema (anti-redness) effect on the skin (Fox *et al*., 2014). The extracts had anti-inflammatory activity and inhibited the growth of *Propionibacterium acnes*, which causes acne and inflammation associated with this skin disease (Jeong and Kim, 2017). In another study, anti-inflammatory activity of *A. ferox* whole leaf aqueous extract was evaluated by rat paw edema that was induced by carrageenan and formaldehyde. A dosage of 400mg/kg had the

highest anti-inflammatory activity with a reduction of 78.2 and 89.3%, respectively (Mwale and Masika, 2010).

Whole-leaf juice preparations of *A. ferox* was found to accelerate the progression of wound closure. Anti-inflammation is the first step in the healing of wounds and the topical application of the leaf preparations played a direct role in facilitating fast healing. Furthermore, the preparations also inhibited bacterial and fungal growth of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853), and fungal spores of *Cryptococcus neoformans* (Jia *et al*., 2008).

Traditionally, *Aloe ferox* is used to treat sexually transmitted infections which is mainly due to the plant's potent anti-bacterial activity. This antibacterial activity of the leaf extracts can be owed to a combination of compounds found within aloe. Three compounds, aloe-emodin, chrysophanol and aloin A, were isolated that had inhibitory activity against a range of bacterial strains. Aloe-emodin and aloin A had inhibitory activities against *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli,* and *Shigella sonnei*. Whereas chrysophanol only had inhibitory activity against *B. subtilis*, *S. epidermidis*, and *E. coli* (Kambizi *et al*., 2008).

1.5.3 *Sutherlandia frutescens*

Sutherlandia frutescens (also known as *Lessertia frutescens*) is an upright, southern African, leguminous shrub, more commonly known as the cancer bush (or kankerbossie in Afrikaans), that forms part of the *Fabaceae* family. This perennial, but a short-lived shrub, reaches up to 2.5 meters in height with bright red flowers that appear from spring to mid-summer (Van Wyk and Albrecht, 2008; Figure 1.13). This plant is restricted to drier areas of southern Africa where it grows in the savannah and hillsides near streams. This plant may also be found on rocky sandy soils along coastal areas (South African National Biodiversity Institute, 2018).

This plant has been traditionally used to treat a variety of ailments - including stomach ulcers, rheumatism, chicken pox, and for the treatment of wounds. Recent studies have found that *S. frutescens* plants have anticonvulsant (Ojewole, 2008), anti-stress, anti-inflammatory and anti-hypertensive properties (Lei *et al*., 2015; Sergeant *et al*., 2017). Some of the important compounds that are produced by these legumes include flavonoids and terpenoids, that are unique to the plant known as sutherlandiosides and sutherlandins, respectively. These two groups of compounds have been linked to Sutherlandia's potential antitumour and anti-diabetic properties (Van Wyk and Albrecht, 2008; Skerman *et al*., 2011). Other compounds

produced by *Sutherlandia* include gamma-aminobutyric acid (GABA), proline, arginine, asparagine, and L-canavanine. These compounds have been linked to the medicinal properties found in this shrub. These wide ranges of medicinal properties make cancer bush a valuable plant to study because new and improved drugs for the treatment of these diseases are always needed.

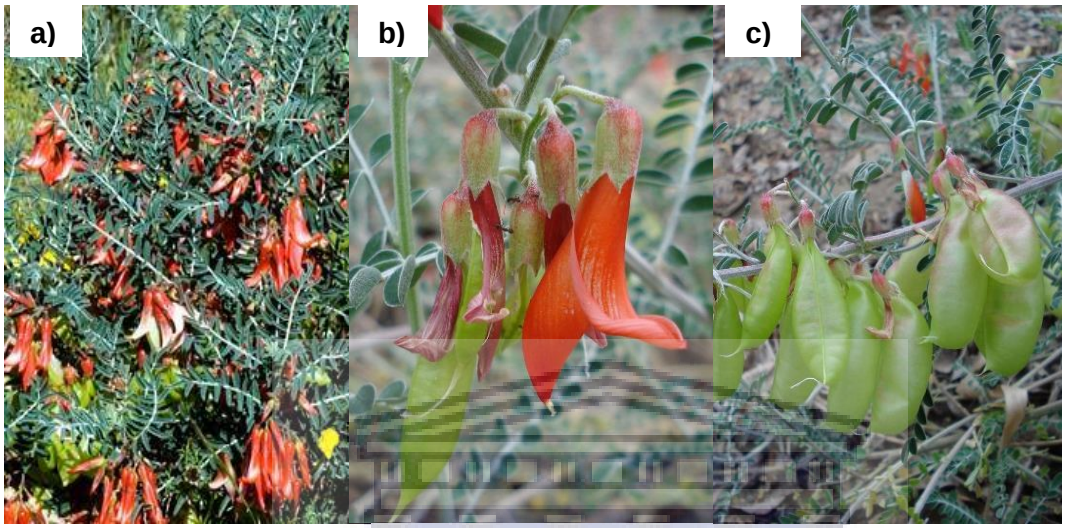


Figure 1.13: *(a)* Sutherlandia frutescens *plant with its (b) bright red flowers and (c) seed pods. Images by (a) Wolfgang Stuppy and (b and c) Oliver Whaley.*

The common name "cancer bush" comes from the fact that traditionally the plant was used by the Khoisan and Dutch people for the treatment of internal cancers (Van Wyk and Albrecht, 2008). Worldwide, about 18 million people have been diagnosed with cancer and more than 9.5 million deaths resulted from this disease in 2018 (Bray *et al.,* 2018). With this alarmingly high fatality and diagnosis rate, it is necessary to develop new drugs to cure or inhibit this deadly disease. Studies on *Sutherlandia* extracts have shown antiproliferative effects on human breast and leukemia tumour cell lines *in vitro* (Tai *et al*., 2004). The plant extracts have been shown to induce apoptosis of neoplastic and Chinese Hamster Ovary (CHO) cells (Chinkwo, 2005), esophageal cancer cell lines (Skerman *et al*., 2011), and tumorigenic breast adenocarcinoma cells (Stander *et al*., 2009).

Extracts made from *S. frutescens* leaves and flowers inhibit HIV-RT and glycohydrolase enzyme activity which subsequently reduces the infectivity of the HIV virion (Harnett *et al*., 2005). A possible compound responsible for this effect is L-canavanine. According to a patent, this amino acid destroyed 95% of HIV-infected lymphocytes *in vitro* (Green, 1992). Sutherlandia capsules were tested on healthy adults and confirmed to be safe to use. The medication also improved the appetite of patients, which could relieve the loss of appetite

experienced by HIV/AIDS patients (Johnson *et al*., 2007). After this study, an adaptive double-blind randomized placebo-controlled trial was conducted to evaluate the consumption of *S. frutescens* by HIV-Seropositive South African adults. The results indicated that the treatment with *Sutherlandia* did not change HIV viral load, and the CD4 T-lymphocyte count. The authors concluded that further studies on individuals with a higher viral load will need to be tested. There was also an indication that treatment may possibly reduce the efficacy of isoniazid preventive therapy (IPT) given to prevent tuberculosis and requires further evaluation (Wilson *et al*., 2015).

Wistar rats that were fed a high-fat diet were administered with *S. frutescens* in their drinking water. After 8 weeks of treatment, the prediabetic rats displayed normal levels of insulin. Glucose uptake into the muscle and adipose tissue increased significantly with a significant decrease in intestinal glucose uptake (Chadwick *et al*., 2007). Canavanine, found in cancer bush, was proved to decrease hyperglycemia in streptozotocin (STZ)-induced type 1-like diabetes rats. Additionally, canavanine increased the B-endorphin level, as well as glucose uptake into the skeletal muscles (Chang *et al*., 2015). *In vitro* insulin resistant liver cells were treated with aqueous *S. frutescens* plant extract and took up significantly more glucose and released less glucose into the culture medium and accumulated less intracellular lipids. The *S. frutescens* extract targeted a total of 27 genes of insulin-resistant cells. These genes encode vesicle transporters, receptors, signaling molecules, transcription factors, and metabolic enzymes that were significantly up or down regulated (Williams *et al*., 2013).

Anti-inflammatory and hypoglycemic properties of *S. frutescens* shoot aqueous extract was tested on rats using fresh egg albumin-induced pedal (paw) edema and streptozotocin (STZ)-induced diabetes mellitus. The analgesic property of the extract was tested on mice using the hot-plate and acetic acid test models of pain. The extracts had significant anti-inflammatory and analgesic activity and significantly increased hypoglycemia (Ojewole, 2004). Ethanolic extracts of *S. frutescens* was evaluated for its anti-inflammatory and antioxidant activity in the brain. The extracts were able to suppress reactive oxygen species (ROS) production in neurons, as well as reduce ROS and nitric oxide (NO) production in microglial cells. The action on microglial cells appears to be mediated through inhibition of the p-ERK1/2 signalling pathway which is central to regulating several intracellular metabolic processes including enhancing STAT1a phosphorylation and filopodia formation. The use of *S. frutescens* can potentially be used for the treatment of stress and inflammatory diseases in the brain (Jiang *et al*., 2014).

Medicinal plants have the potential to host a unique microbiome that may contain a source of novel bacteria that produce new bioactive compounds. These compounds have the potential to be related to the compounds produced by the plant and can even be more potent than those found in the host plant. The plants and their microbes have evolved together and have formed a relationship to help fight off disease, poor nutrition, or climate changes. This synergistic relationship makes the microbiome a valuable resource to research since the potential discovery of medicinally valuable compounds is yet to be unlocked.

1.6 Importance of this project

In 2015 the World Health Organisation (WHO) launched the Global Antimicrobial Surveillance System (GLASS) to monitor the outbreak of antimicrobial resistance. This report revealed that antimicrobial resistance is widespread across 22 countries, with 500 000 people being infected with an antimicrobial-resistant strain of bacteria annually. The bacteria that were most frequently reported to have antimicrobial resistance were *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Salmonella* species.; however, it should be noted that the GLASS does not include data on *Mycobacterium tuberculosis* (MTB) resistance (World Health Organisation, 2017). The WHO has been tracking MTB resistance since 1994 and they provide annual updates in the Global tuberculosis report (World Health Organisation, 2018).

The report on tuberculosis (TB) found that in 2017, there was an estimate of 558 000 new cases of rifampicin-resistant TB (RR-TB) globally, of which almost half were in three countries: India (24%), China (13%) and the Russian Federation (10%). Among these RR-TB cases, an estimated 82% had multidrug-resistant TB (MDR-TB). South Africa has a high outbreak of TB, with 500 people per 100 000 population diagnosed with TB in 2017 (World Health Organisation, 2018).

The need for new and useful drugs that aim to treat, or relieve human conditions is ever-growing. Drug-resistant bacteria and the tuberculosis epidemic is only two examples. Viral and fungal infections, successful organ transplant and prevalence of pain are all medical conditions that can be solved with the discovery of new drugs. Investigating microorganisms from rare environments is a practical approach to isolate novel bacteria that have the potential to produce new and useful compounds. The following four criteria for isolation of bacteria from rare environments associated with plants were identified (Strobel *et al*., 2004):

1. Plants from unique environments, which have developed unique biology to survive in the environment.
2. Plants with an ethnobotanical history.
3. Plants that are endemic to the area and have occupied the area for a long time.
4. Plants growing in areas of great biodiversity.

The investigation of actinobacteria associated with *A. ferox* and *S. frutescens* that are endemic to South Africa and grows naturally in the diverse Fynbos biome aligns to all the above-mentioned criteria. Thus, actinobacteria isolated from these medicinal plants have the

potential to produce unique compounds that can be used for the development of drugs to treat or relieve some of the human diseases and illnesses mentioned.

The aims and objectives of this study includes:
(i) the isolation and identification of actinobacteria associated with *A. ferox* and *S. frutescens* plants
(ii) the screening of the bioactivity of these isolated actinobacteria
(iii) investigating the genetic potential of some the bioactive actinobacteria.

As discussed in Chapter 2, actinobacteria will be isolated and identified using plate culture methods as stipulated by the International *Streptomyces* Project which outlines a standardized protocol for classifying actinomycetia species (ISP; Shirling and Gottlieb, 1966). Phylogenetic analysis will also be used to identify actinobacteria by their genetic sequence of the 16S rRNA and *gyrB* genes, which are both highly conserved in actinobacteria and commonly used for identification. The bioactivity screening of the actinobacteria will be done through plate techniques such as cross-streaking, overlay, and Kirby-Bauer, further described in Chapter 3. Crude extractions of the isolates will be screened for activity using TLC methods, including bioautography to screen for antibacterial activity. Finally, whole genome sequencing of selected isolates will be performed and bioinformatics techniques, such as the screening for biosynthetic gene clusters, will be used to elucidate the genetic potential of the isolates (discussed in Chapter 4).

Combining the isolation of actinobacteria from niche habitats with the advances made in genomics and bioinformatics in recent years will help to better identify compounds of interest. This project combines the aforementioned techniques that have been used in natural product discovery and aims to partially isolate and identify compounds of pharmaceutical value from actinobacteria.

Chapter 2: Isolation, identification and characterisation of actinobacteria

2.1 Introduction

The isolation of actinobacteria from environmental samples can be difficult, due to the presence of other abundant fast-growing microorganisms. Actinobacteria are generally slower growing and can take up to 30 days before growth is observed on an isolation plate. Therefore, specific isolation protocols for actinobacteria, particularly slow-growing or rare actinobacteria, have been developed which includes the pre-treatment of the samples with heat or antibiotics, and the addition of antimicrobials/antifungals to the media. This strategy helps to select for actinobacterial isolates by limiting faster growing organisms. Another strategy for isolation is to use minimal media supplemented with environmental material (e.g. soil- or plant extracts). This helps to facilitate the growth of fastidious actinobacteria by simulating the natural environment, and providing minerals and trace elements at concentrations naturally found within the environment. The use of minimal media helps to only isolate actinobacteria that can utilise the specific nutrient source.

Sampling from unique environments increases the chances of isolating novel bacteria or bacteria producing novel metabolites (Strobel and Daisy, 2003). Utilising this strategy, samples from the Fynbos biome were selected for isolation of actinobacteria. The selection strategy was further focussed by selecting medicinal plants for the isolation of actinobacteria. As discussed previously (Section 1.4.3), actinobacteria associated with medicinal plants may have unique metabolisms, producing the same or similar medicinal compounds as their host plant (Stierle *et al.,* 1993; Castillo *et al.,* 2002; Li *et al.,* 2008; Qin *et al.,* 2009). Isolation of actinobacteria from this niche environment also increases the chances of isolating novel actinobacteria which may possess the ability to produce interesting metabolites. Thus, this strategy was ultimately chosen to increase the likelihood of discovering new medicinal compounds.

Previous studies have shown that medicinal plants constitute a niche environment and the endophytic actinobacteria found within these plants possess great metabolic diversity. Sixteen genera of endophytic actinobacteria were isolated from six medicinal plants species growing in the Gibbon Wild Life Sanctuary in Assam, India. These included *Streptomyces* and also rare genera including *Isoptericola*, *Kytococcus* and *Verrucosispora*. The 76 isolated actinomycetia were screened for antimicrobial activity of which 21 had activity. These active

isolates showed the prevalence of polyketide synthase (PKS) type-II (85%), and PKS type-I (14%) genes. Not only were the authors able to isolate a variety of rare actinobacteria from this unique environment, but they highlighted the bioactive potential of these endophytic actinobacterial isolates (Gohain *et al.,* 2015). The wide-spectrum antibiotics, the munumbicins, were isolated from *Streptomyces* sp. NRRL 30562. This streptomycete was found within the stems of *Kennedia nigricans*, a medicinal plant native to Australia (Castillo *et al.,* 2002). Endophytic actinobacteria with cytotoxic and antifungal activity was isolated from the Chinese medicinal plant *Dracaena cochinchinensis* Lour. (commonly known as dragon's blood). The plant has become a popular ingredient in Traditional Chinese medicine which, unfortunately, has led to a decline in its population size. In an attempt to utilise the resource in a sustainable way, endophytic actinobacteria was isolated from the plant. This was done because actinobacteria from medicinal plants have been found to produce similar medicinal compounds to that of the host plant. In the study, the endophytic actinobacteria were found to possess PKS type-I, PKS type-II and NRPS biosynthetic genes, indicating the potential of these isolates to produce bioactive compounds. The authors concluded that endophytic actinobacteria from medicinal plants can be used as an alternate source of bioactive compounds that were previously obtained from the host medicinal plant (Salam *et al.,* 2017). From these studies, and many others, it is noticeable that medicinal plants host a diverse population of actinobacteria, whether in the roots, stems, leaves or the surrounding soil.

2.1.1 Genus *Streptomyces*

The genus of *Streptomyces*, with the genera of *Kitasatospora and Streptacidiphilus* fall under the order *Streptomycetales* and the family *Streptomycetaceae*. In 2012, over 550 species of *Streptomyces* have been officially recognised (Kämpfer, 2012), with over 800 species cited to this date (http://www.bacterio.net/streptomyces.html; accessed 22-10-2019). Because of the large diversity of this genus, it is difficult to phenotypically classify species. Some of the main characteristics, with some exceptions, is that streptomycetes produce branched vegetative mycelium with multinucleated aerial mycelium that mature into chains of three or more spores. The colonies are leather-like and once they sporulate they take on a granular, powdery appearance. They produce a range of colour pigments as part of their secondary metabolism and thus species in this genus come in a variety of colours. The aerial and substrate mycelium colours, as well as the colour of soluble pigment (if produced) are used to phenotypically characterise species. Generally, *Streptomyces* species use a range of carbon and nitrogen sources and are easy to cultivate on a range of media. *Streptomyces* cells are non-acid fast and Gram-positive, and molecularly they have a high percentage G + C content of their genomic DNA that ranges from 69 - 78%. This genus is well known to produce secondary

metabolites such as antibiotics, pigments and anticancer compounds. Streptomycetes produce more than 7500 bioactive metabolites, which is around 34% of known microbial metabolites (Bérdy, 2005).

Generally, streptomycetes are aerobic, mesophilic, neutrophilic, and non-pathogenic. They play an important role in soil by decomposition of organic matter such as plant material. Largely, streptomycetes have been isolated from soil samples, but have also been isolated from marine sediments and from plant material as endophytes. Some streptomycetes do cause plant diseases, including *Streptomyces scabies, Streptomyces acidiscabies* and *Streptomyces turgidiscabies* that all cause common scab in potatoes (Lambert and Loria, 1989a, 1989b; Miyajima *et al*., 1998).

2.1.2 Genus *Amycolatopsis*

The genus *Amycolatopsis* is one of 22 genera that fall under the order of *Pseudonocardiales* within the family of *Pseudonocardiaceae*. This genus was reclassified from *Nocardia* in 1986 after it was found that it was not susceptible to phages specific to the *Nocardia-Rhodococcus* complex and that this genus had a type PI1 phospholipid pattern (Lechevalier *et al*., 1986). To date, there are 76 *Amycolatopsis* species that are validly published (http://www.bacterio.net/amycolatopsis.html, accessed 22-10-2019). *Amycolatopsis*, like most actinomycetia, are predominantly found within soil environments. *Amycolatopsis* form branching vegetative hyphae which break down into squarish subunits. The aerial mycelium may or may not be present. When formed, aerial mycelium may break down into chains of squarish or oval fragments. They also do not form endospores, sheaths, synnemata, sporangia, or sclerotia. They are non-acid fast, mesophilic, aerobic, catalase positive, and non-motile. The mol% G + C content of this genus ranges between 66 and 69 (Lechevalier *et al*., 1986). Genome sequencing and studies have revealed that these bacteria have relatively large genomes, ranging from ~5 Mb to ~10 Mb, in the form of circular chromosomes, with a fair number of BGCs (Kumari *et al*., 2016). The MiBiG repository contains 21 full or partial BGCs from *Amycolatopsis* (https://mibig.secondarymetabolites.org accessed 22-10-2019). *Amycolatopsis* strains produce a range of useful secondary metabolites including avoparcin, balhimycin, chelocardin, chloroeremomycin, ECO-0501, macrotermycins A–D, pargamicins B–D, ristomycin, rifamorpholines A–E, and rifamycin, (Beemelmanns *et al*., 2017, Hashizume *et al*., 2017, Xiao *et al*., 2017, Chen *et al*., 2016; Kumari *et al*., 2016). Vancomycin, produced by *Amycolatopsis orientalis,* is the most well-known antibiotic produced by this genus.

2.1.3 Genus *Alloactinosynnema*

The genus *Alloactinosynnema* falls under the order of *Pseudonocardiales* and the family *Pseudonocardiaceae*. To date, only two species have been characterised in this genus, *Alloactinosynnema album* (Yuan *et al*., 2010) and *Alloactinosynnema iranicum* (Nikou *et al*., 2014), both isolated from a soil environment. *Alloactinosynnema* is morphologically like the genus *Actinosynnema*, but chemotaxonomically and phylogenetically distinct. *Alloactinosynnema* species are aerobic and form extensively branched white to pink substrate mycelium that may fragment into rod-shaped elements. Yellow-white aerial mycelia that differentiate into long chains of motile and smooth-surfaced spores are produced. Both spore chains and aerial mycelia often cluster. Some media promotes the production of sporangia-like structures. Media composition also stimulates the production of a buff coloured diffusible pigment (Nikou *et al*., 2014; Yuan *et al*., 2010). The cell wall contains *meso*-diaminopimelic acid, and galactose, ribose and arabinose are the diagnostic whole-cell sugars. The predominant menaquinone is MK-9(H4) with the phospholipid pattern consisting mainly of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylcholine. The fatty acid profile consists of straight-chained saturated and monounsaturated and iso- and anteiso-branched fatty acids. The G+C content of the genomic DNA is 68 - 71 mol% (Nikou *et al*., 2014; Yuan *et al*., 2010). No secondary metabolites have been identified from this genus, although *A. album* showed strong inhibition activity against *S. aureus* and *P. aeruginosa* (Yuan *et al*., 2010), unfortunately, *A. iranicum* displayed no activity against these pathogens (Nikou *et al*., 2014).

2.1.4 Genus *Micromonospora*

Micromonospora are widely distributed in nature and are found in different environments. This genus is one of 23 genera within the order of *Micromonosporales* (family *Micromonosporaceae*) and has over 80 species within the genus (http://www.bacterio.net/micromonospora.html accessed 22-10-2019). This genus is well known for its ability to produce secondary metabolites, producing over 700 bioactive compounds (Bérdy, 2005). Micromonospora have also been found to promote plant growth and development (Trujillo *et al*., 2010; Trujillo *et al*., 2015). Micromonospora colonies come in a variety of colours, from white, orange, rose, or brown. But the species are not always easy to differentiate on morphology alone. Genetic screening of the 16S rRNA and *gyrB* genes are used to identify the species (Kasai *et al*., 2000). *Micromonospora* species produce substantial substrate mycelium with an absent or sparse aerial mycelium. They take on a mucoid appearance upon sporulation.

2.1.5 Genus *Actinomadura*

The genus *Actinomadura* is one of five genera in the family of *Thermomonosporaceae*, order *Streptosporangiales*. A total of 86 species (http://www.bacterio.net/actinomadura.html accessed 22-10-2019) fall under this genus. Characteristic features of the genus include a cell wall chemotype (III), sugar pattern B, its aerobic nature, and the formation of branched substrate mycelium with or without secondary mycelium. The secondary mycelium may bear chains of arthrospores (Lechevalier and Lechevalier, 1970). *Actinomadura* has been found to produce a fair number of bioactive compounds, in the range of 345 metabolites (Bérdy, 2005). There are currently 10 BGCs (https://mibig.secondarymetabolites.org accessed 22-10-2019) from this genus on the MIBiG database.

2.2 Materials and Methods

2.2.1 Sampling sites

Aloe ferox plants and the associated soils were collected from farms of Organic Aloe Ltd. in Albertinia, Western Cape Province, South Africa (34°12'40.3"S 21°34'28.6"E; Supplementary Figure B1). Samples were taken in April 2016. Plants from three different sites were included – which were named Top, Bottom and Wild – and two plants were sampled at each site. The Top and Bottom site plants had been replanted and were originally wild plants from a site near to the town and from a more mountainous area, respectively. These samples were labelled as TP1 (Top Plant 1), TP2 (Top Plant 2), BP1 (Bottom Plant 1), BP2 (Bottom Plant 2). Samples were also taken of *A. ferox* plants growing in the wild (i.e. not replanted) and labelled as WP1 (Wild Plant 1) and WP2 (Wild Plant 2).

Isolates associated with *S. frutescens* plants were isolated from soil, leaf, root and seed samples collected from the Parceval Waterkloof farm outside Wellington, Western Cape Province, South Africa (33°40'47.7"S 19°02'38.0"E; Supplementary Figure B1) in October 2016. The plants were all planted in close proximity to each other in a plot. The plot was divided into three sections, section 1, section 2 and section 3. Sections 1 and 3 were located on the outsides of the plot and section 3 was in the middle of the plot. Thus, section 2 was only surrounded by other *S. frutescens* plants, whereas sections 1 and 3 was surrounded by other wild growing plants (mostly grasses) and more exposed to environmental elements. A total of two plants were sampled at each section within the area. These samples were labelled S1P1 (Section 1, Plant 1), S1P2 (Section 1, Plant 2), S2P1 (Section 2, Plant 1), S2P2 (Section 2, Plant 2), S3P1 (Section 3, Plant 1) and S3P2 (Section 3, Plant 2).

2.2.2 Sample collection

Soil samples were collected with a spade (cleaned with ethanol between plants) as close to the plants as possible at a depth of 10 - 20 cm below ground level for both *A. ferox* and *S. frutescens* plants. The soil temperature from *A. ferox* grown plants ranged between 17 °C and 19 °C, with the soil temperature ranging between 23 °C and 25 °C for *S. frutescens* plants. Where possible, the soil was directly associated with the plant roots. Soil samples were placed in labelled plastic resealable bags and placed in a cooler bag and kept cool at ±4 °C during transportation to the laboratory. The soil was stored at -20 °C until it was used for isolation.

Two leaves from each plant from *Aloe* were taken by removing the entire leaf from the stem of the plant with a clean knife. The whole leaves were placed in labelled plastic resealable bags and stored in a cooler bag at ±4 °C for transportation. The leaves were stored at 4 °C until it was processed within 5 days of collection.

The *Sutherlandia* plant leaves and seed pods were cut with clean scissors and stored in labelled resealable bags. The entire plant was also removed and root cuttings were made with clean scissors and stored in 50 mL Falcon tubes. The entire plants were placed in resealable plastic bags and transported at room temperature. The plant samples (leaves, root and seed pods) were stored at 4 °C and the samples were processed within 5 days of harvest. The soil samples were stored at -20 °C and processed within 14 days.

2.2.3 Isolation on selective media

2.2.3.1 Isolation of actinobacteria from soil samples

Actinobacteria were isolated from soil samples by suspending 1g of soil in 10 mL of sterile dH_2O. The tube was vortexed for one minute and left to stand for another minute. The sample was vortexed once again and the sandy mixture was poured into a glass petri dish. The dish containing the sample was microwaved for 5 seconds at full power (850 W) as a pre-treatment for selection of actinobacteria (Wang et al., 2013). One millilitre (1 mL) of this sample was added to 9 mL of sterile dH_2O which constitutes a $1x10^{-2}$ dilution. Subsequent 10-fold dilutions were made up until $1x10^{-6}$ and 100 µL aliquots of these dilutions were spread plated onto various media containing the antifungal agents cycloheximide and nalidixic acid (Table A1).

2.2.3.2 Isolation of actinobacteria from plant material

Leaf, root and seed material from *A. ferox* and *S. frutescens* was washed in sterile dH_2O and this first wash plated on various isolation media (Table A1) to isolate leaf, root and seed epiphytes. The plant material was further washed in 70% EtOH for 3 minutes, followed by a 2-minute wash in a 3% solution of NaClO. The plant material was rinsed three times with autoclaved dH_2O. The last wash water was plated onto LB plates and incubated at 37 °C for 2 days to ensure that the plant material was successfully surface decontaminated.

The surface decontaminated plant material was cut into smaller pieces where necessary using aseptic techniques. Endophytes were isolated by crushing the plant material with a sterile glass rod in 10 mL of 12.5 mM potassium phosphate buffer (pH 7.0). These plant suspensions

were subjected to serial dilutions, up to $1x10^{-4}$, and 100 µL aliquots were spread plated onto the various isolation media.

2.2.4 Cultivation

Isolation plates were incubated at room temperature for up to 4 months. Actinobacteria-like colonies, with distinctive leathery-like or fluffy looking colonies, were picked every 1-2 weeks and numbered accordingly. The selected colonies were streaked onto the medium corresponding to the isolation medium that it was isolated from (Table A1). Isolates were restreaked until colonies were clear from contamination. Purified isolates were grown at room temperature for 2-4 weeks before further evaluations were performed.

2.2.5 Identification

2.2.5.1 Genomic DNA extraction

DNA was extracted using a modified method from Wang *et al*. (2011). Briefly, actinobacterial isolates were grown in ISP-2 broth shaking at 150 rpm for 14 days at room temperature. Cell pellets were harvested by centrifugation of 2 mL actinobacterial cultures at 13 000 x *g* for 5 minutes. The broth was discarded and the cell pellets stored at -20 °C until genomic DNA extraction was performed. The defrosted cell pellets were resuspended in 500 µL of lysozyme buffer (25 mM Tris-HCl at pH 8.0, 50 mM glucose, 10 mM EDTA, 25 mg/mL lysozyme, 0.1 mg/mL proteinase K) and incubated overnight in a 37 °C water bath. After incubation, SDS was added to a final concentration of 1% and vortexed for 10 seconds. The extractions were incubated at 65 °C for 30 minutes. An equal volume of phenol was added to the tubes and mixed by inverting the tubes 10 times. Samples were centrifuged at 13 000 x *g* for 5 minutes at 4 °C and the top aqueous phase was transferred to a sterile 1.5 mL Eppendorf tube. The phenol wash step was repeated if the resulting aqueous layer was turbid. An equal volume of ice-cold chloroform: isoamyl alcohol (24:1; v/v) was added to the samples and mixed by gently inverting. The tubes were centrifuged at 11 000 x *g* for 2 minutes at 4 °C and the top aqueous phase was transferred into a clean tube. The nucleic acids were precipitated by the addition of an equal volume of cold isopropanol. The tubes were gently inverted 10 times to mix and centrifuged at 13 000 x *g* for 5 minutes at 4 °C to pellet the DNA. The supernatant was discarded and the DNA pellet was air-dried for 30 minutes at room temperature. The DNA was resuspended in 50 µL 1 x TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

2.2.5.2 16S rRNA gene amplification

The eubacterial universal primer set, F1 5'-AGAGTTTGATCITGGCTCAG-3' and R5 5'-ACGGITACCTTGTTACGACTT-3' (Weisburg *et al.,* 1991) were used to amplify an approximately 1.4 kb fragment of the 16S rRNA gene. Each PCR reaction (50 µL) contained a final concentration of 1x buffer, 200 µM dNTPs, 0.5 µM of each primer, 0.1 mg/mL BSA, ~0.25 µg template DNA, 1.25 U of Dream*Taq* polymerase (ThermoFisher), and sterile MiliQ water to a final volume of 50 µL. The PCR reaction was initiated with denaturation at 96°C for 2 minutes, followed by 30 cycles of denaturation at 96 °C for 45 seconds, annealing at 56 °C for 30 seconds and extension for 2 minutes at 72 °C. The final extension step was performed at 72 °C for 5 minutes and the reactions were held at 10 °C. Negative controls with no DNA template were included for all PCR reactions. The positive control was genomic DNA from *Streptomyces polyantibioticus* SPR^T.

2.2.5.3 gyrB gene amplification

A 1 kb fragment of the DNA gyrase B subunit *(gyrB)* gene was amplified with the actinobacterial specific primer set, *gyrB*PFA 5'-CTCGAGGGTCTGGACGCGGTCCGCAAGCGACCCGGTATGTA-3' and *gyrB*PRA 5'-GAAGGTCTTCACCTCGGTGTTGCCCAGCTTCGTCTT-3' (Rong *et al.,* 2009). Each PCR reaction contained a final concentration of 1x buffer, 200 µM dNTPs, 0.5 µM of each primer, 0.1 mg/mL BSA, ~0.25 µg template DNA, 1.25 U of Dream*Taq* polymerase (ThermoFisher), and made up to a final volume of 50 µL with sterile MiliQ water. The reaction was initiated with initial denaturation at 95 °C for 5 minutes, followed by 5 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 90 s. This was followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 60 s. Final extension was completed at 72 °C for 10 minutes and was held at 10 °C. A negative control with no DNA template was included in all PCR reactions. The positive control was included as genomic DNA from *Streptomyces polyantibioticus* SPR^T.

2.2.5.4 Sanger sequencing of PCR amplicons

Amplification of PCR fragments were visualised by agarose gel electrophoresis using 1% horizontal agarose gels (w/v) in 0.5 X TAE (Tris-acetate-EDTA) containing 0.5 µL/mL ethidium bromide and ran at 90 V in 0.5 x TAE buffer. The DNA fragments were visualised on a UV transilluminator gel Doc and sized according to their migration in the gel as compared to the molecular weight marker - lambda DNA restricted with *Pst*I.

The PCR amplicons were purified using the NucleoSpin® Gel and PCR Clean-up (MACHEREY-NAGEL) according to manufacturer's specifications. Where necessary, the correct DNA fragment was excised from the gel and purified. Sequencing reactions were carried out by the Central Analytical Facility (CAF) at Stellenbosch using either the 16S rRNA universal primer set (mentioned above) or the *gyrB*FA 5'-GCAAGCGACCCGGTATGTAC-3' and *gyrB*RA 5'-GAGGTTGTCGTCCTTCTCGC-3' (Rong *et al.,* 2009), for the 16S rRNA gene and *gyrB* gene fragments, respectively.

2.2.5.5 Phylogenetic analysis

Sequences were analysed and edited using BioEdit version 7.2.6 software (Hall, 1999). The GenBank database was used to analyse both 16S rRNA and *gyrB* DNA sequences, and perform homology searches. The basic local alignment search tool (BLASTn) was used to determine sequence similarity and identity to known type strains in the GenBank database from the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov). Sequences were aligned using the ClustalW multiple alignment features in MEGA 7.0.21 (Kumar *et al.,* 2016). Phylogenetic and molecular evolutionary analyses were conducted and phylogenetic trees constructed. The phylogenetic trees were constructed using the Neighbour-joining (NJ) tree algorithm with the option of partial deletion (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together was assessed via the bootstrap test based on 1000 replicates (Felsenstein, 1985). The Jukes-Cantor (JC, Jukes and Cantor, 1969) model and the Kimura two-parameter (K2P, Kimura, 1980) models were chosen as substitution models for the construction of NJ trees for 16S rRNA and *gyrB* genes, respectively. All ambiguous positions were removed for each sequence pair. *Frankia elaeagni* was chosen as the outgroup for both gene sequence analyses unless specified otherwise.

2.2.7 Storage of isolates and DNA samples

Isolates were routinely stored on agar plates at room temperature and sub-cultured every 6 to 8 weeks. Isolates were grown in liquid culture for 14 days and the cultures stored in glycerol (20% final concentration) at -20 °C and -80 °C for long term storage. DNA was stored in 1X TE buffer, with short term storage at 4 °C and long-term storage at -20 °C.

2.2.8 Characterisation

2.2.8.1 Phenotype and Morphology

Phenotypic characterisation of the actinobacterial isolates was carried out on ISP (International *Streptomyces* Project) media (Shirling and Gottlieb, 1966; Table A2). Media used for the screening included ISP-2 (Yeast extract - Malt extract agar), ISP-3 (Oats agar), ISP-4 (Inorganic salts - Starch agar), ISP-5 (Glycerol asparagine agar), ISP-6 (Peptone yeast extract iron agar), and ISP-7 (L-Tyrosine agar). Isolates were cultivated on ISP-2 medium for 14 days at 28 °C to evaluate morphological characteristics. The spore chains were observed using a light microscope (Olympus CX21).

2.2.8.2 Physiology

All physiological tests were performed at 28 °C for 14 - 21 days. Sole carbon source utilisation of the isolates was determined by inoculating isolates on ISP-9 medium (Table A2) supplemented with filter sterilised carbon sources at a final concentration of 1%. Controls were prepared by inoculating isolates on ISP-9 medium without a carbon source. Carbon utilisation was measured by comparing growth of isolates on carbon sources to the control plates (Shirling and Gottlieb, 1966). Salt tolerance of the isolates was determined by supplementing Bennett's medium (BM) containing glucose with different concentrations of NaCl (0, 2, 4, 6, 8 %; w/v). The degradation of three polysaccharides was evaluated by supplementing BM containing glycerol with soluble starch (1%, w/v), CMC cellulose (0.3%, w/v), and beechwood xylan (0.4%, w/v). Starch degradation (using soluble starch) was evaluated by flooding the culture plate with Gram's Iodine, which stains the starch a dark blue-black colour, and thus zones of starch hydrolysis are observed. Cellulose-containing culture plates are stained with Congo Red stain (1 mg/mL) for 30 minutes after which the stain was poured off. The plates were destained by flooding with 1M NaCl. Zones of clearing around the culture were used to assess the cellulose degradation (Teather and Wood, 1982). Degradation of xylan was evaluated by zones of clearing around the growth of the isolate.

2.2.8.3 Chemotaxonomy

Wall chemotype was determined by analysing the type of diaminopimelic acid (DAP) and whole cell sugar patterns using a modified version of Hasegawa *et al.* (1983). Briefly, for DAP analysis, 10 mg of dried cells (instead of fresh colonies) were placed in a glass test tube and suspended in 1 mL of 6M HCl. For sugar analysis, 100 mg of dried cells (oven-dried at 40 °C overnight) were placed in a glass test tube suspended in 1 mL of 0.25 M HCl. The test tubes

were covered with a plastic cap and autoclaved at 121 °C for 20 min. One to 5 microlitres of the cooled hydrolysate was spotted on TLC aluminium backed cellulose sheets without fluorescent indicator (Merck). Standards for the DAP analysis included 0.1% DAP standard and 0.1% Glycine. Standards for the sugar analysis included a 1% solution of glucose, mannose and ribose, and a 1% solution of galactose, arabinose and xylose. The DAP cellulose plates were developed in Methanol: dH_2O: 6M HCl: Pyridine (80:26:4:10, v/v/v/v); whereas the cell wall sugar cellulose plates were developed in n-Butanol: dH_2O: Pyridine: Toluene (10:6:6:1, v/v/v/v). Once the chromatograms dried, they were treated for visualisation. The DAP plates were sprayed with 0.1% acetonic ninhydrin and dried for 5 minutes at 110 °C. The sugar plates were sprayed with a reagent consisting of 2 mL aniline, 3.3 g phthalic acid and 100 mL water saturated *n*-butanol. After spraying the plate was allowed to air-dry, and then heated at 100 °C for 5 minutes.

2.2.8.4 Optimum growth conditions

The optimum growth temperature and pH conditions of the isolates were determined by growing isolates on BM agar plates (Table A2) at different temperatures (4, 15, 28, 37, 50 °C) and pH (4.3, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0) ranges. Culture plates were grown for 14 days, with pH plates incubated at 28 °C.

2.3. Results and Discussion

When isolating actinobacteria from environmental samples, some factors need to be taken into consideration such as the pH, temperature, and composition of the natural environment. Recreating these conditions in the laboratory can be difficult, and in an attempt to ensure the widest diversity of actinobacteria was isolated from the samples, a variety of isolation media were used with different compositions ranging from minimal to rich media (Table A1). Fungi are fast growing and are often the most prominent organisms cultured when isolating from soil and plant samples, and thus isolation media were supplemented with cycloheximide to suppress fungal growth. Cycloheximide's mode of action is to inhibit protein synthesis by binding to the E-site of the 60S ribosomal subunit of eukaryotes and thus inhibits the translation elongation (Schneider-Poetsch *et al.,* 2010)

In addition, soil samples were pre-treated with heat to reduce the number of non-spore-forming bacteria. Heat treatment favours the selection of organisms which produce heat-resistant spores such as many actinobacterial genera. Actinobacteria are slow-growing bacteria, and the pre-treatment reduces the number of fast-growing bacteria which typically outcompete the actinobacteria and overgrow the isolation plate before the actinobacteria has had a chance to grow.

2.3.1 Isolation

Actinobacteria with a streptomycete-like colony morphology were the most abundant on all of the isolation media. These colonies were identified by their high spore-forming nature. A total of 241 actinobacterial isolates were successfully isolated from *A. ferox* soil samples. Unfortunately, the five endophytic actinobacteria that were isolated from the leaf samples were lost due to fungal and bacterial contamination. The selection of soil isolates for further study was based on screening to identify their antimicrobial potential. In addition, 172 actinobacteria were isolated from soil, four inside the leaves, six on the surface of the seeds and six within the roots of *S. frutescens* plants.

Humic acid vitamin (HV) agar and complex HV agar were used for the isolation of actinobacteria from soil and root samples. These media contain humic acid as the sole carbon- and nitrogen source. Humic acid is an important component in soil that helps plant roots to take up water and nutrients. Therefore, it is hypothesised that the addition of humic acid in these media would help to replicate the natural soil environment and would allow for the isolation of unique actinobacteria that need humic acid to grow and sporulate. It has been

reported that HV medium enhances the growth of some non-streptomycete genera such as *Dactylosporangium*, *Microbispora*, *Micromonospora*, *Saccharomonospora*, and *Streptosporangium* (Hayakawa and Nonomura, 1987). In the present study the complex HV medium was supplemented with soil extract from the environmental sample in order to mimic the minerals and nutrients found within the original environment. The use of HV medium proved to be successful as isolates belonging to two "rare" genera (*Alloactinosynnema* and *Amycolatopsis),* as well as numerous *Streptomyces* species were isolated using HV and complex HV media (Table 2.1 and 2.2).

2.3.2 Identification and Characterisation

The phenotypic and growth characteristics of the isolates were tested using the International *Streptomyces* Project specified media (Shirling and Gotlieb, 1966). While these media are used for the phenotypic characterisation of mainly *Streptomyces* species, some other actinobacterial genera are also characterised using these tests. These methods are well-established and routinely used to characterise actinobacterial isolates. The various mediums are composed of nutrients that affect the isolates growth, pigment colour, sporulation and the ability to produce a soluble compound. Due to the relative ease of conducting these tests, the ISP was instrumental in organising the relative chaos of *Streptomyces* taxonomy in the 1960s into a classification system which is still used today.

Isolates were identified via sequence analysis of their 16S rRNA gene and their *gyrB* gene. Typically, while sequence analysis of the 16S rDNA of actinobacterial species can identify an isolates' genus correctly, due to high homology within same genera it can be difficult to distinguish isolates to the species level (Labeda *et al.,* 2012). Thus, more recently other housekeeping genes have been used to identify strains to the species level. In this study the *gyrB* gene, which encodes for the gyrase B subunit of the DNA gyrase enzyme which is required for the ATPase activity of the enzyme, was used. The *gyrB* gene is suggested to be a useful alternative to DNA-DNA hybridization and 16S rRNA gene analysis for the identification and phylogenetic analysis of bacterial species, including actinobacterial genera such as *Amycolatopsis*, *Kribbella*, *Micromonospora* and *Streptomyces* (Everest and Meyers, 2011; Hatano *et al.,* 2003; Kasai *et al.,* 2000; Kim *et al.,* 2012; Kirby *et al.,* 2010). The 16S rRNA gene sequences of these actinobacteria from the studies mentioned had high similarity to their closest relatives. However, when the *gyrB* gene was analysed, the genetic similarity to their closest relatives were lower. Thus, the *gyrB* gene was useful in determining the novelty of the species. DNA-DNA hybridisation of these species further indicated that the *gyrB* gene correlated to the DNA-DNA relatedness among species and thus could be used as an

alternative or preliminary method to identify bacteria to a species level. However, unlike the 16S rRNA gene comparison, it is important to note that different genera will have a different genetic distance value used to distinguish between species. *Micromonospora* and *Streptomyces* species that have a *gyrB* gene genetic distance of more than 0.014 are considered to be genetically different (Hatano *et al.,* 2003; Kasai *et al.,* 2000; Kirby *et al.,* 2010), whereas a genetic distance of 0.02 has been proposed for species delineation within the genus *Amycolatopsis* (Everest and Meyers *et al.,* 2009). Unfortunately, this analysis has not been conducted on all actinobacterial genera with high 16S rRNA gene similarities and future studies will need to be done to decide whether *gyrB* analysis of actinobacteria species can conclusively differentiate between species.

Due to the large number of strains initially isolated, isolates for full phenotypic characterisation were selected based on antimicrobial activity and/or interesting morphological characteristics, and only these strains were identified by 16S rRNA gene analysis. Many of these isolates had relatively low similarity to their closest relative (Table 2.1 and 2.2), with the majority of the *Aloe ferox* isolates sequenced (14 out of 21; 66%) and *Sutherlandia frutescens* (9 out of 17; 53%) having similarities lower than 99% (Table 2.1 and 2.2). This may indicate the potential of these isolates to be novel.

Table 2.1: *16S rRNA sequence similarity of isolates associated with* Aloe ferox*. Non-streptomyces species are highlighted in orange. Percentage similarity lower than 99% is highlighted in blue. Isolates that were selected for further screening are highlighted in green.*

Isolate	Isolation Media	Sample	Site	Top-hit taxon	Similarity (%)
A2	HV	Soil	WP1	*Streptomyces lateritius*	98.41
A3	HV	Soil	WP1	*Streptomyces lunaelactis*	97.1
A12	ISP-2 +Aloe	Soil	BP1	*Streptomyces glebosus*	99.11
A30	ISP-2 +Aloe	Soil	WP1	*Streptomyces lincolnensis*	98.88
A32	HV	Soil	WP1	*Alloactinosynnema album*	98.52
A37	HV	Soil	TP1	*Amycolatopsis albidoflavus*	95.99
A44	SCN	Soil	WP1	*Streptomyces lincolnensis*	98.51
A45	AI+Gly	Soil	BP1	*Streptomyces thermospinosisporus*	96.37
A52	ISP-2+Aloe	Soil	BP1	*Streptomyces globisporus*	99.79
A75	AI+Glu	Soil	BP1	*Streptomyces thermospinosisporus*	97.05
A81	SCN	Soil	BP1	*Streptomyces californicus*	99.03
A91	ISP-2+Aloe	Soil	BP1	*Streptomyces griseus*	99.07
A100	SCN	Soil	WP1	*Streptomyces lateritius*	100
A119	SCN	Soil	BP1	*Streptomyces pluricolorescens*	98.25
A120	SCN	Soil	BP1	*Streptomyces globisporus*	98.77
A130	SCN	Soil	BP1	*Streptomyces puniceus*	98.69
A159	AI+Gly	Soil	WP1	*Streptomyces puniceus*	97.9
A160	AI+Gly	Soil	BP1	*Streptomyces griseus*	98.69
A183	SCN	Soil	WP1	*Streptomyces lincolnensis*	97.44
A201	SCN	Soil	WP1	*Streptomyces californicus*	99.93

Table 2.2: *16S rRNA sequence similarity of isolates associated with* Sutherlandia frutescens*. Non-streptomyces species are highlighted in orange. Percentage similarity lower than 99% is highlighted in blue. Isolates that were selected for further screening are highlighted in green.*

Isolate	Isolation Media	Sample	Site	Top-hit taxon	Similarity (%)
S3	SCN	Soil	S1P2	*Streptomyces rubiginosus*	98.74
S17	Suth-Media	Soil	S3P1	*Streptomyces nigrescens*	97.71
S25	SCN	Soil	S1P1	*Streptomyces angusmyceticus*	98.95
S41	AI+Gly	Seed-epi	S3P2	*Streptomyces chattanoogensis*	99.93
S43	ISP-2 +Suth	Soil	S2P2	*Streptomyces glebosus*	98.37
S45	AI+Gly	Seed-epi	S3P1	*Streptomyces angustmyceticus*	98.07
S55	SCN	Root-endo	S1P1	*Actinomadura macra*	97.31
S93	ISP-2 +Suth	Soil	S2P1	*Amycolatopsis rifamycinica*	99.12
S102	AI+Gly	Soil	S2P2	*Streptomyces rubiginosus*	99.86
S106	CHV	Soil	S1P1	*Amycolatopsis lurida*	99.42
S108	AI+Gly	Soil	S2P2	*Streptomyces nigrescens*	99.79
S125	ISP-2+Suth	Soil	S2P2	*Streptomyces nigrescens*	99.86
S148	SCN	Leaf-endo	S1P1	*Micromonospora tulbaghiae*	99.3
S149	SCN	Leaf-endo	S2P1	*Streptomyces canus*	99.73
S170	SCN	Soil	S2P1	*Streptomyces rubiginosus*	98.15
S171	SCN	Soil	S2P1	*Streptomyces viridobrunneus*	99.14
S173	AI+Gly	Seed-epi	S3P1	*Actinomadura bangladeshensis*	98.21

As this study mainly focussed on finding unique antibacterial activity all isolates that did not have significant antibacterial activity were excluded from further identification, which included isolates A2, A32 and S148, S170. Isolate A32 had relatively low similarity to the genus *Alloactinosynnema* genus (which current includes only two species) and further phenotypic and chemotaxonomic will be explored in a future study. However, as it did not have significant antibacterial activity it was excluded from further analysis. Some of the isolates with low similarities, unfortunately, stopped growing, including isolates A45, A75, S17, S25 and S55, and could thus not be included in further phenotypic classifications. Other slow and difficult to grow isolates were also excluded from further identification, which included isolates A32, S3 and S93. These isolates did not produce sufficient cell mass for genotypic/antimicrobial screening. To grow these so-called "unculturable" or fastidious bacteria, the natural environment may need to be simulated. This proves difficult as there are variable conditions (nutrients, pH, osmotic conditions, temperature etc.) that will need to be properly replicated to ensure growth of these organisms. Addressing all of these varying conditions results in a multidimensional matrix of possibilities that would be difficult to perform with reasonable time and effort (Stewart, 2012).

2.3.2.1 *Streptomyces* strain A3

Isolate A3 was isolated from soil associated with *Aloe ferox* roots (WP1; Table 2.1). It forms well developed substrate mycelium with the colony colour ranging from pale cream, vellum, light stone to light beige, with scarce aerial mycelium that only developed on ISP-4. The colonies ranged in texture, from tough and leathery to brittle and flaky (Figure 2.1). The isolate was able to grow in all media tested with good growth observed on ISP-3, ISP-4, ISP-5, and ISP-6 after 1 – 2 weeks at 28 °C (Table 2.3). The isolate furthermore becomes mucoid with age. No diffusible pigment or production of melanin was noted.

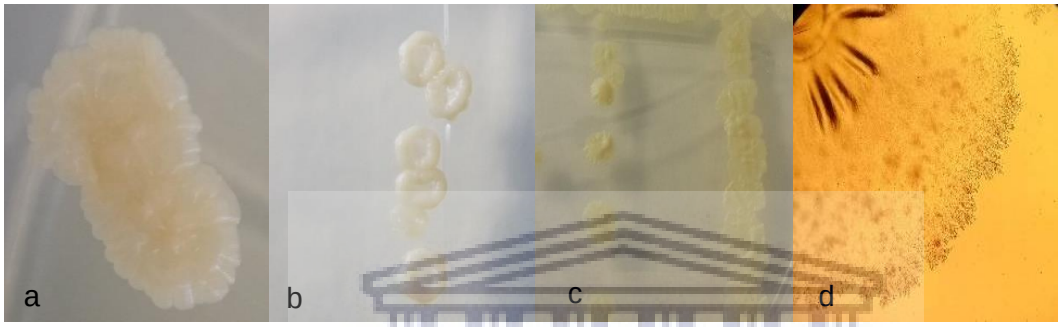


Figure 2.1: *a) Tough, leathery colony on ISP-2 medium; b) small, crater-like colonies on Bennett's medium containing glucose; c) flaky, brittle colonies on ISP-5 medium; d) colony under microscope at 100X magnification show branched vegetative mycelia with no spores.*

Table 2.3: *Growth and phenotypic characteristics of isolate A3 under various culture conditions.*

Media	Growth	Substrate mycelium	Aerial mycelium	Diffusible pigments
ISP-2	++	pale cream	none	none
ISP-3	+++	vellum	none	none
ISP-4	+++	vellum	white	none
ISP-5	+++	pale cream	none	none
ISP-6	+++	light stone	none	none
ISP-7	++	light beige	none	none

+++: good; ++: moderate; +: weak.

The isolate grew at temperatures ranging from 4 to 50 °C, with optimum growth at 15 °C. The pH range for growth was determined to be from pH 6.0 – 10.0, with an optimum growth at pH 8.0 (Table 2.4). The isolate was able to withstand a salt concentration of up to 2% NaCl (w/v). Isolate A3 was positive for the hydrolysis of carboxymethylcellulose and xylan, but negative for the hydrolysis of soluble starch. The strain was able to utilise sucrose, D-fructose, D-raffinose, maltose and citric acid as sole carbon sources. Isolate A3 differed phenotypically to its closest relative *Streptomyces lunaelactis* (Maciejewska *et al.,* 2015) by being more sensitive to salt, not producing melanin, and utilising D-fructose but not inositol as a sole

carbon source. The cell wall sugars detected were glucose, galactose and ribose. Glucose and ribose were also detected for one of its closest relatives, *Streptomyces maoxianensis* (Guan *et al.,* 2015; Table 2.4).

Table 2.4: *Differential characteristics of isolate A3 and the closest related* Streptomyces *species. Optimum growth conditions indicated in brackets.*

Characteristic	***A3***	***S. lunaelactis (Maciejewska et al., 2015)***	***S. tanashiensis (Compendium of Actinobacteria, Wink)***	***S. maoxianensis (Guan et al., 2015)***
16S rRNA similarity	**-**	97.1 %	97 %	97.01 %
Spores				
Spore surface	n/t	smooth	smooth	smooth
Spore chains	RF	RA	RF	long
Temperature (°C)	4 - 50 (15)	10 - 30 (18-28)	26	10 - 40 (28)
pH	6 - 10 (8)	6-8 (6)	n/t	6-11 (7)
Salt tolerance (%)	0 - 2 (0)	1 – 4 (3)	5	0 - 5
Polysaccharide degradation				
Starch	-	n/t	n/t	+
Cellulose	+	n/t	n/t	-
Xylan	+	n/t	n/t	n/t
Carbon utilisation				
Sucrose	+	+	-	+
myo-inositol	-	+	-	+
D-fructose	+	-	-	+
D-raffinose	+	+	-	+
Maltose	+	+	n/t	+
Citric acid	+	n/t	n/t	n/t
Malic acid	-	n/t	n/t	n/t
DAP	None detected	LL-diaminopimelic acid (chemotype 1)	n/t	LL-diaminopimelic acid, glycine (chemotype 1)
Sugar	glucose, galactose, ribose	n/t	n/t	glucose and ribose

RA: retinaculum-apertum*; RF:* recti flexibilis*; n/t: not tested or not reported; +: carbon utilisation; (+): weak carbon utilisation; -: negative carbon utilisation*

Streptomyces lunaelactis was isolates from a moonmilk deposit inside the "Grotte des Collemboles" cave in Comblain-au-Pont, Belgium. This species was found to produce a green pigment, identified as ferroverdin A, which is an iron-chelating molecule (Tomoda *et al.,* 1999; Maciejewska *et al.,* 2015). No anti-microbial activity of this species was reported. *Streptomyces lunaelactis* grew poorly on ISP-2 medium with greyish yellow substrate

mycelium and no aerial mycelium or diffusible pigments produced. On ISP-3, -4, -5, -6 and -7 a greenish substrate mycelium was observed, with white/ grey aerial mycelium on ISP-3 and -4. No aerial mycelium was observed on ISP-5, -6 and -7. Diffusible pigments were observed on ISP-6 and ISP-7 indicating the production of melanoid and melanoid-like pigments. Phenotypically, *S. lunaelactis* differed from *Streptomyces* strain A3 in that strain A3 produced no aerial mycelia, diffusible pigments or greenish substrate mycelia on any of the ISP-media tested. Sequence analysis of isolate A3 (1120 bp) showed that it belonged to the genus of *Streptomyces* with relatively low sequence similarities to *S. lunaelactis* (97.1%), *Streptomyces tanashiensis* (97%) and *S. maoxianensis* (97.01%) (Figure 2.2).

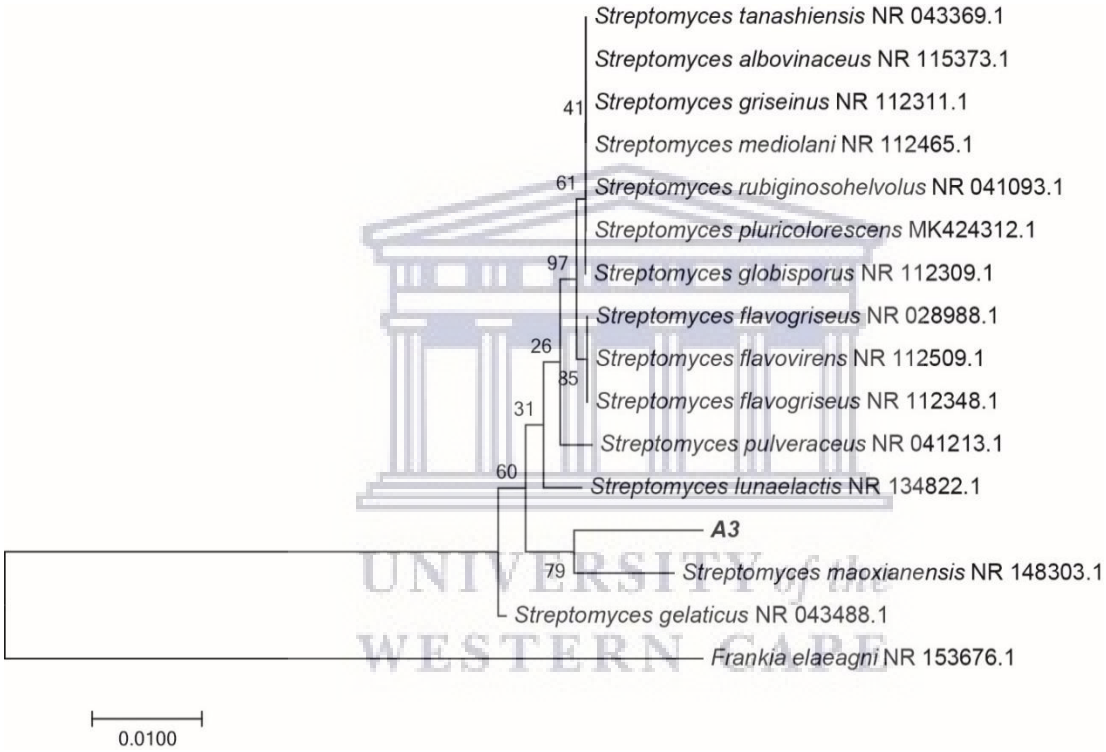


Figure 2.2 *Neighbour-Joining tree of the 16S rRNA sequences of the closest relatives to isolate A3 is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis included 16 nucleotide sequences and was based on 1138 bp.*

The *gyrB* gene analysis of isolate A3 had an even lower sequence similarity to *S. lunaelactis* (89.76%) with a high bootstrap score (97%) to support the association between these bacteria (Figure 2.3). Isolate A3 had a genetic distance of 0.105 (± 0.011) to *S. lunaelactis*, thus indicating that this isolate is genetically different to *S. lunaelactis* and possibly a novel *Streptomyces* species due to the *gyrB* gene genetic distance being higher than 0.014 (Hatano *et al.,* 2003).

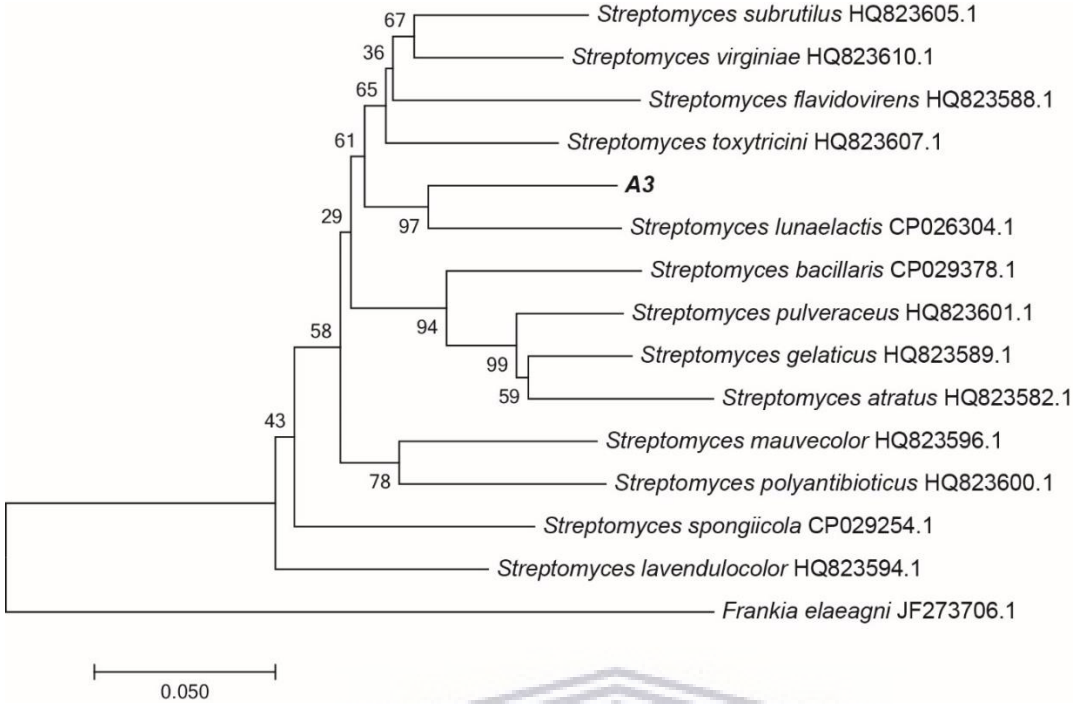


Figure 2.3: *The Neighbour-Joining tree of the* gyrB *sequences of the closest relatives is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis included 15 nucleotide sequences with a total of 1049 positions in the final dataset.*

Based on the physiological and phylogenetic properties of isolate A3, it can be distinguished from its closest relatives. We suggest that isolate A3 should be placed within the genus of *Streptomyces* as the type strain of a novel species, for which we propose the name *Streptomyces albertinaensis*^{T}, referring to Albertinia, South Africa, where the type strain was isolated.

2.3.2.3 *Amycolatopsis* strain A37

Isolate A37 was isolated from soil associated with *A. ferox* roots (TP1; Table 2.1) and had good growth on all media tested. It formed yellow-toned vegetative mycelium ranging from bold yellow to manila, with abundant white aerial mycelia. The isolate produced a non-melanoid soluble pigment on each media, except for ISP-6 (Table 2.5). The colonies are soft, brittle and raised, and form a cluster. When stabbed inoculated the colonies grow on top of each other forming a 'popcorn-like' structure (Figure 2.4).

Table 2.5: *Growth and phenotypic characteristics of isolate A37 under various culture conditions.*

Media	Growth	Substrate mycelium	Aerial mycelium	Diffusible pigments
ISP-2	+++	golden yellow	white	golden yellow
ISP-3	+++	bold yellow	white	bold yellow
ISP-4	+++	manila	white	manila
ISP-5	+++	golden yellow	white	light buff
ISP-6	+++	light stone	white	none
ISP-7	+++	golden yellow	white	golden yellow

+++: good; ++: moderate; +: weak.

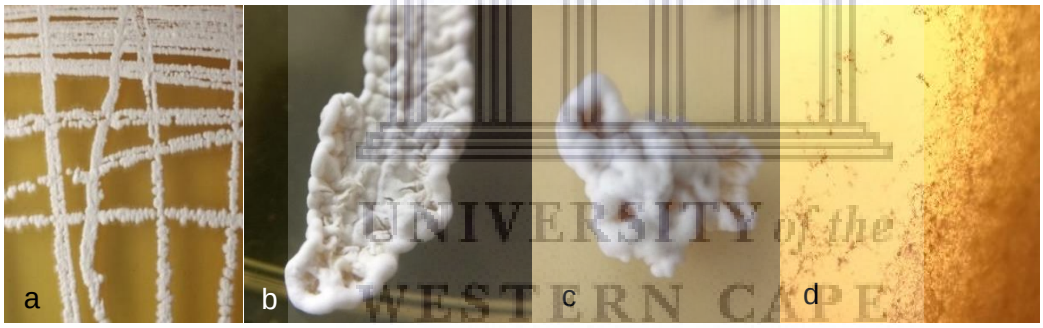


Figure 2.4: *a) Soft, brittle colonies with abundant white aerial mycelia b) after more than 2 weeks of growth can form raised colonies that clump together c) and can grow on top of each other to form a popcorn-like structure on ISP-2; d) an abundance of fragmented short spore chains was detected when viewed under 100X magnification.*

The isolate grew at temperature ranges of 4 – 50 °C, with optimal growth occurring over a wide range (15 – 37 °C). The isolate grew well in both alkaline and acidic environments, with optimal growth between pH 5.0 and 10.0. The isolate could tolerate salt up to a concentration of 8% NaCl (w/v), and was able to utilise sucrose, *myo*-inositol, D-fructose, D-raffinose and lactose as carbon sources. Isolate A37 was unable to degrade starch, xylan and cellulose. Arabinose and xylose were observed as cell wall sugars (Table 2.6).

The three closest relatives of strain A37 was identified as *Amycolatopsis albidoflavus* (Lee and Hah, 2001), *Amycolatopsis echigonensis* and *Amycolatopsis niigatensis* (Ding *et al.,*

2007). *Amycolatopsis albidoflavus* was isolated from soil and forms well-developed white aerial mycelium with yellow vegetative mycelium. *Amycolatopsis echigonensis* and *A. niigatensis* were isolated from the filtration substrate of volcanic soil and form well-developed aerial and vegetative mycelia. The aerial mycelium of *A. echigonensis* is white and light-yellow vegetative mycelium, whereas *A. niigatensis* forms white or light-yellow aerial mycelium and purple or purple-brown vegetative mycelium. Strain A37 is mostly similar to *A. albidoflavus*, in that it also forms bright yellow vegetative mycelium with well-developed white aerial mycelium. Isolate A37 was able to tolerate a higher salt concentration than its three closest relatives. It was also able to utilise D-raffinose as a sole carbon source, but was unable to degrade soluble starch. Strain A37 was able to grow at higher (50 °C) and lower (4 °C) temperatures than its closest relatives. No antimicrobial activity or production of bioactive compounds was reported for the closest relatives.

Table 2.6: *Differential characteristics of isolate A37 and the closest related* Amycolatopsis *species. Optimum growth conditions indicated in brackets*

Characteristic	***A37***	***Amycolatopsis albidoflavus (Lee and Hah, 2001)***	***Amycolatopsis echigonensis (Ding et al., 2007)***	***Amycolatopsis niigatensis (Ding et al., 2007)***	
16S rRNA similarity		95.99 %	95.99 %	95.91 %	
Temperature (°C)	4 - 50 (15 - 37)	10-37	5 - 45 (30)	5 - 45 (30)	
pH	4.3 - 10 (5 - 10)	n/t	6 - 11 (9)	6 - 11 (9)	
Salt tolerance (%)	0 - 8	7	3 - 7	3 - 7	
Polysaccharide degradation					
Starch	-	+	n/t	n/t	
Cellulose	-	n/t	n/t	n/t	
Xylan	-	n/t	n/t	n/t	
Carbon utilisation					
Sucrose	+	+	n/t	n/t	
myo-inositol	+	+	+	-	
D-fructose	+	n/t	n/t	n/t	
D-raffinose	+	-	n/t	n/t	
Lactose	+	+	+	-	
Citric acid	-	n/t	n/t	n/t	
Malic acid	-	n/t	n/t	n/t	
DAP	Not detected	*meso*-DAP	*meso*-DAP	*meso*-DAP	
Sugar	arabinose and xylose	arabinose and galactose (type IV)	arabinose and galactose (type IV)	arabinose and galactose (type IV)	

RA: retinaculum-apertum*; RF:* rectiflexibilis*; n/t: not tested or not reported; +: carbon utilisation; (+): weak carbon utilisation; -: negative carbon utilisation*

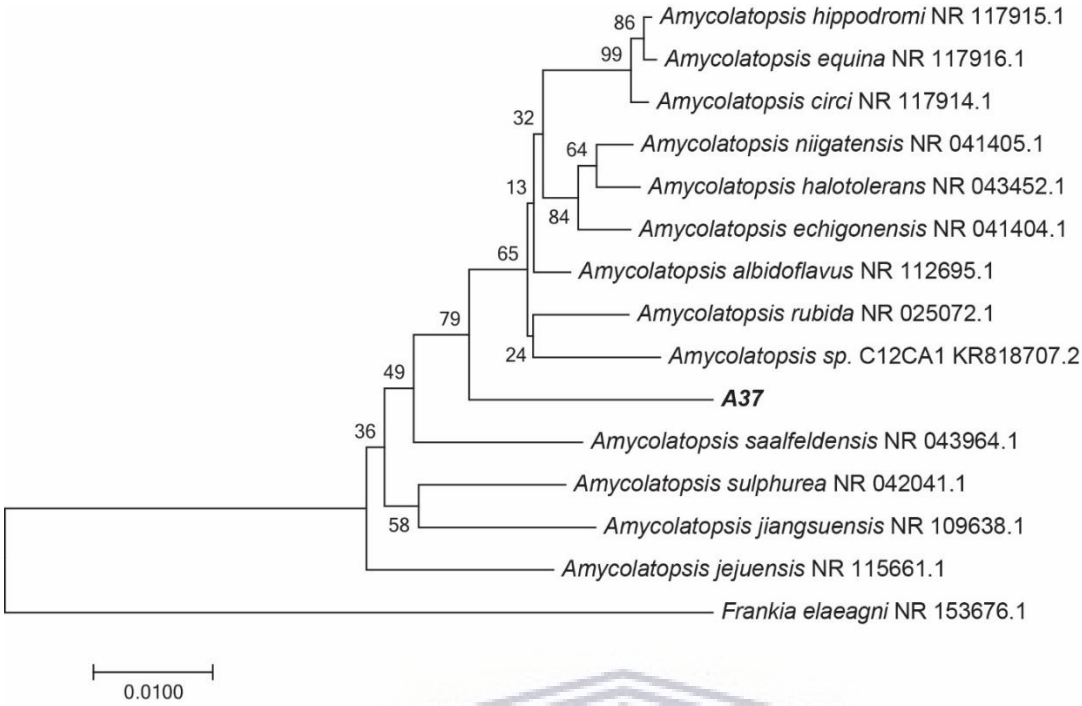


Figure 2.5: *Neighbour-Joining tree of the 16S rRNA sequences of the closest relatives to isolate A37 are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 15 nucleotide sequences. There were a total of 1206 positions in the final dataset.*

Sequence analysis of the 16S rRNA gene with BLASTn analysis on the GenBank database revealed that isolate A37 had low similarity to other *Amycolatopsis* species, with the highest similarity of 95.99% to *A. albidovlavus* and *A. echigonensis* (Figure 2.5). The *gyrB* sequence analysis revealed an even lower sequence similarity of 94.27% to *A. albidoflavus* with a genetic pairwise distance of 0.059 (Figure 2.6). This large genetic distance is significantly higher than the 0.02 value which has been proposed for the genus (Everest and Meyers *et al.,* 2009). A37 may be a novel species within the genus of *Amycolatopsis* or potentially a novel species in a different genus due to the different cell wall characteristics of this isolate. *Amycolatopsis* species have a cell wall type IV, whereas isolate A37 contained xylose and arabinose as cell wall sugars, giving it a cell wall type II. Genera that contain these sugars as well as *meso*-DAP include *Actinoplanes*, *Ampullariella*, *Cattellatospora*, *Dactylosporangium*, *Frankia*, *Glycomyces*, *Micromonospora*, and *Pilimelia* (Barka *et al.,* 2016). This may indicate that strain A37 belongs to one of these genera and not *Amycolatopsis*.

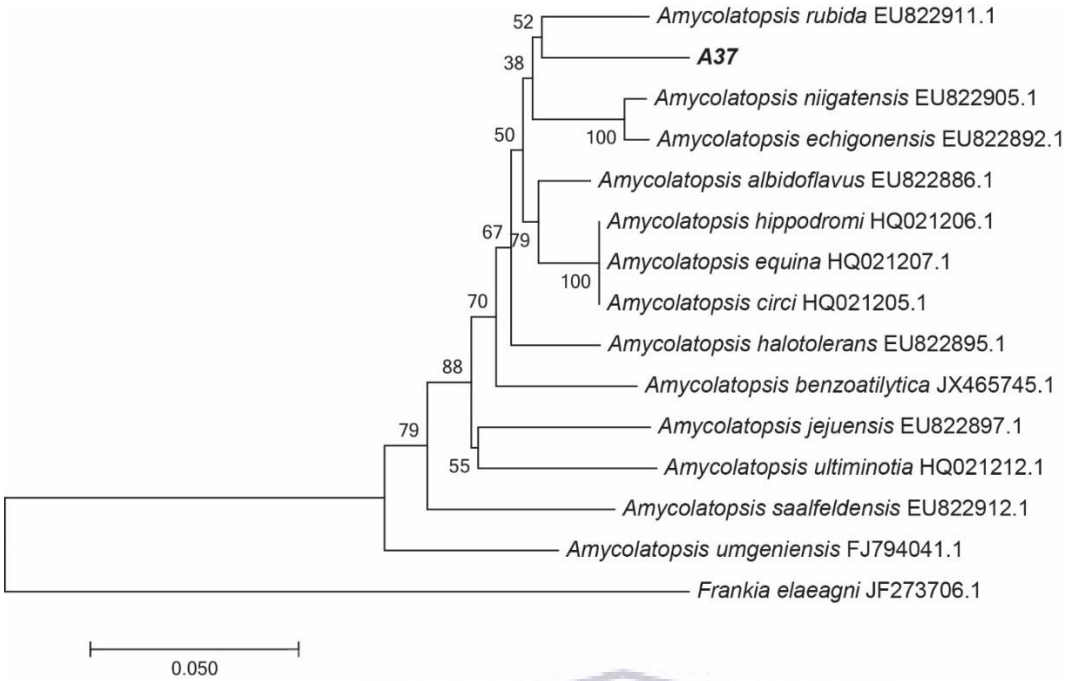


Figure 2.6: *The Neighbour-Joining tree of the gyrB sequences of the closest relatives to isolate A37 is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 15 nucleotide sequences with a total of 1076 positions in the final dataset.*

Additional work is required to confirm the taxonomic placement of this strain but based on the current physiological and phylogenetic characteristics from this study, it is proposed that isolate A37 can be distinguished from its closest relatives. It is suggested that isolate A37 be added in the genus of *Amycolatopsis* as a novel type strain with the name of *Amycolatopsis maiziuminfatum*^{T} (referring to the popcorn-like structure of the colonies).

2.3.2.4 *Streptomyces* strains A81 and A159

Streptomyces strain A81 and A159 were isolated from soil associated with *A. ferox* roots (plants BP1 and WP1, respectively; Table 2.1). Isolates A81 and A159 have similar phenotypic characteristics and are genotypically related to similar species within the *Streptomyces puniceus* clade. The BLASTn analysis of the 16S rRNA gene identified that the two isolates are 99.12% similar to one another. The BLASTn analysis of the *gyrB* gene had a lower percentage identity at 98.49%. These results may indicate that the isolates are possibly closely related species or different strains of the same species. It has been shown that several *Streptomyces* species with identical 16S rRNA genes have distinct secondary metabolomes (Antony-Babu *et al.,* 2017). Thus, a high 16S rRNA gene similarity does not eliminate the possibility of finding different metabolites that may be of pharmaceutical importance. The isolates both have purple to red substrate mycelium and form white to violet aerial mycelium (Figure 2.7). Both produce brown to purple diffusible pigments on all media tested, however melanoid pigments were not detected on ISP-6. Phenotypically, only slight differences in colour were observed on the different media tested (Table 2.7, Figure 2.7).

Table 2.7: *Growth and phenotypic characteristics of isolate A81 and A159 under various culture conditions.*

Isolate	Media	Growth	Substrate mycelium	Aerial mycelium	Diffusible pigments
A81	**ISP-2**	+++	dark purple-brown	white to light violet	middle brown
	ISP-3	+++	crimson	white	light stone
	ISP-4	+++	violet	light violet	very light purple-brown
	ISP-5	+++	maroon	light violet	light purple-brown
	ISP-6	+++	transparent	white	none
	ISP-7	+++	maroon	white	camouflage beige
A159	**ISP-2**	+++	maroon	white	light purple-brown
	ISP-3	+++	ruby	light violet	very light purple-brown
	ISP-4	+++	violet	vellum	very light purple-brown
	ISP-5	+++	dark violet	white	very light purple-brown
	ISP-6	+++	middle buff	white	none
	ISP-7	+++	maroon	vellum	dark camouflage dessert sand

+++: good; ++: moderate; +: weak.

Nonetheless, there are some characteristic differences between the isolates (Table 2.8). Isolate A159 was able to grow at very low pH of 4.3 whereas isolate A81 was only able to grow from a pH of 6.0. Isolate A81 is able to degrade soluble starch whereas isolate A159 was not able to degrade any of the polysaccharides tested. Isolates A81 and A159 have higher tolerance to salt (8%) than their closest relatives (5%), and have an optimum growth temperature between 16 and 37 °C. The cell wall sugars contained glucose, galactose, ribose and xylose.

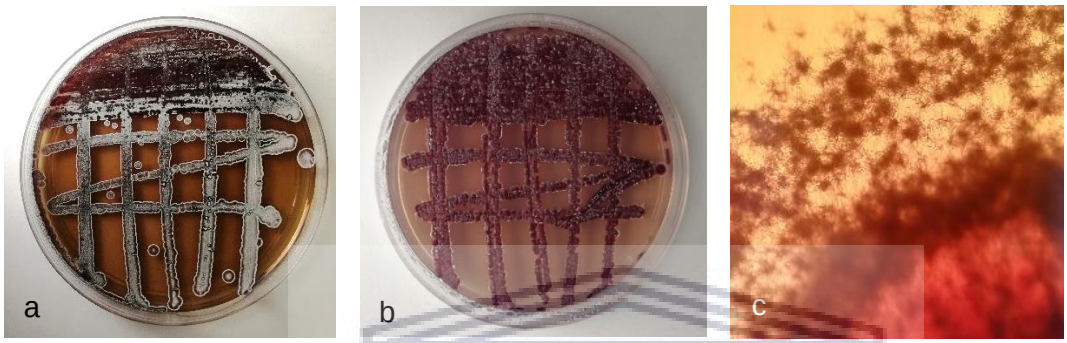


Figure 2.7: *Isolate A81 grown on ISP-2 media for 14 days a) had dark purple-brown substrate mycelium, with white to light violet spores and produces a middle brown coloured diffusible pigment; b) purple vegetative mycelium was observed when grown on ISP-5 medium with a light brownish purple diffusible pigment being produced; c) an abundance of spores was observed under 100X magnification with long* Rectiflexibiles *type spore chains.*

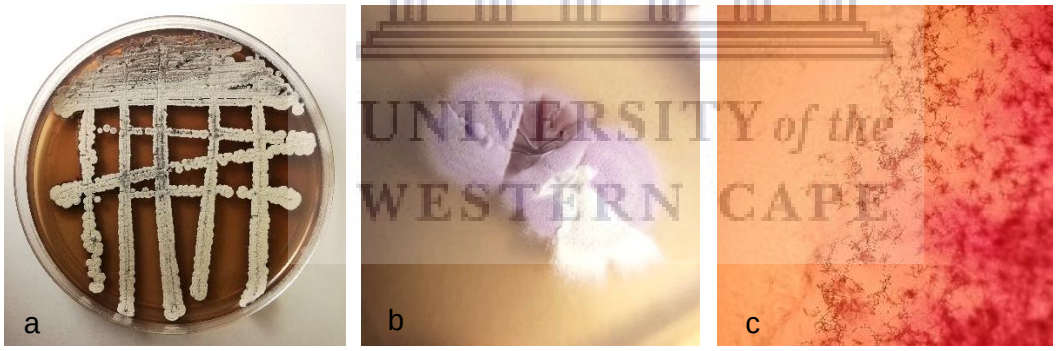


Figure 2.8: *Isolate A159 grown on ISP-2 media a) had maroon substrate mycelium with abundant white aerial mycelia and also produces a purple-brown soluble pigment; b) and forms violet aerial mycelium when grown on Bennett's medium supplemented with glucose; c) spores were organised on long spore* Rectiflexibiles *type spore chains under 100X magnification.*

The most closely related species to strains A81 and A159, *Streptomyces puniceus*, *Streptomyces floridae* and *Streptomyces californicus* all form purplish-red vegetative mycelia and were isolated from soil samples. Additionally, these strains are all known to produce potent antimicrobial and antitumor compounds. *Streptomyces puniceus* produces the well-known antibiotic, viomycin, that was used to treat *Mycobacterium* infections (Finlay *et al.,* 1951). Recently, *S. puniceus* strain AS13 was found to produce an antitumor antibiotic,

dinactin, that also exhibited strong activity against *M. tuberculosis* and other pathogenic Gram-positive and -negative bacteria. Dynactin was found to inhibit cell proliferation of the lung (A-549), breast (T47D), colon (HCT-116) and liver (HepG2) cancer cell lines. Furthermore, the compound had no cytotoxicity towards the normal (HEK-293) cell line tested (Hussain *et al.,* 2018). *Streptomyces floridae* also produces viomycin (Ehrlich *et al.,* 1951). *Streptomyces californicus* is a potent bioactive compound producer with strain BS-75 producing antitumor antibiotics active against P388 murine leukemia cells, named pyrisulfoxin A and B (Tsuge *et al.,* 1999). KS-619-1, an anthraquinone antibiotic that inhibits Ca^{2+} and calmodulin-dependent cyclic nucleotide phosphodiesterase, is also produced by *S. californicus* (Matsuda and Kase, 1987). An *S. californicus* strain from India was found to produce the antibiotic compound borrelidin which displayed activity against vancomycin- and methicillin resistant clinical pathogenic strains (Saisivam *et al.,* 2008). Griseorhodins are red pigments produced by *S. californicus* with antibiotic activity against Gram-positive and -negative bacteria (Suetsuna and Osajima, 1989). *Streptomyces tanashiensis* forms brown-coloured vegetative mycelium with strain Kala producing the antibiotic, kalafungin. This non-polyene antibiotic exhibits inhibitory activity against a range of pathogenic fungi, yeasts, protozoa, Gram-positive and -negative bacteria (Johnson and Dietz, 1968).

Table 2.8: *Differential characteristics of isolates A81 and A159 and the closest related* Streptomyces *species. Optimum growth range indicated in brackets. Species data was obtained from the Compendium of Actinobacteria (Wink).*

Characteristic	**A81**	**A159**	***Streptomyces californicus***	***Streptomyces tanashiensis***	***Streptomyces puniceus***	***Streptomyces floridae***
Spores						
Spore surface	n/t	n/t	Smooth	Smooth	Smooth	Smooth
Spore chains	RF	RF	RF	RF	RF	RF
Temperature (°C)	4 - 50 (15 - 37)	4 - 50 (15 - 37)	28	26	28	28
pH	6 - 10 (8)	4.3 - 10 (6 - 9)	n/t	n/t	n/t	n/t
Salt tolerance (%)	8	8	n/t	5	5	5
Polysaccharide degradation						
Starch	+	-	n/t	n/t	n/t	n/t
Cellulose	-	-	-	-	-	-
Xylan	-	-	n/t	n/t	n/t	n/t
Carbon utilization						
Sucrose	-	-	-	-	-	-
myo-inositol	-	-	-	-	-	-
D-fructose	+	+	+	-	-	+
D-raffinose	-	n/t	-	-	-	+
Lactose	+	+	n/t	n/t	n/t	n/t
Citric acid	-	-	n/t	n/t	n/t	n/t
Malic acid	-	-	n/t	n/t	n/t	n/t
DAP	Not detected	Not detected	n/t	n/t	n/t	n/t
Sugar	Glu, Gal, Rib, Xyl	Glu, Gal, Rib, Xyl	n/t	n/t	n/t	n/t

RF: Rectiflexibiles *type; n/t: not tested; +: carbon utilisation; (+): weak carbon utilisation; -: negative carbon utilisation; Glu: Glucose; Gal: Galactose; Rib: Ribose; Xyl: Xylose*

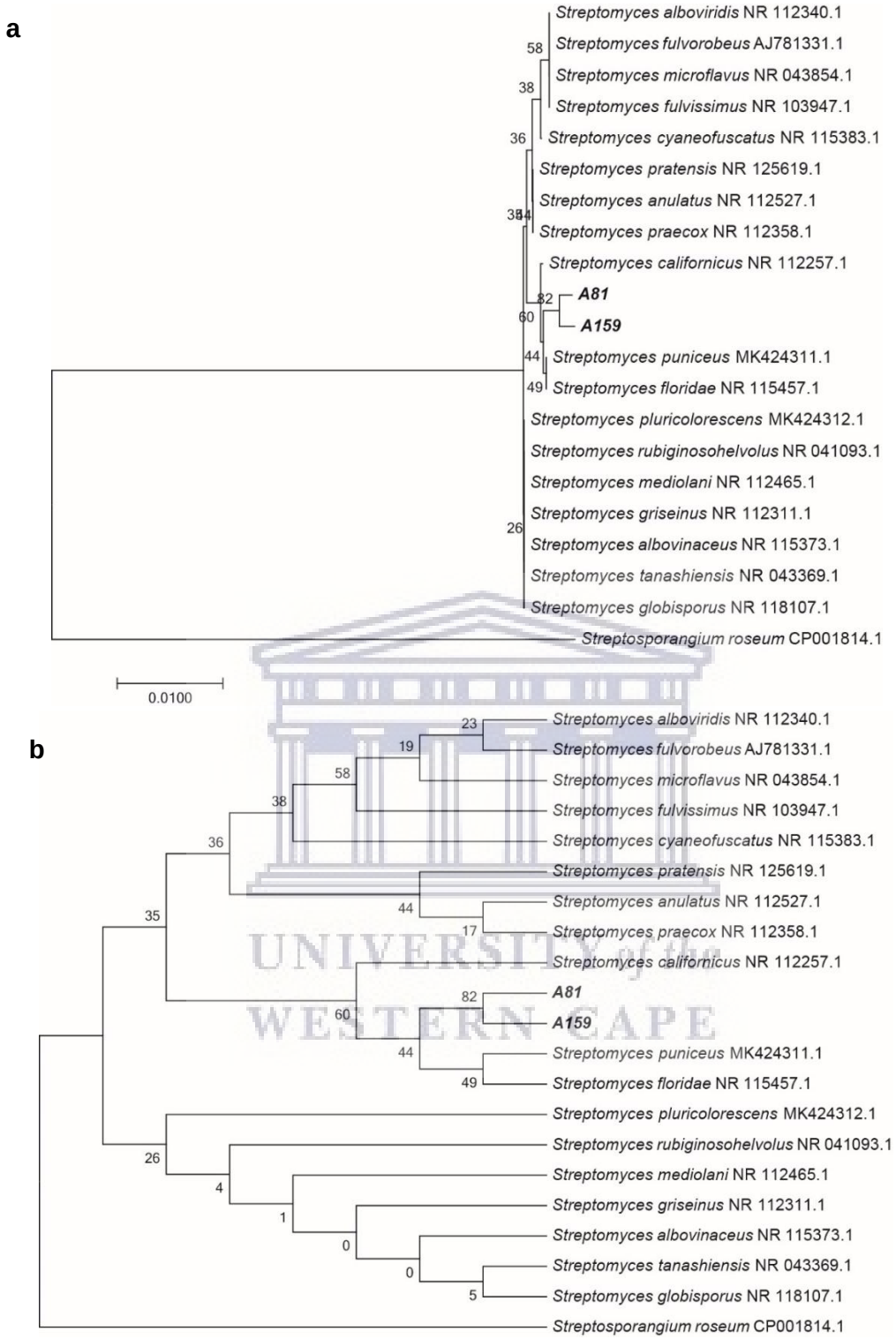


Figure 2.9: *Neighbour-Joining tree of the 16S rRNA sequences of the closest relatives to isolate A81 and A159 are a) drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. b) The topology tree is also shown to better present the bootstrap values. The analysis involved 21 nucleotide sequences. There were a total of 1138 positions in the final dataset.*

Based on 16S rRNA gene analysis isolate A81 closest related *Streptomyces* species (Figure 2.9) is *Streptomyces californicus* (99.03%), *Streptomyces tanashiensis* (98.94%), and

Streptomyces puniceus (98.94%). Isolate A159 had lower similarity to its nearest neighbours, and analyses revealed it to be related to *S. californicus* (97.9%), *S. puniceus* (97.9%), and *Streptomyces floridae* (97.82%). *Streptomyces puniceus, S. californicus* and *S. floridae* are genetically closely related, having DDH values of 84.1–89.6%, and it was suggested that these species be reclassified as a single species, namely *S. puniceus* (Rong and Huang, 2009). The *gyrB* gene analysis of the closely related species revealed a pairwise distance of 0.013 between isolates A81 and A159. This further suggests that these isolates are most likely different strains of a single species due to having a genetic distance lower than 0.014 (Hatano *et al.,* 2003). However, these isolates had a genetic distance of 0.027 (A159) and 0.03 (A81) to *S. californicus*, *S. puniceus* and *S. floridae*, thus indicating that these isolates are genetically distinct from their closely related relatives (Figure 2.10).

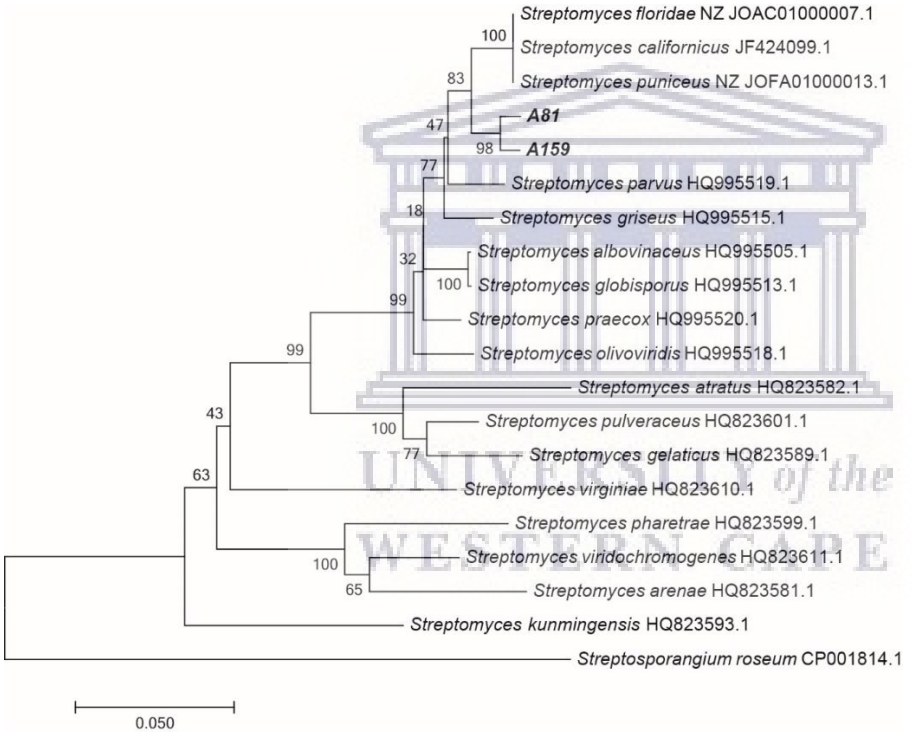


Figure 2.10: *The Neighbour-Joining tree of the gyrB sequences of the closest Streptomyces relatives to isolate A81 and A159 is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 20 nucleotide sequences with a total of 650 positions in the final dataset.*

The genetic and phenotypic analysis of isolates A81 and A159 indicates that these species are similar. Genetic analysis also indicates that these two isolates are distinct from the *S. puniceus* clade due to the high *gyrB* genetic distance (>0.027) and are potentially novel species within the *Streptomyces* genus. The name *Streptomyces aloensis*^{T} (referring to *Aloe ferox*, from which the type strain was isolated) is suggested for these isolates, with strain A81 being the type strain.

2.3.2.5 *Streptomyces* strain S149

This leaf endophyte was isolated from the leaves of the *S. frutescens* plant (S2P1; Table 2.2) and had good growth on ISP-2 – 6, with moderate growth on ISP-7. The isolate formed camouflage beige, leathery and hard vegetative mycelium with abundant white to grey aerial mycelia. The isolate produced a very light beige soluble pigment on ISP-2, -3 and -5, as well as melanin on ISP-6 (Table 2.9; Figure 2.11).

Table 2.9: *Growth and phenotypic characteristics of isolate S149 under various culture conditions.*

Media	Growth	Substrate mycelium	Aerial mycelium	Diffusible pigments
ISP-2	+++	camouflage beige	camouflage beige	camouflage beige
ISP-3	+++	camouflage beige	camouflage beige	camouflage beige
ISP-4	+++	camouflage beige	vellum	none
ISP-5	+++	beige	vellum	beige
ISP-6	+++	dark brown	light aircraft grey	dark brown
ISP-7	++	middle buff	white	none

+++: good; ++: moderate; +: weak.

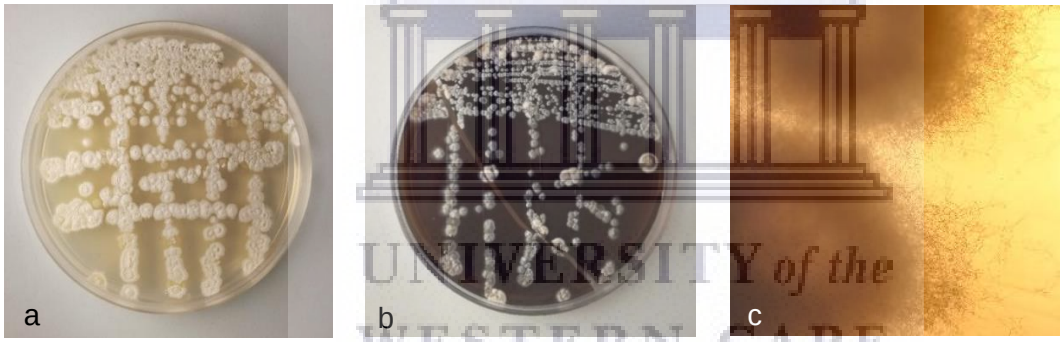


Figure 2.11: *Isolate S149 grown on a) ISP-5 and b) ISP-6 media for 14 days. Isolate forms abundant aerial mycelia on all media and also produced melanin on ISP-6; c)* Rectiflexibilis *type spore chains are seen under 100X magnification.*

The isolate was sensitive to high temperatures and grew from 4 to 37 °C, with optimum growth between 28 and 37 °C. The isolate grew under both acidic and alkaline pH conditions and was able to grow at pH range from pH 4.3 – 10.0, with an optimum growth at pH 7.0 - 8.0 (Table 2.10). The isolate was able to withstand a salt concentration of up to 8% NaCl (w/v). Isolate S149 was positive for the hydrolysis of starch, but not of carboxymethylcellulose and xylan. The strain was able to utilise sucrose, *myo*-inositol, D-fructose, D-raffinose, maltose and lactose as sole carbon sources. Isolate S149 differed phenotypically from its closest relative *Streptomyces canus* (Heinemann *et al.,* 1953) by having a higher salt and temperature tolerance. The cell wall sugars detected were glucose and ribose (Table 2.10). 16S rRNA gene sequence analysis revealed that strain S149 is very closely related to *S. canus* (99.73%), *Streptomyces cinnabarigriseus* (99.46%) and *Streptomyces chartreusis* (99.55%). These

closely related strains have been shown to produce a variety of bioactive compounds, including antibacterials, antifungals and proteasome inhibitors. *Streptomyces canus* was originally isolated from soil and produced the antibiotic, amphomycin (Heinemann *et al.,* 1953). *S. canus* strain FIM-0916 was found to produce analogues of amphomycin namely aspartocin D and E (Yang *et al.,* 2014). A different strain isolated from termites, strain BYB02, produced the antifungals resistomycin and tetracenomycin D (Zhang *et al.,* 2013). A draft genome of *S. canus* ATCC 12647 revealed the presence of multiple BGCs, including those for the antibiotic telomycin effective against Gram-positive organisms (Liu *et al.,* 2016). *Streptomyces cinnabarigriseus* was isolated from soil samples collected in Japan and forms brick-red mycelium with grey spores. This streptomycete produces the proteasome inhibitors, cinnabaramides A-G (Landwehr *et al.,* 2018). *Streptomyces chartreusis* was first isolated from African soil and found to produce the Gram-positive and mycobacterium antibiotic, chartreusin (Leach *et al.,* 1953). The nucleoside antibiotics, tunicamycins are also produced by *S. chartreusis* (Doroghazi *et al.,* 2011). A recent study performed on *S. chartreusis* NRRL 3882 found that the genome harboured 128 predicted BGCs. The researchers also found over a thousand distinct secreted metabolites in the culture supernatants, with only 22 that could be identified on standards and public libraries. The production of a number of these metabolites was iron dependent, including desferrioxamine siderophores that aid in iron acquisition (Senges *et al.,* 2018).

Table 2.10: *Differential characteristics of isolate S149 and the closest related* Streptomyces *species. Optimum growth indicated in brackets*

Characteristic	***S149***	***Streptomyces canus (Heinemann et al., 1953)***	***Streptomyces cinnabarigriseus (Landwehr et al., 2018)***	***Streptomyces chartreusis (Leach et al., 1953)***
16S rRNA similarity	**-**	99.73%	99.46%	99.55%
Spores				
Spore surface	n/t	spiny	n/t	spiny
Spore chains	RF	SP	n/t	SP
Temperature (°C)	4 - 37 (28 - 37)	28	15 - 40	30
pH	4.3 - 10 (7 - 8)	n/t	n/t	n/t
Salt tolerance (%)	0 - 8 (0)	5	7.5	n/t
Polysaccharide degradation				
Starch	+	n/t	n/t	n/t
Cellulose	-	n/t	-	n/t
Xylan	-	n/t	n/t	n/t
Carbon utilization				
Sucrose	+	+	+	n/t
myo-inositol	+	-	+	n/t
D-fructose	+	+	+	n/t
D-raffinose	+	-	+	n/t
Maltose	+	n/t	n/t	n/t
Lactose	+	n/t	n/t	n/t
Citric acid	-	n/t	n/t	n/t
Malic acid	-	n/t	n/t	n/t
DAP	Not detected	n/t	LL-DAP	n/t
Sugar	glucose, ribose	n/t	n/t	n/t

SP: Spira*; RF:* rectiflexibilis*; n/t: not tested or not reported; +: carbon utilisation; (+): weak carbon utilisation; -: negative carbon utilisation*

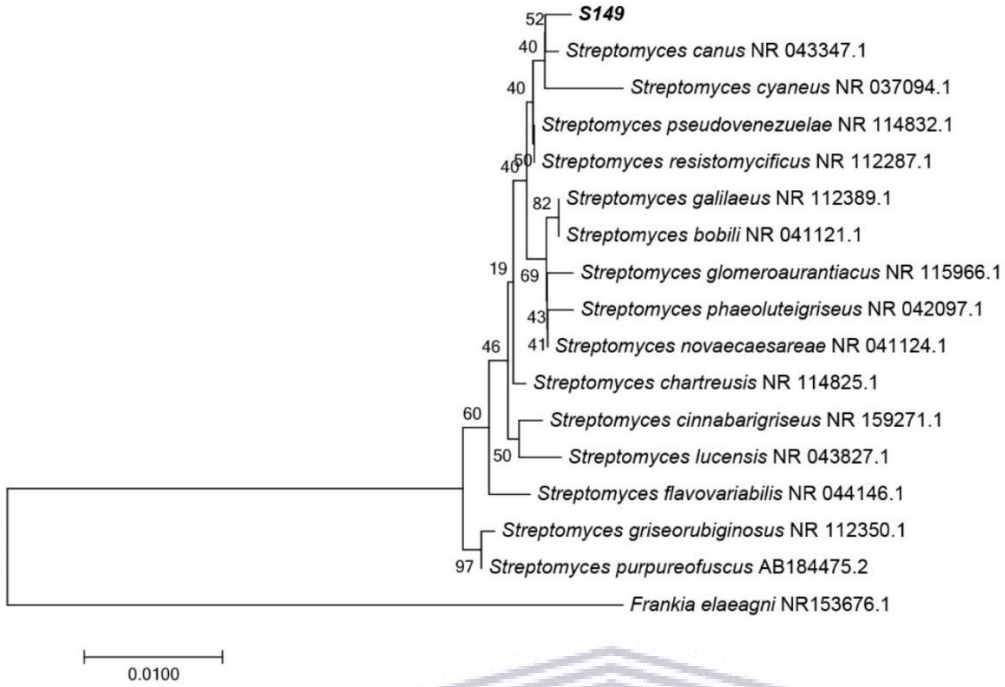


Figure 2.12: *Neighbour-Joining tree of the 16S rRNA gene sequences of the closest relatives to isolate S149 are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 17 nucleotide sequences. There were a total of 1069 positions in the final dataset.*

Sequence analysis of the 16S rRNA gene with BLASTn analysis on the GenBank database revealed that isolate S149 belonged to the genus *Streptomyces* with the highest similarity of 99.73% to *S. canus, S. cinnabarigriseus* (99.46%) and *S. chartreusis* (99.55%) (Figure 2.12). Phylogenetic analysis revealed a relatively low bootstrap confidence value of 52% to *S. canus*. This low confidence value may indicate that the isolate is not as closely related to its highest match and may potentially be related to another *Streptomyces* species. However, a low bootstrap value can also be indicative of very closely related sequences. Thus, gyrase B gene analysis was also performed to further evaluate the phylogenetic relationship.

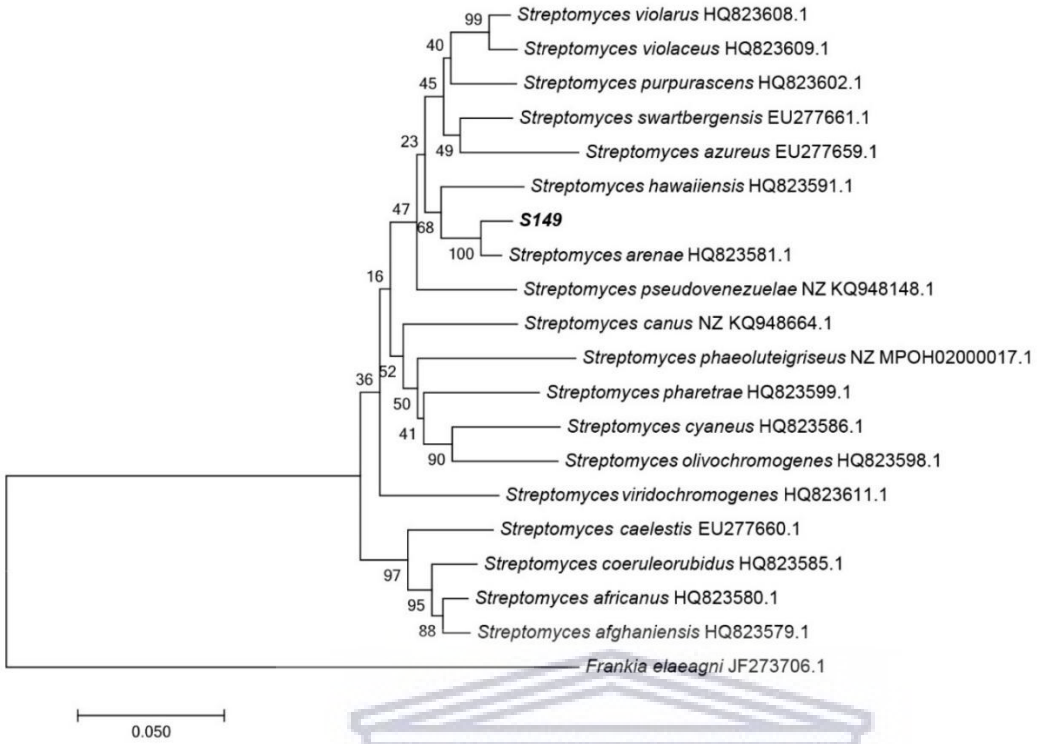


Figure 2.13: *The Neighbour-Joining tree of the gyrB sequences of the closest Streptomyces relatives to isolate S149 is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 20 nucleotide sequences with a total of 1044 positions in the final dataset.*

Analysis revealed that the S149 *gyrB* sequence shared an 98.03% similarity to *S. arenae* with a genetic pairwise distance of 0.018 (Figure 2.13). The *gyrB* gene had a large genetic distance of 0.087 to *S. canus*. This genetic distance of the *gyrB* gene of more than 0.014 may indicate that this isolate is genetically distinct from its closest match, *S. canus* and *S. arenae*, and may be a novel species within the genus of *Streptomyces.* Based on phenotypic and genotypic analysis it is not possible to elucidate strain S149's closest relative/degree of novelty. Further screening of other housekeeping genes such as *rpoB*, *recA*, *atpD*, and *trpB* will be needed to resolve this relationship.

2.3.2.6 *Actinomadura* strain S173

Isolate S173 was isolated from the surface of the *S. frutescens* seeds as an epiphyte (S3P1; Table 2.2). Due to this association with the seed it is speculated that the isolate may help the plant with germination. Plant growth and seed germination was out of the scope of this project and it is suggested to evaluate this isolate's potential for plant growth promotion in future investigations. This strain was able to grow on all media tested and forms weak to moderate substrate mycelium with no aerial mycelium (Table 2.11). The colonies' texture is tough and leathery, and they form small colonies, some of which are pink and others that appear beige in colour (Figure 2.14). No diffusible pigment or production of melanin was noted.

Table 2.11: *Growth and phenotypic characteristics of isolate S149 under various culture conditions.*

Media	Growth	Substrate mycelium	Aerial mycelium	Diffusible pigments
ISP-2	++	pink and vellum	none	none
ISP-3	++	pink and vellum	none	none
ISP-4	++	pink	none	none
ISP-5	+	white	none	none
ISP-6	+	beige	none	none
ISP-7	+	white and salmon pink	none	none

+++: good; ++: moderate; +: weak.

Figure 2.14: *Growth of isolate S173 on a) ISP-2 media forms pink and vellum colonies and on b) ISP-4 medium the isolates only form pink colonies. C) Isolate S173 is able to degrade starch. D) Under 100X magnification some short spore chains can be seen.*

The isolate was sensitive to high temperatures and grew from 4 to 37 °C, with optimum growth at 37 °C. The isolate grew under both acidic (pH 4.3) and alkaline (pH 10.0) conditions with optimum growth at pH 7.0 - 9.0 (Table 2.12). The isolate was able to withstand a salt concentration of up to 4% NaCl (w/v). Isolate S173 was able to hydrolyse starch, but not carboxymethylcellulose or xylan. Sparse growth was observed on the negative control ISP-9 plates containing no carbon source with no increase in growth when tested on various carbon sources. Thus, the strain was unable to utilise any of the sole carbon sources tested. Isolate S173 differed phenotypically to its closest relative *Actinomadura bangladeshensis* by not being able to grow at temperatures higher than 37 °C and did not produce spores. It was similar to its closest relative in that it had the same salt tolerance and the ability to hydrolyse starch as the other relatives were not. The cell wall sugars detected for isolate S173 was

xylose (Table 2.12). *Actinomadura* species are known to contain madurose in their cell walls. The presence of madurose in isolate S173's cell wall will need to be confirmed to classify whether this isolate forms part of the *Actinomadura* genus. *Actinomadura bangladeshensis* was isolated from sandy soil collected in Bangladesh and forms light wheat to bamboo substrate mycelium with pale pink to white aerial mycelium (Ara *et al.,* 2008). *Actinomadura darangshiensis* was isolated from a volcanic rock collected from the peak of Darangshi Oreum in Jeju, Republic of Korea. This species forms well-branched, non-fragmented, yellow-brown or cream substrate mycelia with whitish aerial mycelia (Lee and Kim, 2015). *Actinomadura macra* was first isolated from a soil sample collected in Okayama, Japan and produces the polycyclic ether antibiotics, CP-47,433 and CP-47,434 (Huang, 1980). The species produced cream, pale yellow, pale greyish, faint pink, pinkish orange, lavender, greyish or black vegetative mycelium depending on the growth medium with none to sparse white, pale grey or greyish aerial mycelia. This strain different morphologically from strain S173 in that it formed greyish black vegetative mycelium with white to grey aerial mycelium and a brown soluble pigment on ISP-2, whereas strain S173 formed pink and vellum substrate mycelia with no aerial mycelia or soluble pigment (Table 2.11; Figure 2.14a).

Table 2.12: *Differential characteristics of isolate S173 and the closest related* Actinomadura *species.*

Characteristic	***S173***	***Actinomadura bangladeshensis (Ara et al., 2008)***	***Actinomadura darangshiensis (Lee and Kim, 2015)***	***Actinomadura macra (Huang, 1980)***
16S rRNA similarity		98.21 %	98.06 %	97.99 %
Spores Spore surface Spore chains	 Short	 rough curved to hooked	 warty-surfaced Spiral	 smooth straight, flexuous, hooked, or coiled
Temperature (°C)	4 - 37 (37)	20 - 45	20 – 35 (30)	21 - 37
pH	4.3 - 10 (7 - 9)	5 - 9	7 - 8 (7)	n/t
Salt tolerance (%)	4	4	1	n/t
Polysaccharide degradation Starch Cellulose Xylan	 + - -	 + n/t n/t	 - n/t n/t	 - - n/t
Carbon utilization Sucrose *myo*-inositol D-fructose D-raffinose Maltose Lactose Citric acid Malic acid	 - - - - - - - -	 (+) - (+) - - n/t n/t n/t	 + + + - - - - -	 + - (+) - n/t - n/t n/t
DAP	Not detected	*meso*	*meso*	*meso*
Sugar	Xylose	Mad, Gal, Glu, Man	Glu, Gal, Man, Mad, Rib	Mad

n/t: not tested or not reported; +: carbon utilisation; (+): weak carbon utilisation; -: negative carbon utilisation

Sequence analysis of the 16S rRNA gene revealed that isolate S173 had highest similarity to species within the *Actinomadura* genus with similarities to *Actinomadura bangladeshensis* (98.21%), *Actinomadura darangshiensis* (98.06%) and *Actinomadura macra* (97.99%) (Figure 2.15). Phylogenetic analysis revealed a relatively low bootstrap confidence value of 39% to *A. darangshiensis*. This low confidence value may indicate that the isolate is not as closely related to its highest match and may potentially be related to another *Actinomadura* species. Gyrase B gene analysis was performed to further evaluate the relationship to the closest relatives.

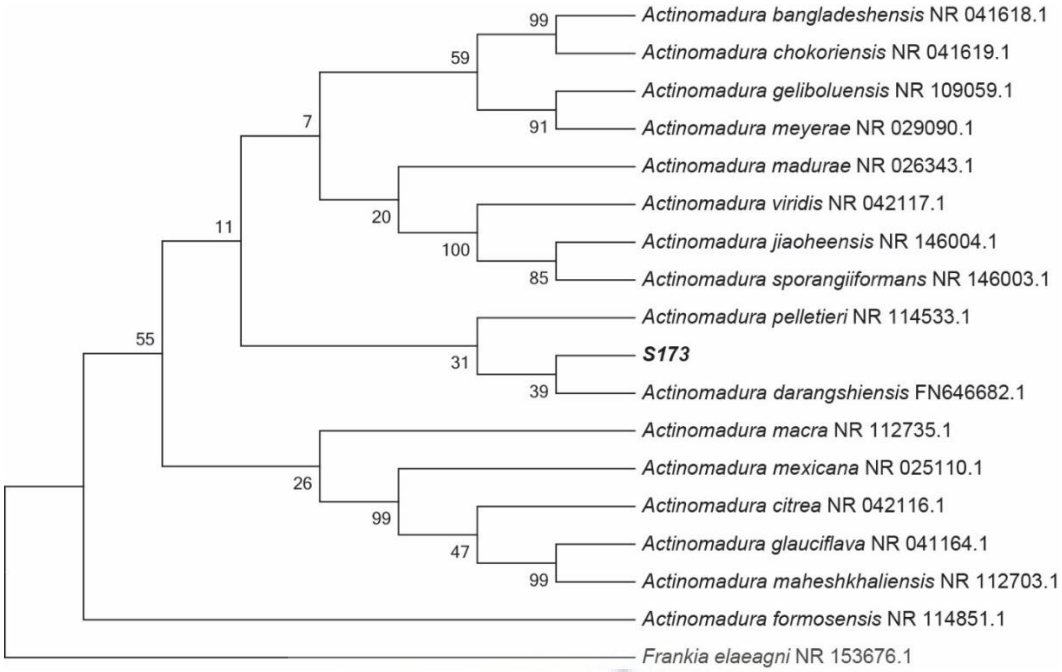


Figure 2.15: *Neighbour-Joining tree of the 16S rRNA sequences of the closest relatives to isolate S173 are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 18 nucleotide sequences. There were a total of 1398 positions in the final dataset.*

Sequence analysis of the *gyrB* gene with BLASTn analysis on the GenBank database revealed that isolate S173 had similarity to species from a range of different genera and not only *Actinomadura*. The closest match was *Actinomadura amylolytica* (85.05%), *Thermomonospora curvata* (81.57%), and *Planomonospora alba* (81.33%). However, this is likely a reflection that although the genus *Actinomadura* included 86 validly published species, only a small number of the *gyrB* gene sequences are available on the GenBank database. This can explain the relatively low similarity of the gyrase B gene to other *Actinomadura* species. It is also possible that the *gyrB* gene is not a good housekeeping gene to use to differentiate between species in this genus.

A search for the *gyrase B* gene sequences of *Actinomadura* species was performed and phylogenetic analysis was done to evaluate how closely related isolate S173 *gyrB* gene is to the closest matches and other *Actinomadura* species (Figure 2.16). The closest pairwise distance to isolate S173 was *Actinomadura formosensis* (0.053). The most closely related species, from the 16S rRNA gene analysis, *Actinomadura bangladeshensis* and *A. darangshiensis* had larger *gyrB* genetic distances at 0.084 and 0.055, respectively.

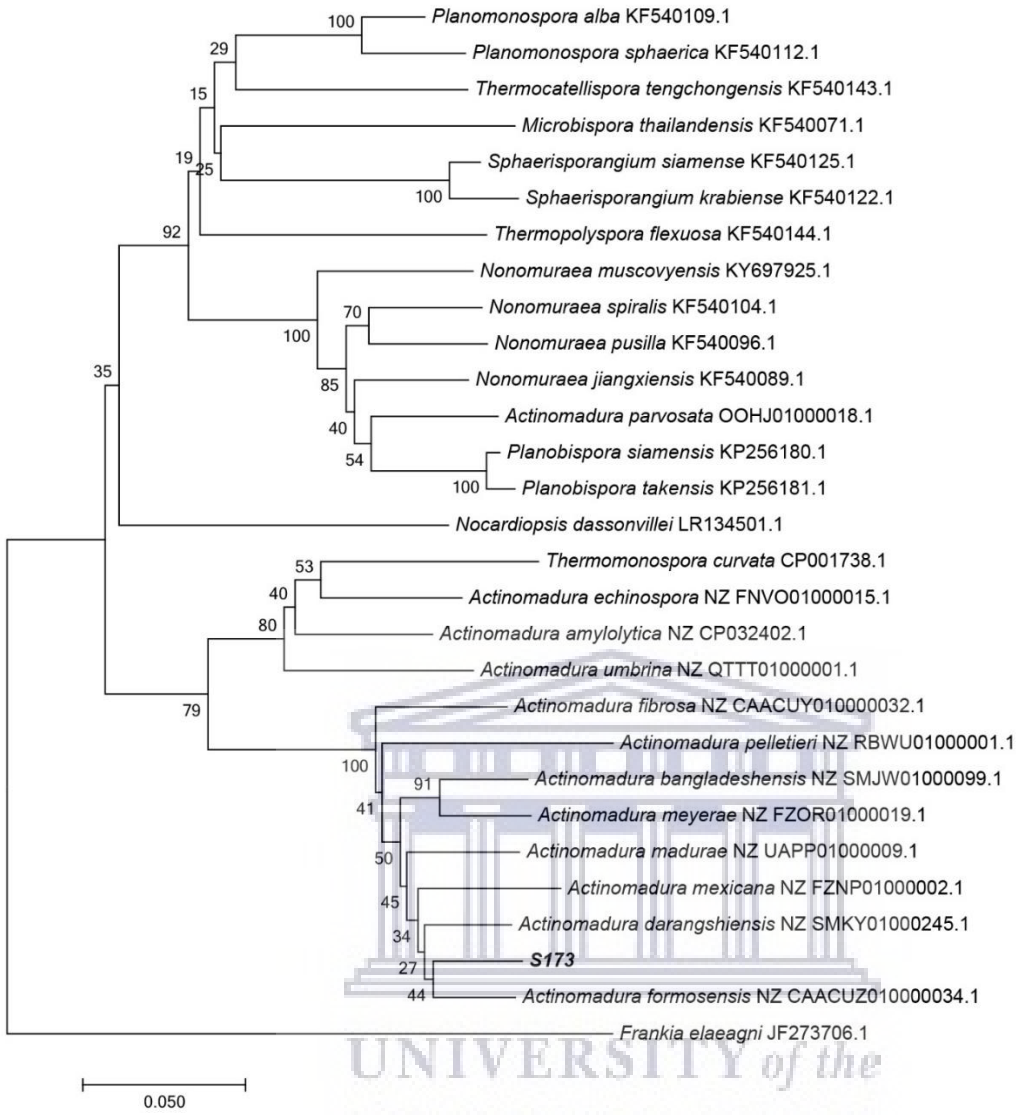


Figure 2.16: *The Neighbour-Joining tree of the gyrB sequences of the closest relatives to isolate S173 is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 29 nucleotide sequences with a total of 1045 positions in the final dataset.*

Unfortunately, the analysis of the gyrase B gene of *Actinomadura* species have not been previously performed and concluding the differentiating value of the genetic distance of the gyrase B gene is unavailable. Previous studies on the genetic distances of the gyrase B gene in other actinobacteria genera indicate that distances greater than 0.02 in the case of *Amycolatopsis* (Everest and Meyers, 2011) and 0.014 for *Streptomyces* (Hatano *et al.,* 2012) and *Micromonospora* (Kirby *et al.,* 2010) are indications of genetically distant species. Thus, from previous work on the gyrase B gene of actinobacterial species, it can be hypothesised that a genetic distance greater than 0.02 can be used to determine genetically distinct species within the genera of *Actinomadura* as well. Further studies will need to be conducted to confirm

this hypothesis. The large genetic distance in this case (0.053) indicates that even if the gyrase B genetic distance between the *Actinomadura* species are larger than 0.02, that there is a high possibility of isolate S173 being a novel species within the genus of *Actinomadura*. It is suggested that strain S173 be added within the genus of *Actinomadura* as a novel type strain as *Actinomaudura sutherlandiae*^{T} (referring to *Sutherlandia frutescens*, the medicinal plant from which the type strain was isolated).

2.4 Conclusion

The medicinal plants investigated in this study were found to host a wide range of actinobacteria, most of which were *Streptomyces* species. Generally, *Streptomyces* species are associated with plants, for example, 619 actinobacteria were isolated from different tomato cultivars and all of these were *Streptomyces* species (Tan *et al*., 2006). In a study focussing on 36 medicinal plants from Thailand, 330 actinobacterial species were isolated belonging to *Streptomyces*, *Microbispora*, *Nocardia* and *Micromonospora* genera (Taechowisan *et al*., 2003). The Fynbos biome is a unique environment and the plants in this biome have the potential to host a range of bacteria, including previously undiscovered species. This study isolated a range of actinobacteria, and of those that were subjected to 16S rRNA sequencing, the majority (60%) were found to have similarities lower than 99% to their closest phylogenetic neighbour. This highlights the potential of this research methodology to isolate novel actinobacteria associated with medicinal plants.

As the primary focus of the study was to assess the chemical newness of metabolites produced by these strains only six were selected for further investigation based on their antimicrobial activities. However, given that the probability of finding new compounds is increased if the producing organism is also novel the six isolates selected for full characterisation were genetically and phenotypically dissimilar to their closest relatives. In the case of isolates A3, A37 and S173, may even be classified into other genera based on their phenotypical and genetic traits. Future work will include the further analysis of the other likely novel isolates including strains A32, A183, and S170 (and other isolates highlighted in blue from Table 2.1 and 2.2) which were not characterised due to their limited antimicrobial activity.

This study was successful in isolating novel species of actinobacteria from either soil, leaves, roots, or seeds associated with medicinal plants. This discovery highlights that medicinal plants from the Fynbos environment have the potential to host a range of unique bacteria that may also have different metabolomes which can be the focus for future studies. The isolates discussed in this study were all related to isolates previously discovered in soil or rock samples, making these isolates unique in that they form relationships with medicinal plants, particularly those that were isolated from the leaves and seeds. *Streptomyces* strains A81, A159 and S149, and *Actinomadura* strain S173 are related to antimicrobial producing actinobacteria, whereas *Streptomyces* strain A3 and *Amycolatopsis* strain A37 was not related to antibiotic-producing actinobacteria.

The limitations of culture-based screening methods used in this study for the discovery of new bioactive compound-producing actinobacteria include that only isolates that can be cultured under the limited conditions tested can be evaluated for their novelty and potential to produce metabolites. Some of the strains stopped growing on the culture plates and this may be due to the isolates not being in contact with its host. Furthermore, only isolates that grew on the selected media were evaluated. Due to the large amount of actinobacteria isolated from this study, samples were dereplicated based on antibacterial activity against a range of Gram-positive and -negative pathogenic bacteria (as discussed in Chapter 3). Additionally, actinobacteria that displayed interesting morphologies, had high antimicrobial activity, or were isolated as an endo- or epiphyte were subjected to 16S rRNA gene sequencing. Thus, potential novel isolates that are plain-looking or that may only produce bioactive compounds in low amounts or under specific conditions may have been missed during the screening.

Even though these limitations of using culture-based screening exist, it is still used as the main method to discover novel, bioactive actinobacteria from the environment. The advantages of these methods being that isolation from environmental samples is easier, faster and more cost-efficient as compared to using sequencing-based discovery methods where DNA extraction from environmental samples can prove difficult, especially from plants rich in metabolites such as medicinal plants, as well as soil samples. The other advantage of culture-based screening versus sequence-based for the search of novel bacteria, is that scarce bacteria may have the potential to be discovered using selective media, whereas these bacteria may be underrepresented or difficult to identify using sequence-based screening due to limitations of sequence databases. Thus, using 16S rRNA gene metagenomic screening methods can result in not identifying bacteria present in minute quantities within the sample. Sequence-based screening is very dependent on the quality of the original sample as well as the quality of DNA extracted from the sample, which can prove difficult with plant and soil samples.

One major limitation of sequence-based screening for the identification of bioactive compounds, is the heterologous expression of the large PKS and NRPS pathways that can be composed of over 20 genes distributed between multiple polycistronic transcriptional units. Thus, the chance of the expression of the entire pathway in a heterologous host is significantly lower (Trindade *et al.,* 2015). These secondary metabolite pathways are regulated by a variety of enzymes and need certain intermediate compounds to produce a specific metabolite. Thus, a different metabolite may be produced than what is expected or none at all.

The focus of this study was to evaluate the production of antimicrobial compounds of actinobacteria, and thus, using a sequence-based approach for the discovery of antimicrobials would be difficult as these compounds may be toxic and potentially fatal to the heterologous host. This makes it very difficult to represent these gene clusters in metagenomic libraries. The study rather focussed on isolation and screening of actinobacteria using the culture-based approach. Sequencing was used of unique actinobacteria that showed the potential to produce a range of metabolites (Chapter 4).

Chapter 3: Bioactivity of Actinobacterial Isolates

3.1 Introduction

Actinobacteria are likely to play a significant role in the future of biotechnology, due to their ability to produce a wide range of compounds, including enzymes, anticancer agents, antioxidants, anti-inflammatories, and antimicrobial compounds (Abdelmohsen *et al*., 2015). Actinobacteria produce roughly 65% of all known natural bioactive compounds used in pharmaceuticals (Bérdy, 2005; Lee *et al*., 2018). Although there are a lot of pharmaceuticals on the market, the need for new drugs that treat or relieve human conditions is ever-growing. The growing number of drug-resistant bacteria and increasing prevalence of tuberculosis is only two examples of the need for new drugs. Other ailments and medical conditions such as viral and fungal infections, successful organ transplants and prevalence of pain can be solved through the discovery of new pharmaceuticals. As discussed in Chapter 1, exploring novel habitats has been recommended for the discovery of new bioactive compounds from actinomycetia (Strobel and Daisy, 2003). The selection of the plants in this study conforms to all of the criteria recommended. The actinobacteria were isolated from two medicinal plant species, *A. ferox* and *S. frutescens*, with known ethnobotanical uses and grows within the biodiverse rich Fynbos biome. Relatively few South African medicinal plants have been screened for the presence of bioactive endophytic actinobacteria. These as yet discovered bacteria could hold the potential for the discovery of new bioactive compounds that can ultimately be used as drug leads.

Bioactive actinobacteria have been successfully isolated from novel environments. By using the ethnobotanical history of the Australian snakevine plant, *Kennedia nigriscans*, the bioactive endophytic actinobacteria, *Streptomyces* NRRL 3052, was isolated. This endophyte produces the peptide antibiotics, munumbicins (Castillo *et al*., 2005). Unique soil environments have also shown promise for the isolation of bioactive actinobacteria. For example, *Streptomyces* strain 606 was isolated from Brazilian tropical soil, and had high activity against pathogenic bacteria, phytopathogenic fungi and the human pathogenic yeast, *Candida albicans*. The isolate also had high antiviral activity against an acyclovir-resistant herpes simplex virus type 1 strain on HEp-2 cells at non-cytotoxic concentrations (Sacramento *et al*., 2004). *Streptomyces swartbergensis* was isolated from a local unique soil environment, from the banks of the Gamka river in the Western Cape Province of South Africa, which had tyrosinase activity as well as high antimicrobial activity against a range of human pathogenic strains (Le Roes-Hill *et al*., 2018). Novel antibiotic-producing *Amycolatopsis* species were also

isolated from South African soil samples (Everest *et al*., 2013) which further validates the potential of unique environments for the isolation of antimicrobial producing actinobacteria.

Antimicrobial activity screening of actinobacterial isolates can be conducted with a few different techniques. The methodology is dependent on several factors: (1) the number of isolates to be screened; (2) the target species and (3) the pathogenic test organism used. In this study, primary antimicrobial screening was performed using the cross-streak method due to the large numbers of strains isolated. In this method, the actinobacterial isolate is streaked in the centre of the plate in a straight line and allowed to grow for 2 weeks. Multiple pathogenic test strains are then streaked perpendicularly to the actinobacterial isolate. After 2 days incubation, inhibition of growth of the pathogenic test strains can be observed indicating antimicrobial activity. After this initial screening, isolates that had high antimicrobial activity were further screening via the overlay method, which is more time and labour intensive. With this method, antimicrobial activity can be quantified by measuring the zone of inhibition. This allows for easy observation and selection of highly bioactive actinobacterial isolates. Crude extractions of the actinobacterial isolates were further subjected to disc diffusion plate screening to evaluate to ability of the isolate to produce the antimicrobial compound in broth. The crude extracts were separated via thin layer chromatography (TLC) and subjected to bioautography screening to identify the compounds that are responsible for the activity. During bioautography, MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] tetrazolium dye is used to assesses cell metabolic activity. When cells are metabolically active, they are able to reduce yellow MTT to its insoluble formazan form which is purple. When there is no cellular activity, the tetrazolium is not reduced and remains yellow. This colorimetric assay can determine the viability of the pathogenic test strains, with yellow zones indicating activity of the separated compound.

In this study, the actinobacterial isolates were tested against a range of Gram-positive and -negative human pathogenic microorganisms. These pathogens included *Bacillus cereus*, *Staphylococcus aureus*, *Mycobacterium aurum, Mycobacterium smegmatis, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. *Bacillus cereus* is a soil-dwelling, Gram-stain positive, rod-shaped bacterium. It is also well adapted to grow within the intestinal tract of insects and mammals. From these environments, it can easily spread to food where, when ingested, can cause an emetic or diarrheal-type of food-associated illnesses. Its ability to form endospores makes it difficult to control the contamination of food products by the bacteria because the endospores can withstand normal cooking and sterilization methods. These endospores are commonly present on food products and easily spreads from one food source to another (Arnesen *et al*.,

2008). *B. cereus* is one of the most common food-borne pathogenic bacteria and produces a range of virulence factors that can cause unpleasant disease in humans when ingested. The actual number of food poisonings caused by this pathogen is not known, since food poisoning is not a highly reported illness and is not always diagnosed since the effects normally last between 24 and 48 hours. Normally, only outbreaks which can be linked to a single source are reported. For instance, 173 cases were reported in Italy from contaminated cake in 2000 (Ghelardi *et al*., 2002). The toxicity of *B. cereus* ranges between strains, with certain strains being used as probiotics (Hong *et al*., 2005), and others causing food-related fatalities (Mahler *et al*., 1997; Lund *et al*., 2000; Dierick *et al*., 2005). Some *B. cereus* strains produce tissue-destructive exoenzymes including hemolysins, phospolipases, emesis-inducing toxin, and enterotoxins such as hemolysin BL, nonhemolytic enterotoxin and cytotoxin K. Additionally, enzymes such as beta-lactamases, proteases and collagenases are known potential virulence factors. Clinically beta-lactamases, cause beta-lactam antibiotic resistance, and three different forms (I, II and III) have been detected among *B. cereus* strains (Kotiranta *et al*., 2000). A close relative to *B. cereus*, *Bacillus anthracis* is known for its potential to cause the life-threatening disease, anthrax (Spencer, 2003). Antimicrobial activity against *B. cereus* will improve treatment against this foodborne pathogen as well as indicate the potential of the antimicrobial compound to have antagonistic effects against *B. anthracis* and other disease-causing *Bacillus* species.

Staphylococcus aureus is a Gram-positive, coccoid-shaped human commensal that is naturally found on the skin and in the nostrils (Kluytmans *et al*. 1997; Coates *et al*., 2013). This opportunistic pathogenic bacterium causes a range of clinical infections, including bacteraemia, food poisoning, and infective endocarditis. It also causes osteoarticular infections, skin- and soft tissue infections, pleuropulmonary infections, and device-related infections (Tong *et al*., 2015). Due to the increase of infections by methicillin-resistant S. *aureus* (MRSA) strains, traditional treatment has become problematic. It was reported that up to 42.6 % of healthcare-associated *S. aureus* infections were resistant to oxacillin, methicillin, or cefoxitin (Weiner *et al*., 2016). *Staphylococcus aureus* produces an extensive number of virulence factors, with structural and secreted products forming part of its pathogenicity of infection. Some of the virulence factors may have several functions in pathogenicity, whereas other virulence factors serve the same function (Gordon and Lowy, 2008). Once *S. aureus* infects host tissues, it can form biofilms (slime) that enables it to persist by avoiding host defence systems and antibiotics (Donlan and Costerton, 2002). Not only is the infection difficult to treat, but is also costly due to hospitalisation, barrier precautions, antimicrobial treatment and laboratory investigations (Goetghebeur *et al*., 2007). Improved

prevention and treatment strategies are needed to effectively treat infections caused by MRSA.

Mycobacterium is a genus within the order *Actinobacteria.* These bacteria are acid fast, Gram-positive and form straight or slightly curved rods. Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* infection, with an estimated 10 million new infections and 1.6 million deaths globally in 2017. South Africa is one of the top 20 high TB-burdened countries in the world, with an estimated 300 000 people having TB, and 62 000 reported deaths in 2018 (World Health Organisation, 2018; 2019). Due to the rise of antimicrobial resistant strains there is a need for new drugs and drug regimes. Model systems are used to speed up discovery of antimicrobials and circumvent biosafety issues. Models such as *Mycobacterium aurum* and *Mycobacterium smegmatis* are used, as they can be handled in biosafety-level 2 laboratories and are faster growing, making high throughput screening possible without safety concerns (Namouchi *et al*., 2017). These model systems do unfortunately have some limitations; thus, it is suggested to use more than one model species for inhibition screening.

Enterococcus faecalis are nonmotile, Gram-positive commensal bacteria that inhabit the gastrointestinal tract of humans and other mammals. Even though they are found in healthy humans, they can cause hospital related infections due to the high number of antibiotic resistant strains found in this environment. Morbidities include endocarditis, bacteraemia, as well as intra-abdominal, pelvic, wound, and urinary tract infections (Murray, 1990; Kau *et al*., 2005). *E. faecalis* is also the main cause of re-infection in root canal-treated teeth, being found in 24 – 77% of cases (Stuart *et al*., 2006). The production of biofilms and its ability to survive extreme environments, including high alkaline and salt environments, make it difficult to control and treat infections. Antibiotic resistant enterococci are important nosocomial pathogens and are becoming an increasing problem in clinical settings. *Enterococcus faecalis* strains can develop resistance to vancomycin and in 2014 the National Health Service (UK) reported that 9.8% of the *E. faecalis* species found were resistant to vancomycin (Weiner *et al*., 2016).

Escherichia coli is a Gram-negative, rod-shaped bacterium that is naturally found in the lower intestine of warm-blooded animals. Most *E. coli* strains are harmless, but some types are pathogenic and can cause food poisoning and urinary tract infections (Croxen and Finlay, 2010). Pathogenic *E. coli* infections result in severe diarrhoea, with 1.7 billion cases reported worldwide every year. Diarrheal infection is the second largest cause of death in children under the age of 5, with approximately 760 000 deaths every year (World Health Organisation, 2013; Chowdhury *et al*., 2015). Diarrheal infections can be successfully treated with antibiotics and rehydration, but in some cases the *E. coli* may become resistant to antibiotics (Tadesse *et al*.,

2012). In 2011, a multi-drug resistant Shiga toxin-producing *E. coli* O104:H4 outbreak occurred in Germany from contaminated fenugreek seeds. The outbreak caused 4 321 infections, with 53 deaths reported (Robert Koch Institute, 2011). Recently, in June 2019, an outbreak by the Shiga toxin-producing *E. coli* O103 in ground beef was reported by the Centres for Disease Control and Prevention (USA) (https://www.cdc.gov/ecoli/2019/o103-04-19/index.html, accessed 25 June 2019). Thus, finding the correct treatment for the different *E. coli* pathotypes are needed to control outbreaks and lessen the mortality rates.

Klebsiella pneumoniae is a Gram-negative, rod-shaped bacterium that naturally forms part of the human mouth, skin, and intestinal flora. This bacterium is an important multi-drug resistant pathogen and is the causative agent of community and nosocomial pneumonia, bacteraemia, urinary tract infections, and liver abscesses (Siu *et al*., 2012; Broberg *et al*., 2014). *Klebsiella pneumoniae* represents one of the most concerning multi-drug resistant pathogens, and together with other multi-drug resistant organism, is classified as an ESKAPE pathogen which includes ***E****nterococcus faecium*, ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumanii,* ***P****seudomonas aeruginosa*, and ***E****nterobacter* species (Boucher *et al*., 2009). Antibiotic resistant strains of *K. pneumoniae* in Asia were° evaluated and were found to be resistant to several first-line antibiotics, including cefotaxime (79.2%), ceftazidime (75.7%), imipenem (65.6%), meropenem (62.7%) and gentamicin (58%) (Effah *et al*., 2020). Given the multidrug resistance of these bacteria, therapeutic options are limited, with high failure rates using monotherapy. Combination-therapy with carbapenem is generally used which has had higher success rates (Tzouvelekis *et al*., 2012).

Pseudomonas aeruginosa is an encapsulated, Gram-negative, rod-shaped bacteria and is found in soil, water, and most man-made environments due to its ability to utilize a wide range of organic materials for energy. *Pseudomonas aeruginosa* is part of normal skin flora but is also an opportunistic pathogen and infects immunocompromised individuals, particularly people who suffer from cystic fibrosis (CF). It is detected in around 50% of CF patients, with 20% of patients harbouring multi-drug resistant strains (Papp-Wallace *et al*., 2017). Its natural antibiotic resistance and its ability to form biofilms makes it difficult to treat infections, with the potent antimicrobial treatment required having adverse effects on individuals. Combination-therapy with ibuprofen and ceftazidime antibiotic enhanced the survival rate of *P. aeruginosa*-infected mice and shows promise as a treatment in CF patients (Papp-Wallace *et al*., 2017). Ceftolozane-tazobactam treatment of multi-drug resistant *P. aeruginosa* strains in CF patients was successful, treating 71% of patients (Haidar *et al*., 2017). Combination therapies hold the most promise for treatment of *P. aeruginosa* infections, with alternative strategies being

researched, including phage treatment, quorum sensing inhibitors, probiotics, vaccines, anti-microbial peptides, and antimicrobial nanoparticles (Chatterjee *et al*., 2016).

The production of bioactive compounds by *Actinobacteria* is influenced by the nutrients available, and mainly influenced by the nitrogen and carbon sources. *Actinobacteria*, and *Streptomyces* specifically, have a unique and complex life cycle. The morphological differentiation of the different life stages, from vegetative mycelium production to spore formation, involves the complex regulation of genes which often coincides with the production of various secondary metabolites. In their natural environment, actinobacteria compete for nutrients with other microorganisms to produce essential biomolecules used for growth. In the laboratory, the fermentation media can be adjusted to optimally stimulate the production of bioactive metabolites.

Bacteria use multiple sources of carbon for growth. These carbon sources can either be co-metabolised or the bacteria can use a preferred carbon source that is easily utilised and allows for fast growth. The presence of glucose often prevents the use of other carbon sources. This preference of a preferred carbon source over other secondary carbon sources which can lead to the reduced activities of the corresponding enzymes, is known as carbon catabolite repression (CCR) (Görke and Stülke, 2008). No common CCR mechanism have been found in *Actinobacteria*. In *Streptomyces coelicolor* A3(2), glucose kinase plays a key role in CCR. Glucose kinase mutants (Δ *glk*), did not exert CCR with glucose or other readily available carbon sources, indicating that glucose kinase has a regulatory role in catabolite repression (Van Wezel *et al*., 2007). Glucose is a common carbon source used for fermentative production and is a precursor to many secondary metabolites. Due to glucose being preferred by bacteria, this may interfere with the use of other carbon sources that are precursors for the formation of other secondary metabolites. Recently, the effect of carbon and nitrogen sources on the production of tacrolimus by *Streptomyces tsukubaensis* DSM-42081 was evaluated. The researchers found that using glucose or maltose at a concentration of 3% resulted in the highest production of tacrolimus. Media containing more than 3% carbon content exerted CCR, and thus decreased the production of tacrolimus (Moreira *et al*., 2020). Therefore, when screening for antimicrobials both the type(s) of carbon source and the concentration in the media should be considered.

Actinobacteria are able to assimilate nitrogen from various organic and inorganic sources, with ammonium being the preferred inorganic nitrogen source, as it can be taken up directly from the environment with ammonium transporters. Additionally, ammonium can be made available through the reduction of nitrate to nitrite and subsequently, ammonium, through nitrate and

nitrite reductases (Amin *et al*., 2012). Two main pathways, depending on the nitrogen levels, have been identified in *Streptomyces* species for the assimilation of ammonium. The first pathway, the glutamate dehydrogenase pathway, is active under high nitrogen (>1 mM) and synthesises glutamate from oxoglutarate and ammonium without the consumption of ATP. The second pathway is active under low nitrogen conditions, and ammonium assimilation is performed by glutamine synthetase (GS) and glutamine 2-oxoglutarate aminotransferase (GOGAT) enzymes. In most natural environments, nitrogen availability is low, and thus this GS/GOGAT pathway is the predominant pathway used for ammonium assimilation (Reuther and Wohlleben, 2006).

Actinobacteria are known producers of siderophore-antibiotic complexes (Wang *et al*., 2014). Siderophores are low-molecular-weight chelating agents that facilitates the uptake of iron and are produced by bacteria, fungi and plants (Schalk *et al*., 2011). These agents play an important role in iron solubilisation from minerals to make it available for microorganisms (Haas, 2003). These non-ribosomal peptides are classified based on their chemical nature as catecholate, hydroxamate, carboxylate or mixed types (Khan *et al*., 2018). Siderophores have various chemical structures, some of which include antibiotics, known as sideromycins, and includes albomycins, ferrimycins, danomycins, salmycins, and tetracyclines (Ferguson *et al*., 2000; Schalk, 2018). These conjugates are used as a "Trojan Horse" to transfer antibiotics into pathogenic bacteria. In this strategy, the outer-membrane receptors of the pathogenic bacterium bind to the siderophore-antibiotic complex and transports it into the periplasmic space. In the periplasm, the siderophores are sequestered and delivered to ATP-dependent transporters which facilitates their entrance into the cell. This strategy allows antibiotics that would otherwise not be able to pass cellular membranes to be taken up by pathogenic organisms (De Carvalho and Fernandes, 2014; Mislin and Schalk, 2014). To screen for siderophores, a plate assay was developed using chrome azurol S (CAS) and hexadecyltrimethylammonium bromide (HDTMA) as indicators. The CAS/HDTMA binds tightly with ferric iron to produce a blue colour. A blue to orange colour change is observed when a strong iron chelator, such as a siderophore, removes iron from the dye complex (Schwyn and Neiland, 1987). This simple plate assay allows for fast screening of general siderophore activity.

Actinobacteria produce a vast number of antioxidants. Antioxidants are compounds that remove or inactivate potentially harmful reactive oxygen species (ROS). These free radicals are produced in the body as a consequence of normal aerobic metabolism. Antioxidants help to destroy, prevent the generation of and/or scavenge ROS. Thus, minimizing oxidative stress-induced tissue damage (Gutteridge,1994). Antioxidant assays measure the depletion of

antioxidants as a change in signal (Chapple *et al*., 1997). The main assay used to measure the antioxidant potential of actinobacterial extracts is the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. In this assay the DPPH scavenging activity of the extract is measured by the reduction of the radical as a change in colour. The purple DPPH ethanolic solution is reduced to a yellow zone in the presence of antioxidants.

This chapter describes the bioactive screening of actinobacteria isolated from two South African medicinal plants. Various techniques were employed to evaluate the bioactive potential of these isolates. Due to the large number of actinobacteria isolated from this study, plate assays were mostly used to evaluate activities, due to the general low cost, simplicity and high screening throughput of plate assays.

3.2 Materials and Methods

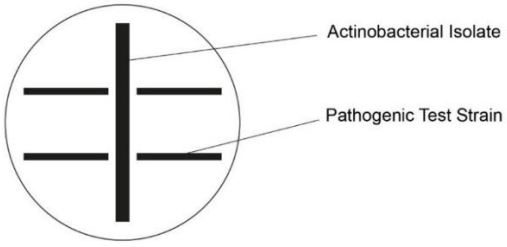
3.2.1 Pathogenic test strains

Human pathogenic test strains were used to test the antimicrobial activity of the actinobacterial isolates. The test strains used were *Bacillus cereus* ATCC 10876, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae subsp. pneumoniae* ATCC 700603, *Mycobacterium aurum* A+ (National Institute for Communicable Diseases (NICD) clinical isolate), *Mycobacterium smegmatis* (NICD clinical isolate), *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* subsp. *aureus* ATCC 29213. These pathogens were routinely cultured in LB broth and stored as glycerol stocks at -20 °C until use.

3.2.2 Cross-streak method

The cross-streak method was used as a primary mass screening method to evaluate the antibiotic activity of the actinobacterial isolates. The majority of the isolates were screened for activity against *B. cereus*, *E. faecalis*, *E. coli* and *S. aureus*. Isolates were grown on ISP-2 media plates for ten days at 28 °C. These isolates were then streaked using a sterile toothpick on cross streak media (Table A2) in the centre of the plate in a straight line. The actinobacterial isolates were incubated at 28 °C for 2 weeks. The pathogens were inoculated in Luria Bertani (LB) broth and incubated overnight at 37 °C shaking at 250 rpm. Twenty microlitres (20 µL) of the pathogenic strains (OD_{600} adjusted to 0.5 with LB) were applied in a straight line perpendicularly to the actinobacterial isolate (Figure 3.1) using a pipette. The inoculated plates were incubated at 37 °C for two days after which the level of activity was recorded. The isolates that had high activity against one or more of these pathogens were selected for further antibiotic screening via the overlay method.

Figure 3.1: *Actinobacterial isolates were streaked in the middle of an agar plate with pathogenic test strains streaked perpendicularly to the isolate.*



3.2.3 Overlay method

Actinobacterial isolates with proven bioactivity were subjected to overlay screening method. A variety of media was selected for the screening of antibiotic activity of the isolates, which included ISP-2, SCN, ISP-1 and Oats (Table A2). The isolates were stab inoculated with a sterile toothpick in the middle of the plate and incubated at 28 °C for two weeks after which overlays were performed. Pathogenic test strains were cultured in 10 mL of LB broth (Table A3) and grown for 24 hours at 37 °C. These cultures were used as starter cultures to inoculate sloppy LB agar (0.7% agar). The appropriate volume of test strain to be used per 10 mL of sloppy agar was calculated using the following formula:

OD_{600} .x µL = 160

The formula to calculate the volume of *E. coli* to be used was calculated as follow:

OD_{600} .x µL = 4

Where x is the volume of culture to add to the sloppy agar and OD_{600} is the optimal density at 600nm.

The seeded sloppy agar was poured over the actinobacterial isolate plates and incubated at 37 °C for 48 hours. After this incubation period, zones of clearing were measured (i.e. area of clearing in mm^2) to determine the antibiotic activity of the actinobacterial isolates. The experiment was repeated three times (n = 3).

3.2.4 Fermentation of isolates

Isolates were inoculated into 100 mL Erlenmeyer flasks containing 20 mL of either ISP-2 or ISP-1 broth, grown for seven days at room temperature, shaking at 150 rpm, and used as seed cultures. The seed cultures were transferred to one-litre flasks containing 100 mL of either ISP-2 or ISP-1 broth and grown shaking (150 rpm) for 7 days at room temperature. After fermentation, the cells were separated from the broth by centrifugation at 4000 x *g* for 10 min. The broth was divided in two and filtered through coffee filters into clean flasks. An equal volume (± 60 mL) of ethyl acetate (EtAc) or chloroform was added to the broth; 10 mL of methanol (MeOH) was added to the cells and the extractions were incubated shaking at 150 rpm for 2 hours at room temperature. After the extraction, the EtAc and chloroform extractions were separated using a separation funnel. The solvent layer was collected into clean glass petri dishes and dried in a fume hood overnight. Methanol extracts were filtered through coffee filter paper (House of Coffees, 1x4 size) and dried overnight in a fume hood. The dried extracts were resuspended in 2 mL of the respective solvents and transferred to a weighed glass

McCartney flask and dried once again. The dried extract was weighed and stored at 4 °C for further experiments. Uninoculated blank media controls of the solvents were also prepared for all experiments.

3.2.5 Kirby-Bauer disk diffusion method

Isolates that had high antibacterial activity were subjected to antibiotic screening via the Kirby-Bauer disk method. The dried solvent extracts were resuspended in the corresponding solvents to a final concentration of 2 mg/mL. Ten microlitres (10 µL) aliquots of the extracts was applied to 6 mm filter paper disks in five separate applications and allowed to dry between each application. To increase the uptake of the extracts into the cells, 4 % DMSO was added to each disk. Thus, the final amount of extract per disk was approximately 100 µg. Positive controls used were either ampicillin or kanamycin (for mycobacterium test strains). The concentration of the positive controls was the same as the samples (i.e. 100 µg per disk). Negative controls were the blank media extractions. Disks were allowed to dry overnight in the fume hood to ensure all solvents evaporated. LB agar plates were spread plated with 100 µL of the pathogenic test strains (OD_{600} = 0.5) and dried for 30 minutes. The impregnated disks were placed on the inoculated plates and incubated at 37 °C overnight; or at 28 °C for 2 days for the mycobacterium test strains. The zone of clearing around each disk was measured (i.e. area of clearing in mm^2) after incubation. The experiment was repeated two times, each having three replicas.

3.2.6 Bioautography

The solvent extracts which

had antibacterial activity from the Kirby-Bauer disk method were subjected to bioautography against the pathogenic test strains it had activity against. The solvent extracts (30 µL) were applied to thin layer chromatography (TLC) silica gel 60 UV 254 nm aluminium plates (Merck). The extracts were separated in TLC tanks with a mobile phase consisting of methanol and chloroform (70: 30; v/v). The plates were dried for a few hours, and the separated compounds viewed under 254 nm UV light, marked and the R_f values calculated as follows:

$$R_f = \frac{\text{Distance from start to centre of substance spot (mm)}}{\text{Distance from start to solvent front (mm)}}$$

The TLC plates were then cut into individual strips and dipped into the pathogenic test strain (OD_{600} = 0.5). The inoculated plates were placed in petri dishes with wet tissue paper and incubated at 37 °C overnight, or at 28 °C for 2 days for mycobacterium test strains. After incubation, the plates were dipped in 0.25 % MTT tetrazolium dye (prepared in phosphate buffer) and incubated at 37 °C for 30 minutes. Yellow zones indicate cell death and thus activity of the separated compound. A duplicate plate that was not subjected to the test strains was prepared for each extract and compared to the developed bioautography plate. The area on the duplicate plate containing the putative activity was scraped and dissolved in MeOH. These samples were stored at 4 °C until further analysis.

3.2.7 Siderophore screening

Siderophore activity of the isolates was measured using the CAS colorimetric plate assay (Schwyn and Neiland, 1987). Isolates were stabbed inoculated into the CAS medium (Table A4) plates and incubated at 28 °C for 14 days. After incubation, orange zones were measured and recorded as siderophore activity of the isolate. The experiment was done in triplicate (n=3).

3.2.8 Antioxidant assay

Five microlitres (5 µL) of ethyl acetate extract from ISP-2 broth was spotted onto TLC F_{254} silica plates. The mobile phase consisted of chloroform : ethyl acetate : formic acid (5 : 4 : 1; v/v/v). One millimolar (1 mM) L-ascorbic acid (prepared in dH_2O) was used as the positive control. The plates were developed in an airtight glass tank for 20 – 30 minutes. The plate was air dried and bands were visualised under 254 nm UV light and marked. The plates were stained with 100 µM 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical solution in methanol (similarly to the technique described by Jesionek *et al*., 2015). The plates were allowed to dry and colour changes were recorded.

3.2.9 Data and statistical analysis

Quantitative analysis is represented as mean values and the reproducibility of the results is conveyed as standard deviation. The normality of the data was determined using a Shapiro Wilk's W test. Normal distributed data was analysed using the one-way analysis of variance (ANOVA) with a Tukey honest significant difference (Tukey HSD) post-hoc test. For abnormally distributed data, a Kruskal-Wallis test was performed. The differences between

means reaching a minimal confidence level of 95% were considered as being statistically significant. All data were analysed with the use of Statistica Version 8 program (TIBCO Software Inc. 2007). Graphs were drawn using Microsoft Excel 2016 (Microsoft Corporation 2016).

3.3 Results and Discussion

3.3.1 Cross-streak

The cross-streak method was used to screen each actinobacterial isolate against four pathogenic test strains, *B. cereus*, *S. aureus*, *E. coli* and *E. faecalis*. This method was used as a primary screening method to determine the antibacterial potential of the isolate. The majority (79%) of the isolates associated with *A. ferox* (Supplementary Table C1) and 70% of the isolates associated with *S. frutescens* (Supplementary Table C2) had activity against one or more of the pathogens. Isolates with high inhibitory activity (i.e. clearing of 5 mm or more around the streaked actinobacterial isolate) against one or more of the pathogens (Figure 3.2) were selected for further antibacterial screening (Table A2 and A3). The top isolates selected for further screening had high activities against the tested pathogens (Table 3.1). The exceptions were isolates S149 and S173, which were selected because S149 is a plant endophyte and S173 is a seed epiphyte that may represent unique metabolomes based on their close association with *S. frutescens* plants.

Table 3.1: Cross-streak results of *Aloe ferox* and *Sutherlandia frutescens* isolates. - : growth not inhibited; +: growth inhibited up to 2 mm from isolate; ++: growth inhibited up to 5 mm from isolate; +++: growth inhibited up to 10 mm from isolate; ++++: complete growth inhibition.

Isolate	*B. cereus*	*S. aureus*	*E. coli*	*E. faecalis*
A3	++++	++++	++	++++
A37	+++	+++	-	+
A81	++	++	-	+
A159	+++	+++	+++	+++
S149	++	-	-	-
S173	++	+	-	-

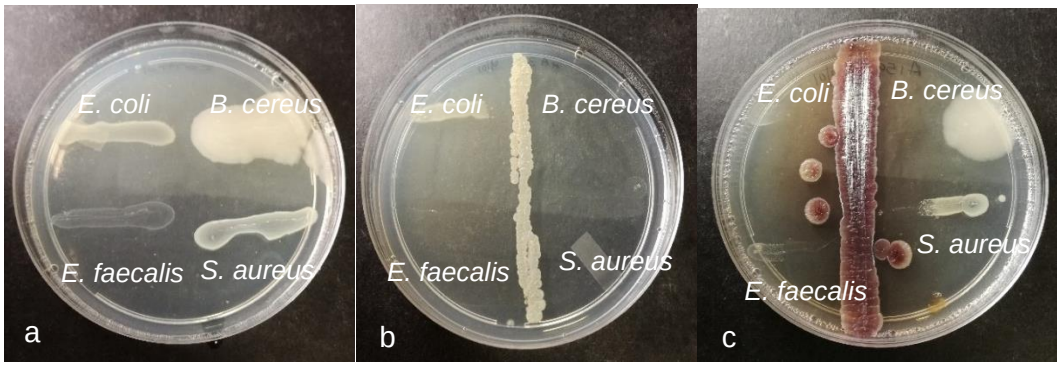


Figure 3.2: *Cross-streak method for screening of one actinobacterial isolate against multiple pathogenic strains. a) Pathogenic test strains are streaked horizontally on a plate and used to*

determine activity of the isolates. Isolates b) A3 and c) A159 was determined to have high activities against multiple of the pathogens.

3.3.2 Overlay

The antibacterial activity of the isolates was quantified using the overlay method. Isolates had activity against both Gram-positive and -negative bacteria. Furthermore, the media the actinobacterial isolate were grown on effected antibacterial activity of the isolate. For instance, an isolate would have activity against a specific pathogen on one media and lose activity or have better activity on a different media against the same pathogen.

Streptomyces strain A3 had high activity against *B. cereus* (6017.98 mm^2), *E. faecalis* (3856 mm^2), *M. aurum* (6062.49 mm^2) and *S. aureus* (2801.52 mm^2). The isolate had low activity against *E. coli* (150.53 mm^2) which was only observed when cultured on Oats medium. Activity against *B. cereus* was significantly more when the strain was grown on ISP-1 (0.003 or 0.0009) media compared to ISP-2 or Oats media. *Mycobacterium aurum* growth was significantly inhibited when strain A3 was grown on either ISP-1 or SCN media, resulting in the complete clearing of the 90 mm petri dish plate. Activity against *S. aureus* was highest when the isolate was grown on either Oats or SCN media (Figure 3.3). Some of *Streptomyces* strains A3 is most related to do have antimicrobial activities and produce secondary metabolites. *Streptomyces lunaelactis* (97.1% 16S rRNA similarity) produces ferroverdin A, an iron-chelating molecule, with no reported antimicrobial activity (Maciejewska *et al*., 2015). *Streptomyces tanashiensis* (97% 16S rRNA similarity) strain Kala UC-5063 produces the antibiotic kalafungin with inhibitory activity against pathogenic fungi, yeasts, protozoa, Gram-positive bacteria and, lesser activity against Gram-negative bacteria. The minimal inhibitory concentrations of kalafungin were measured against *Bacillus subtilis* (1 µg/mL), *S. aureus* (2 µg/mL), *P. aeruginosa* (125 µg/mL), *K. pneumoniae* (250 µg/mL) and *E. coli* (250 µg/mL). Similarly, high inhibitory activity against Gram-positive bacteria, with lesser activity against Gram-negative bacteria was observed for *Streptomyces* strain A3. The luteomycin-like antibiotic produced by *S. tanashiensis* strain AZ-C442 has inhibitory effects against fungi, yeasts, Gram-positive and Gram-negative bacteria, including *S. aureus, K. pneumoniae*, and *E. coli.* Optimal antimicrobial production was observed when the strain was grown in media containing glycerol (carbon source) and $NaNO_3$ (nitrogen source) supplemented with vitamin B12 (Afifi *et al*., 2012). A different study optimised the growth and metabolite production of a salt-tolerant, alkaliphilic strain of *S. tanashiensis* (strain A2D). This strain had inhibition activities against *B. subtilis*, *S. aureus*, *E. coli*, *K. pneumoniae* and *Mycobacterium phlei*, with lower inhibitory activities against yeast and fungi. Production of the antimicrobial compounds (20.2 µg/mL) were highest when 1% glucose was used as a carbon source. Other carbon

sources, such as fructose, starch and sucrose also favoured antibiotic production (12 - 16 µg/ml), but not as high as glucose. Metabolite yield was also influenced by the nitrogen source, with soybean meal (20.6 µg/mL) having the greatest effect, followed by peptone, yeast extract and casein (Singh *et al*., 2009). Similarly, growth of strain A3 on different media containing different carbon and nitrogen sources effected antimicrobial production of the strain. SCN media containing both starch and casein stimulated the production of compounds with inhibitory activity against *B. cereus*, *E. faecalis*, *M. aurum* and *S. aureus*. Another *S. tanashiensis* strain (IM8442T) produces the anticancer antibiotics, lactoquinomycin A and B (Okabe *et al*., 1985). *S. maoxianensis* (97.01%; Guan *et al.,* 2015) produces two aliphatic acid amides, maoxianamides A and B. These compounds had weak antimicrobial activities, but maoxianamide A and B exhibited cytotoxic activities against human lung carcinoma A549 (IC_{50} = 55.3 and 60.4 µg/mL, respectively), human hepatoma carcinoma HepG2 (IC_{50} = 32.4 and 37.7 µg/mL, respectively) and human leukaemia K562 (IC_{50} = 33.2 and 40.6 µg/mL, respectively) cell lines (Li *et al*., 2017). Anticancer and cytotoxic activity of strain A3 was not screened for in this study, but it may be of interest to screen the strain for these activities in future work.

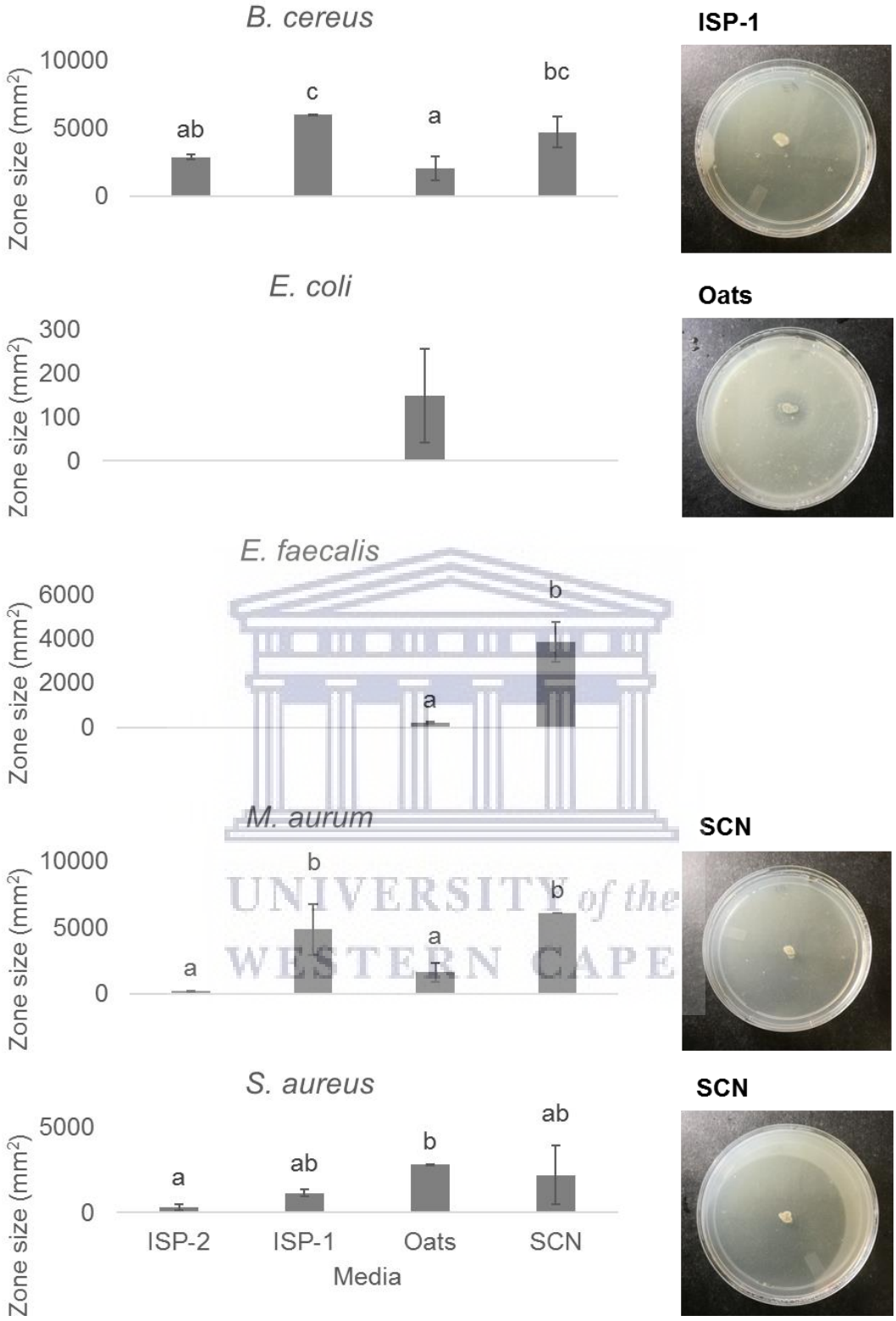


Figure 3.3: *The effect of media on the antibacterial activity of* Streptomyces *strain A3* via *the overlay method. Zone size indicated in mm^2. Data is means ± SD; n=3. Different letters indicate a statistically significant difference at the 95% confidence level.*

Amycolatopsis strain A37 had very high potent activity (cleared the 90 mm petri dish plate) against *B. cereus* (6242 mm^2), *E. faecalis* (6151.5 mm^2), *M. aurum* (6160.1 mm^2).

M. smegmatis (6176.9 mm^2), and *S. aureus* (4818.2 mm^2), when grown on ISP-2 media. The isolate had significantly lower activity against these human pathogens when grown on media other than ISP-2. Thus, the composition of the media has a large effect on the antibacterial activity of strain A37. ISP-2 medium is a rich complex media and contains high levels of minerals, vitamins, protein, nitrogen, and carbon. This complex media was able to provide strain A37 with important components for the production of metabolites that have activity against these pathogenic test strains (Figure 3.4).

No antimicrobial activity or the production of specific bioactive compounds was reported for *Amycolatopis* strain A37's closest relatives, *Amycolatopsis albidoflavus*, *Amycolatopsis echigonensis* or *Amycolatopsis niigatensis*. Nonetheless, other *Amycolatopsis* species do produce potent antimicrobial compounds such as rifamycin. Previously, the effect of carbon and nitrogen source on the production of rifamycin by *Amycolatopsis mediterranei* (MTCC14) was studied (Venkateswarlu *et al*., 1999). Inorganic and organic nitrogen sources at 1% and 2% concentrations, respectively, were evaluated and included sources like potassium nitrate, ammonium sulphate, ammonium carbonate and more. Ammonium sulphate resulted in the highest production of rifamycin (960 mg/L). Potassium nitrate, also found in SCN medium, stimulated the production of rifamycin (560 mg/L). Organic nitrogen sources like peptone, malt extract and soybean meal were also evaluated. Rifamycin production was highest (1020 mg/L) when soybean meal was used. Peptone and malt extract stimulated rifamycin production to 690 mg/L and 825 mg/L, respectively. Nitrogen metabolite regulation was observed when the concentration of nitrogen was increased, i.e. the production of rifamycin increases and then starts to decrease with more nitrogen. Carbon sources (at 2.5% concentrations) such as glucose, lactose and starch also stimulated the production of rifamycin at 1080 mg/L, 1020 mg/L and 730 mg/L, respectively. An increase in the concentration of carbon sources did not result in an increase of rifamycin which may be due to catabolite repression. Productivity of rifamycin is greatly influenced by these nitrogen and carbon sources (Venkateswarlu *et al*., 1999).

In this study, organic nitrogen sources including peptone, malt extract, yeast extract, oats, and casein is used. The results indicate that the use of ISP-2 medium greatly stimulates the production of antimicrobial compound(s) of *Amycolatopsis* strain A37, possibly due to the yeast and malt extract that serve as nitrogen sources. Furthermore, glucose serves as the carbon source in ISP-2 medium and may further stimulate the production of bioactive compounds. Similarly, as in the aforementioned study, the effect of carbon and nitrogen sources should be studied in more detail to determine the optimal concentration and source

of carbon and nitrogen for the maximum stimulation of antimicrobial production in *Amycolatopsis* strain A37.

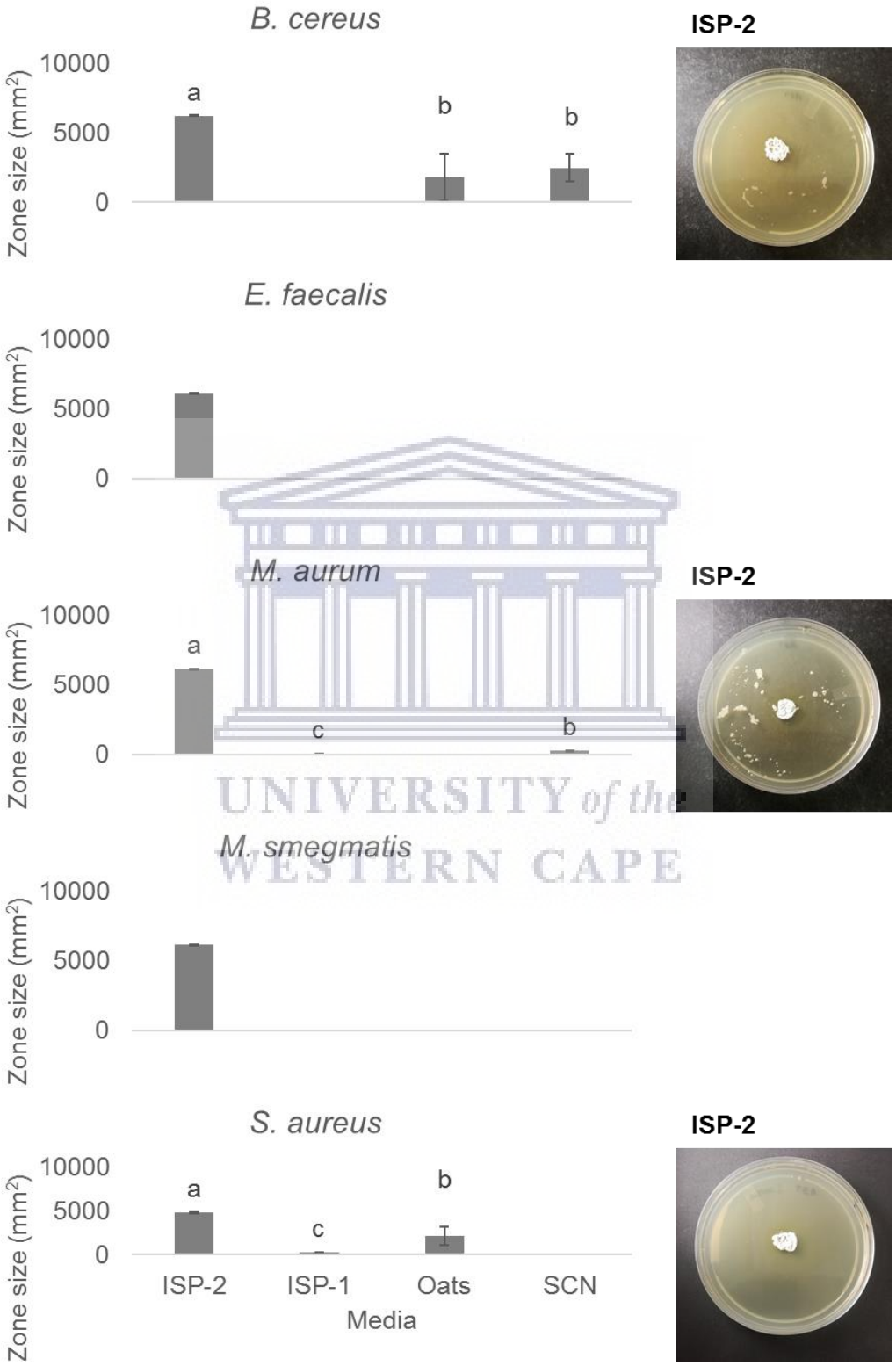


Figure 3.4: *The effect of media on the antibacterial activity of* Amycolatopsis *strain A37* via *the overlay method. Zone size indicated in mm². Data is means ± SD; n=3. Different letters indicate a statistically significant difference at the 95% confidence level.*

Streptomyces strain A81 had weak activity against *P. aeruginosa* (233.5 mm^2) when grown on ISP-2 media. Low activity was observed against *B. cereus* on ISP-1 (594.8 mm^2) and *E. faecalis* (729.6 mm^2) on ISP-2. The isolate had moderate activity against *M. aurum* (1009.2 mm^2) and *S. aureus* (1273.7 mm^2) when grown on SCN. Moderate antimicrobial activity against *M. smegmatis* (1663.7 mm^2) was observed on ISP-2 media (Figure 3.5). Inhibition activity of this isolate against both *M. aurum* and *M. smegmatis* makes it a candidate for further studies against *M. tuberculosis*. Media composition had a great effect on strain A81 and stimulated the isolate to produce compounds that were otherwise not produced. For example, activity against *E. faecalis* and *P. aeruginosa* was only observed when cultured on ISP-2 media and not on any of the other media. Thus, the antibacterial metabolite(s) is/are produced in the presence of specific media components. SCN media was able to stimulate the production of antibacterial compound(s) that had specific antagonistic activity against the Gram-positive strains, *B. cereus*, *M. aurum* and *S. aureus*.

Streptomyces strain A81 is closely related to *Streptomyces californicus* (99.03%), *Streptomyces tanashiensis* (98.94%), and *Streptomyces puniceus* (98.94%) (Section 2.3.2.4). These bacteria are known bioactive metabolite producers, with *S. californicus* BS-75 producing the antitumor compounds, pyrisulfoxin A and B, which are active against P388 murine leukemia cells (Tsuge *et al*., 1999). Other strains of *S. californicus* produced more antibiotic compounds: KS-619-1, an anthraquinone antibiotic that inhibits Ca2+ and calmodulin-dependent cyclic nucleotide phosphodiesterase (Matsuda and Kase, 1987); borrelidin, an antibiotic compound active against vancomycin- and methicillin-resistant clinical pathogenic strains (Saisivam *et al*., 2008); and griseorhodins, red pigmented compounds with antibiotic activity against Gram-positive and -negative bacteria (Suetsuna and Osajima, 1989). Fermentation of *S. californicus* in SCN medium stimulated the production of borrelidin (Saisivam *et al*., 2008). The effect of carbon and nitrogen sources on the production of griseorhodins was previously evaluated. As a carbon source, soluble starch optimally stimulated the production of griseorhodins. The use of peptone and yeast extract as nitrogen sources led to maximum griseorhodin production, with a combination of these two nitrogen sources leading to an even further increase in griseorhodin production (Suetsuna and Osajima, 1989).

In this study, SCN had the ability to stimulate the production of antimicrobial compounds active against *B. cereus*, *M. aurum* and *S. aureus*. Soluble starch is used in SCN medium as the main carbon source. The production of borrelidin and griseorhodins was stimulated using soluble starch and thus it is possible that these or similar compounds may be produced by

Streptomyces strain A81 as well. Furthermore, amylase activity of *Streptomyces* strain A81 was previously confirmed (Section 2.3.2.4) and may indicate the strain's ability to effectively use starch as its carbon source as amylases are able to break down starch into maltose and subsequently into glucose. Effective use of this carbon source may influence the production of secondary metabolites.

In ISP-2 medium, glucose is used as a carbon source, with yeast extract and malt extract as nitrogen sources. These carbon and nitrogen sources may stimulate the production of antimicrobial metabolites, albeit not in the quantities that SCN media can with regards to antimicrobial activities against *B. cereus*, *M. aurum* and *S. aureus*. ISP-2 media however did stimulate the production of antimicrobials active against *E. faecalis*, *M. smegmatis* and *P. aeruginosa* that could not be stimulated by SCN or any of the other media used. The carbon and nitrogen sources in ISP-2 media may have stimulated the production of other antimicrobial compounds not stimulated by the carbon and nitrogen sources found in the other media used.

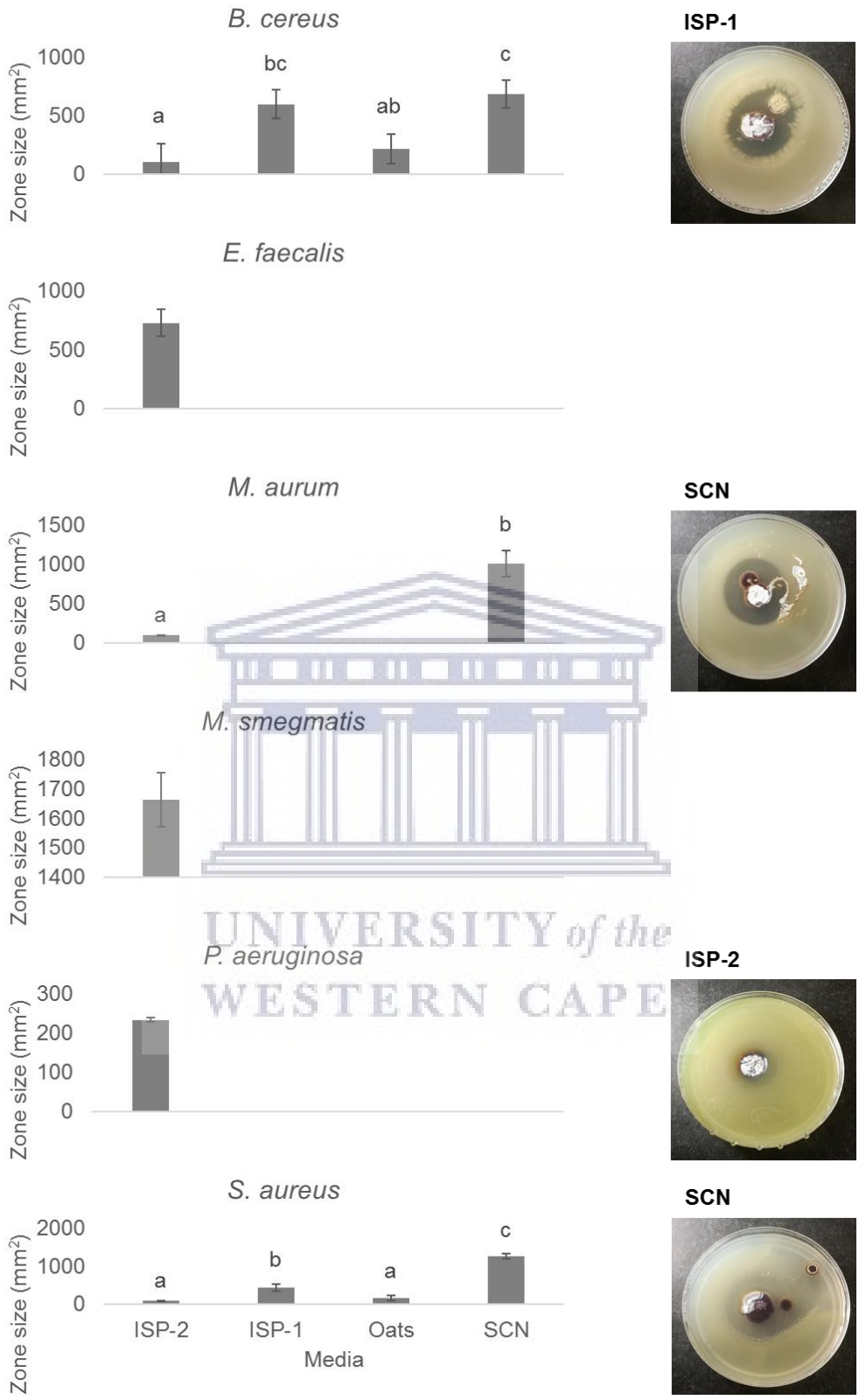


Figure 3.5: *The effect of media on the antibacterial activity of* Streptomyces *strain A81* via *the overlay method. Zone size indicated in mm². Data is means ± SD; n=3. Different letters indicate a statistically significant difference at the 95% confidence level.*

Streptomyces strain A159 grown on ISP-1 media had antibacterial activity against *B. cereus* (897.2 mm²), *E. faecalis* (232.2 mm²) and *M. aurum* (840.1 mm²). Activity against

M. smegmatis (5806.4 mm^2) on ISP-2 was very high, with the isolate producing antimicrobial compounds that were able to clear the petri dish. Activity against *P. aeruginosa* (240.9 mm^2) on ISP-2 varied between tests, with some having activity and others having no detectible activity. In some cases, the expression of the antibacterial compound was stimulated and others not. This may be due to the genetic machinery involved in the transcription and expression of the antibacterial compound. Media composition appeared to have little effect on the activity against *S. aureus* a and weak activity was detected against this pathogen on all media ranging from 213.4 mm^2 (Oats) to 554.8 mm^2 (SCN).

Streptomyces strain A159 had similarities to *S. californicus* (97.9%), *S. puniceus* (97.9%), and *Streptomyces floridae* (97.82%). These three species were suggested to be reclassified as different strains of a single species, namely *S. puniceus* (Rong and Huang, 2009). As discussed previously, *S. californicus* produces a range of bioactive compounds. With griseorhodin production being stimulated by different carbon and nitrogen sources. ISP-1 medium stimulated antimicrobial production of compounds active against *B. cereus*, *E. faecalis*, *M. aurum* and *S. aureus* (Figure 3.6). Griseorhodin production was optimally stimulated when both peptone and yeast extract was used in combination as the nitrogen source. Since ISP-1 contains peptone and yeast extract, this combination may stimulate the production of griseorhodin or similar compounds in *Streptomyces* strain A159. The yeast extract and glucose in ISP-2 may also stimulate production of antimicrobial compounds like griseorhodins and may explain the inhibitory activity observed against *M. aurum*, *M. smegmatis*, *P. aeruginosa* and *S. aureus*. SCN media stimulated the production of antimicrobial compounds active against *B. cereus*, *M. aurum* and *S. aureus*. The stimulation may be due to the soluble starch that serves as a carbon source. The production of griseorhodin was optimally stimulated with soluble starch, and the production of borrelidin was stimulated using SCN media. Thus, SCN medium may stimulate the production of these or similar compounds in *Streptomyces* strain A159.

Streptomyces puniceus produces the anti-mycobacterial compound viomycin. The production of viomycin was stimulated on nutrient media, containing peptone and yeast extract (Finlay *et al*., 1951). Thus, the high inhibitory activity against *M. aurum* and *M. smegmatis* on ISP-1 and ISP-2 media, respectively, may be explained by the presence of peptone and yeast extract to stimulate the production of anti-mycobacterial compounds like viomycin. *Streptomyces puniceus* strain AS13 was recently found to produce dinactin, an antitumour antibiotic with antimicrobial activities against Gram-positive and -negative bacteria, including *M. tuberculosis* (Hussain *et al*., 2018). Dinactin production was stimulated on SC media. This media consists of soluble starch as a carbon source, and tryptone and potassium nitrate as nitrogen sources.

As such, the inhibitory activity of *Streptomyces* strain A159 against *M. aurum* on SCN may be due to the soluble starch that stimulates the production of dinactin or similar compound(s).

Carbon and nitrogen source have an effect on the antimicrobial activity of *Streptomyces* strain A159 as can be seen by the inhibition activities observed on different media. The carbon and nitrogen sources need to be further investigated and optimised for peak production of the antimicrobial metabolites.

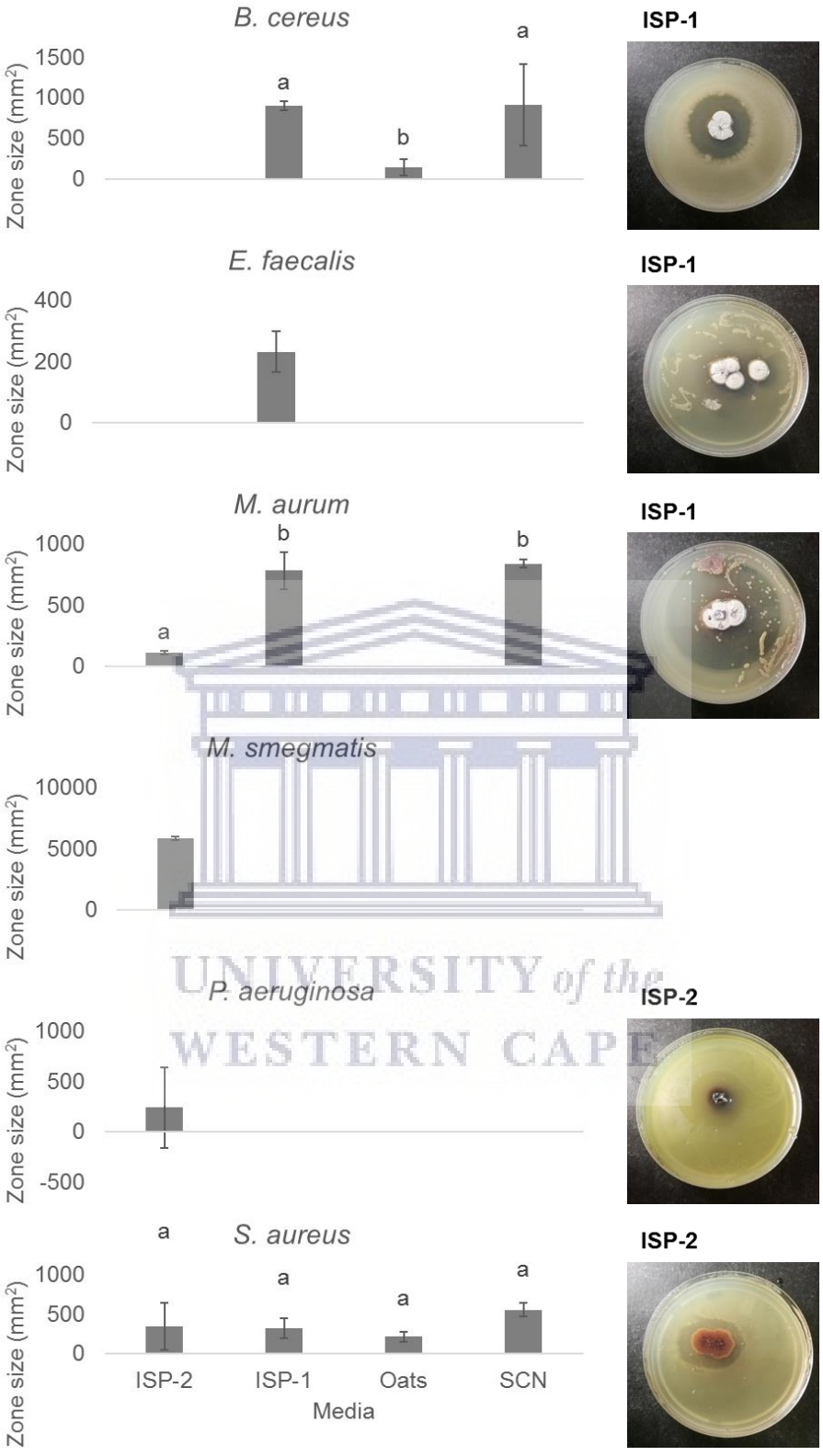


Figure 3.6: *The effect of media on the antibacterial activity of* Streptomyces *strain A159* via *the overlay method. Zone size indicated in mm². Data is means ± SD; n=3. Different letters indicate a statistically significant difference at the 95% confidence level.*

Streptomyces strain S149 had significantly higher antibacterial activity against *E. faecalis* when grown on ISP-1 (848.23 mm²) media compared to when grown on Oats media (264.9 mm²). Antagonistic activity against *M. aurum* (663.1 mm²) was observed when the isolate was

grown on SCN. No activity against any of the test strains was observed when the isolate was grown on ISP-2 media (Figure 3.7).

Streptomyces strain S149 is very closely related to *Streptomyces canus* (99.73%), *Streptomyces cinnabarigriseus* (99.46%) and *Streptomyces chartreusis* (99.55%). The effect of media composition on kanamycin production in *S. canus* was investigated with the addition of peptone, glucose and molasses stimulating kanamycin production (Abou-Zeid *et al*., 1971). Peptone and yeast extract are the nitrogen sources in ISP-1 media and may enhance the production of a kanamycin-like compound and explain the high inhibitory activity against *E. faecalis* on SCN media. Production of amphomycins, lipopeptide antibiotics active against *B. subtilis* and *S. aureus*, was stimulated in *S. canus* strain FIM-0916 using sucrose as a carbon source, with a mixture of yeast and beef extract as the organic nitrogen sources. The activity of these compounds was calcium dependent and thus calcium was added to the susceptibility testing medium (Yang *et al*., 2014). The production of these compounds may be stimulated in *Streptomyces* strain S149, but activity may not have been observed due to the lack of calcium in the medium. The only medium containing calcium, in the form of calcium carbonate, is SCN medium. Though this medium does contain calcium, it does not contain the optimal carbon and nitrogen sources to stimulate the production of amphomycins or similar compounds. This may explain why no antimicrobial activity was observed against *B. cereus* and *S. aureus*. *Streptomyces canus* produces the Gram-positive antibiotic, telomycin, which is stimulated through a carbohydrate rich medium, such as tryptone soya media (Hooper *et al*., 1962; Fu *et al*., 2015). *Streptomyces* strain S149 had amylase activity and thus is able to degrade soluble starch. The ability of this isolate to use soluble starch may indicate that it can effectively use this carbon source for the production of bioactive compounds. This isolate may be utilising the soluble starch in SCN media to produce an antimicrobial compound active against *M. aurum* (Figure 3.7). Further investigation on the media components to stimulate production of the antimicrobial compounds will need to be investigated. In future, the addition of calcium to the medium should be investigated as the production of some of the antimicrobials could potentially be stimulated in the presence of calcium.

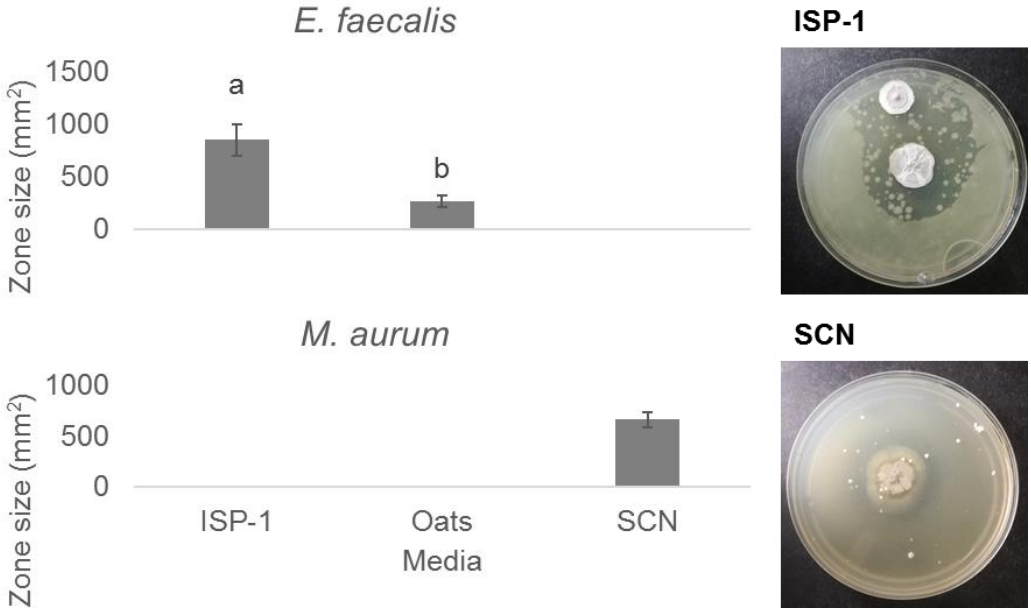


Figure 3.7: *The effect of media on the antibacterial activity of* Streptomyces *strain S149* via *the overlay method. Zone size indicated in mm². Data is means ± SD; n=3. Different letters indicate a statistically significant difference at the 95% confidence level.*

Actinomadura strain S173 had very high activity against *B. cereus* (3104.2 mm²), *E. faecalis* (6324.6 mm²), and *S. aureus* (880.7 mm²) when grown on ISP-2 media. Moderate activity against the Gram-negative *K. pneumoniae* (1008.2 mm²) was observed in the presence of SCN media. Media influenced the antagonistic activity against *S. aureus*, with ISP-1 (67.81 mm²) and Oats (24.09 mm²) having a similar effect on activity, SCN (502.65 mm²) having better activity and ISP-2 (880.69 mm²) media having the best activity (Figure 3.8). Phylogenetic analysis revealed that strain S173 had similarities to *Actinomadura bangladeshensis* (98.21%), *Actinomadura darangshiensis* (98.06%) and *Actinomadura macra* (97.99%). *A. bangladeshensis* and *A. darangshiensis* have no reported bioactive activities. *A. macra* strain Pfizer FD 25934 (ATCC 31286) produces the antibiotics, CP-47, 433 and CP-47, 434 (Huang, 1980). No further information on the production or activity of these antibiotics is available. *Actinomadura* species have not been extensively screened for bioactive compounds, but of the 86 species identified in the genus thus far, they do produce 345 different bioactive metabolites (Bérdy, 2005). Due to the lack of research into bioactive metabolite production of *Actinomadura* species, very few studies have investigated the effect of carbon and nitrogen sources on the stimulation of bioactive compound production. One study investigated the effects of calcium and nitrogen on the production of the glycopeptide antibiotic A40926 by *Actinomadura* species ATCC 39727. The production of the antibiotic was stimulated on ISP-2 (28 mg/L), Oatmeal-Yeast Extract (OMY; 33 mg/L) and Yeast Extract-Soluble starch (YS; 38 mg/L) media. These media contained yeast extract with glucose, oatmeal and soluble starch as carbon sources respectively. The nitrogen sources on defined

media were investigated, with glutamine and asparagine stimulating antibiotic production dramatically compared to ammonium nitrate. The researchers furthermore found that ammonium and calcium suppress the production of the antibiotic (Technikova-Dobrova *et al*., 2004). The study focussed on optimising the production of a specific antibiotic, specifically focused on the effects of nitrogen, carbon and calcium on the antibiotic production. Future studies on *Actinomadura* strain S173 should perform a similar optimisation process. *Actinomadura* strain S173 was able to degrade soluble starch and thus is able to utilise the carbon source in SCN media successfully. The effective use of soluble starch and the nitrogen sources in SCN media, it was able to produce the antimicrobials active against *B. cereus*, *K. pneumoniae* and *S. aureus* (Figure 3.8).

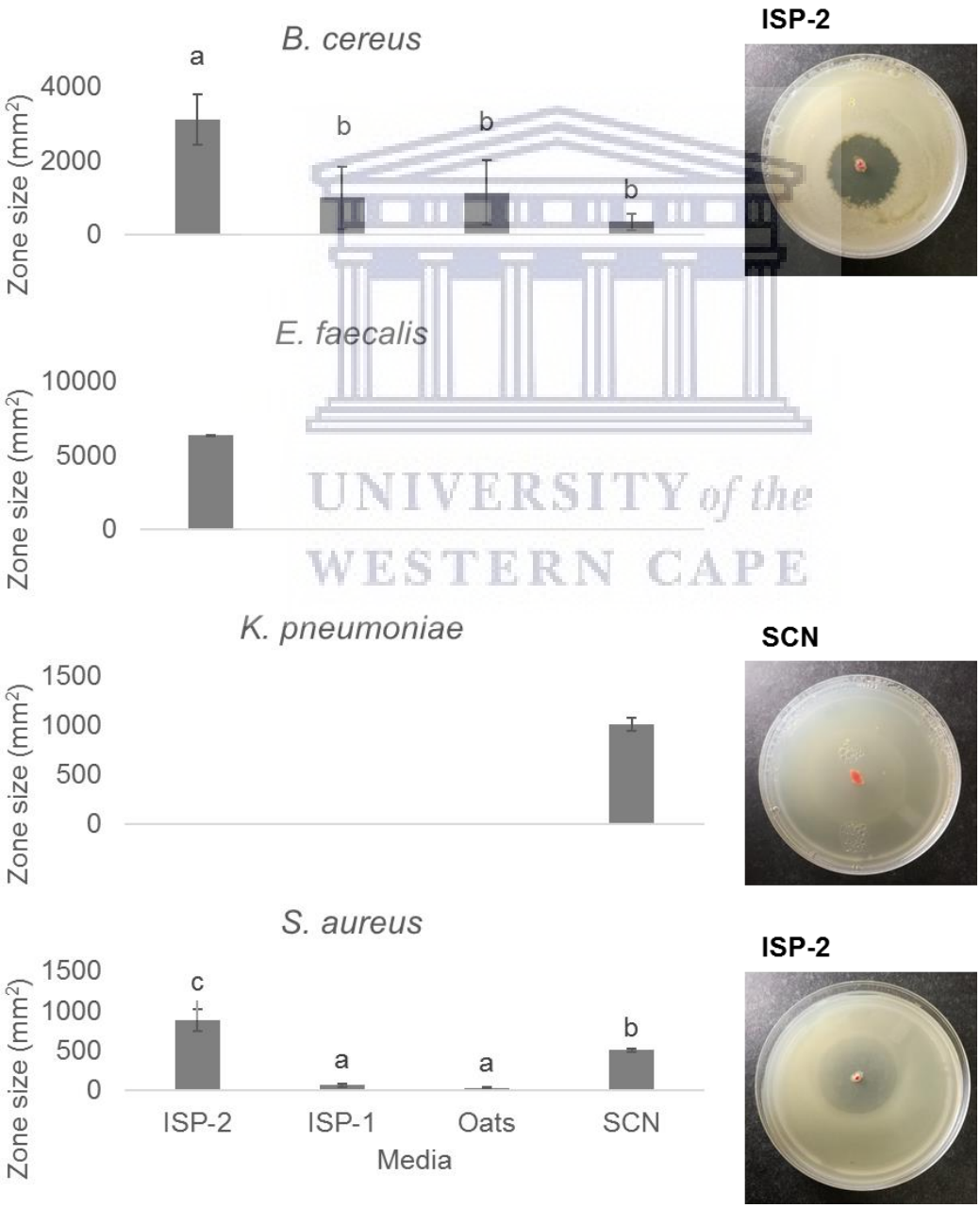


Figure 3.8: *The effect of media on the antibacterial activity of* Actinomadura *strain S173* via *the overlay method. Zone size indicated in mm^2. Data is means ± SD; n=3. Different letters indicate a statistically significant difference at the 95% confidence level.*

The majority of the isolates (80%) had high activity against Gram-positive bacteria, including *B. cereus*, *E. faecalis*, *M. aurum*, *M. smegmatis* and *S. aureus*. Four of the six isolates tested are part of the *Streptomyces* genus, which are known producers of antimicrobial compounds with streptomycetes producing more than 7500 bioactive compounds (Bérdy, 2005). In general, antimicrobial compounds are more likely to be active against Gram-positive bacteria, because these bacteria lack an outer membrane as compared to Gram-negative bacteria (Epand *et al*., 2016). The outer membrane of Gram-negative bacteria houses structural components that help protect the cell from antibiotics or any other environmental determents (Epand *et al*., 2016). The outer membrane of Gram-negative bacteria can also be toxic to its host. It also possesses porin channels that inhibit the entry of harmful chemicals such as antibiotics. These channels are also able to expel antibiotics out of the cell (Figure 3.9).

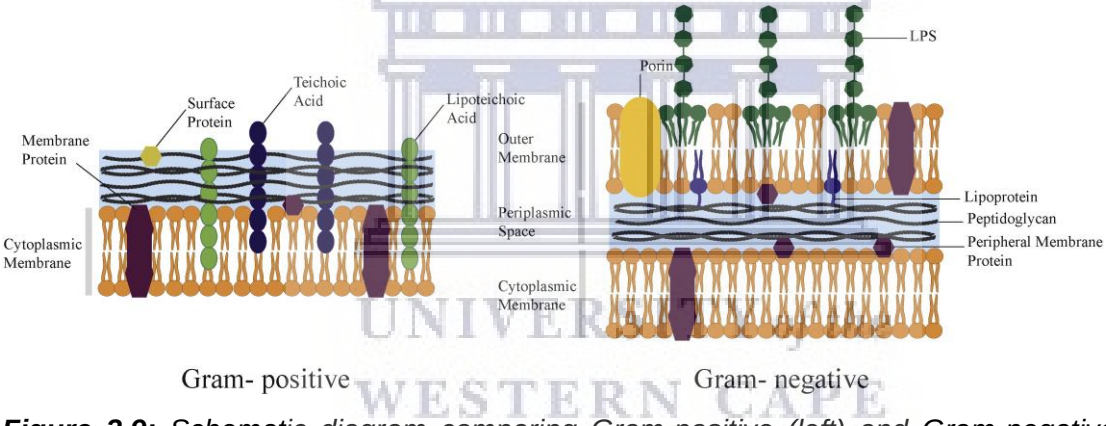


Figure 3.9: *Schematic diagram comparing Gram-positive (left) and Gram-negative (right) bacterial cell membranes. Image taken from Epand et al., 2016.*

A simplified OSMAC (one strain many compounds) approach was taken to stimulate the expression of compounds by altering the nutrient content. From this data, it is observed that media composition plays an important role in the activity of the isolates. The ISP-2 media stimulated antimicrobial compound production for most isolates except *Streptomyces* strain S149 which had no inhibitory activity on ISP-2 media. Good activity was also exhibited on ISP-1 and SCN, with Oats having the lowest effect on antimicrobial compound production. ISP-2 media is composed of yeast extract which provides the strains with B-complex vitamins, nitrogen, carbon and other nutrients. The malt extract serves as a source of carbon, nitrogen, protein and other nutrients, with the glucose serving as a carbon source. The strains are able to utilise these nutrients to stimulate and promote the production of secondary metabolites including antimicrobial compounds.

The production of many antimicrobial compounds is linked to sporulation, thus, stimulating sporulation of actinobacteria is important. Starch casein nitrate (SCN) media contains calcium carbonate that stimulates sporulation and serves as a calcium and carbon source (Salas *et al*., 1983). The media also contains soluble starch as a complex carbon source, with casein as a nitrogen source. Potassium nitrate serves as a nitrogen and potassium source, and magnesium sulphate is a macro nutrient and used as a co-factor for enzyme activity. Due to these factors, SCN media can be used to stimulate the production of compounds that require some of these nutrients. Yeast extract and tryptone in ISP-1 media provides the strains with B-complex vitamins, nitrogen, carbon, and amino acids. Oats media is used by the strains as a source of nitrogen, carbon, protein, electrolytes, minerals, and other nutrients. These media provide the strains with a complex mix of nutrients for the production of secondary metabolites.

As discussed previously, the nitrogen and carbon source play an important role in stimulating secondary metabolites, with certain metabolites only stimulated with specific carbon and nitrogen sources. Optimal stimulation of the secondary compounds can be obtained by varying the concentrations of nitrogen and carbon sources and/or using a mix of different sources. Furthermore, compounds such as calcium, can also influence the activity and stimulation of bioactive compound production. Further investigations to optimally stimulate the production of the antimicrobial compounds from this study will need to investigated. The use of these different media gives a guideline and starting point for further refinement. Additional factors such as growth temperature, aeration, pH and addition of trace elements will also be investigated.

3.3.3 Kirby-Bauer disc diffusion assay

Crude extractions of the antimicrobial compounds were performed using three solvents (chloroform, ethyl acetate and methanol) and tested for inhibition activity using the Kirby-Bauer disc diffusion method. Ampicillin was used as a positive control. It should be noted that the crude extracts would contain a mixture of compounds, some of which are bioactive and others not. The crude extract may also contain one or more bioactive compounds. Thus, observed activity, however small, indicates that a bioactive compound is present but provides no information about purity or number of bioactives. Isolation and further purification of the compounds in the extract may result in even higher observed activities. Furthermore, some compounds may not diffuse into the agar plates very well and thus zone size isn't always a reflection of the amount of compound being produced. Activities of crude extracts similar to that of the ampicillin control indicate that the compound(s) present in the extract is very effective and that further purification will almost always increase observed activity. The disc diffusion assay helps to establish which solvent extraction methods to use for further downstream purification and identification of the bioactive compounds of interest. It is suggested that future studies should be done using the minimum inhibitory concentration (MIC) assay to more accurately determine the inhibitory effect of the extracted compounds.

The solvent extracts of *Streptomyces* strain A3 had activity against *B. cereus* when grown in ISP-2 broth, and *S. aureus* when grown in ISP-1 broth. The activity against *B. cereus* was similar to that of the ampicillin control. This antimicrobial activity may be increased further through purification of the crude extract. Activity against *S. aureus* was significantly lower than the positive control. *Streptomyces tanashiensis* (strain Kala), closely related to *Streptomyces* strain A3, produces kalamycin and was extracted from the culture filtrate with methylene chloride. Kalamycin was found to be soluble in various solvents including aliphatic esters (e.g. ethyl acetate, amyl acetate, butyl acetate), lower alkenones (e.g. acetone, methyl ethyl ketone, isopropyl butyl ketone), halogenated hydrocarbons (e.g. chloroform, methylene chloride) and dimethyl sulfoxide. And can be extracted using these solvents as well. Lower solubility was observed in alkanols, e.g., methanol, ethanol, isopropanol, the butanols, and the like. It is relatively insoluble in water, cyclohexane and ether (Bergy *et al*., 1967). Interestingly, in this study, extraction with methanol from *Streptomyces* strain A3 mycelia yielded no antimicrobial activity when tested against *B. cereus,* but inhibitory activities were observed when the filtrate was extracted with chloroform or ethyl acetate (Figure 3.10a). A luteomycin-like antibiotic was extracted from *S. tanashiensis* AZ-C442 filtrate with ethyl acetate (Afifi *et al*., 2012). A similar antibiotic may have been extracted from *Streptomyces* strain A3 with activity against *B. cereus*

using ethyl acetate. Optimising the growth conditions and the extracting solvents may further increase the observed inhibition activity of the strain.

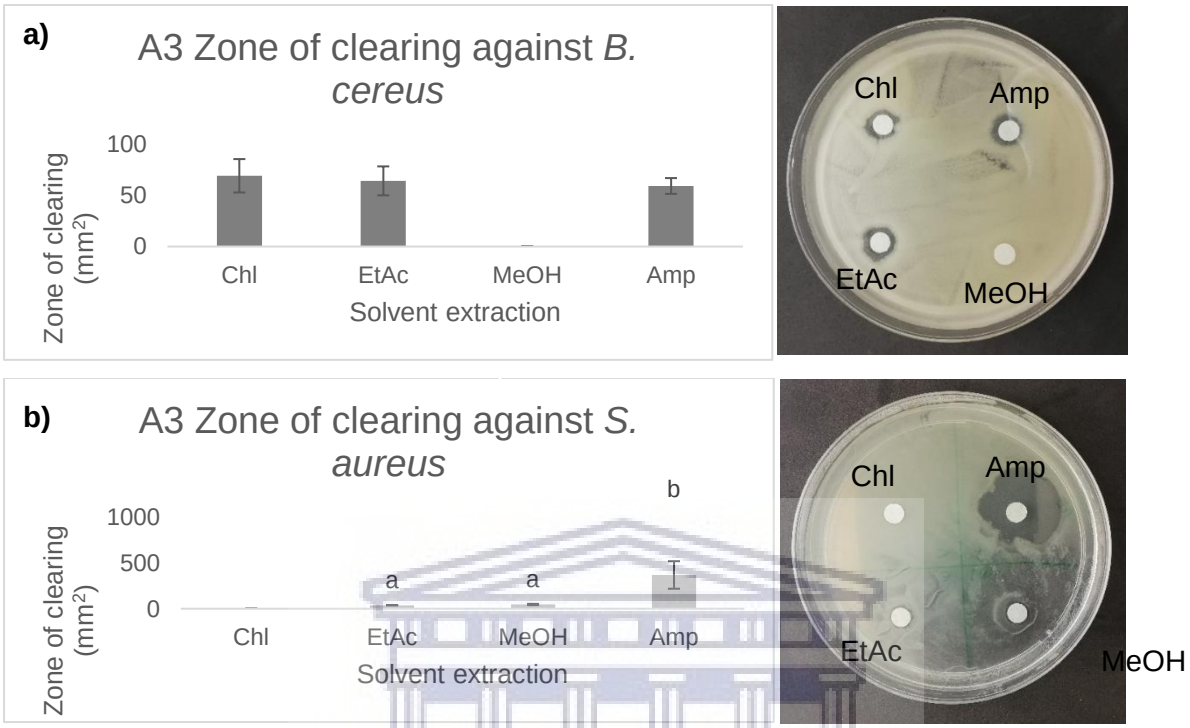


Figure 3.10: *The effect of different solvent extractions on the antimicrobial activity of* Streptomyces *strain A3 grown in a) ISP-2 media against* B. cereus *and b) ISP-1 media against* S. aureus*. Ampicillin (Amp) was used as a positive control. Zone size indicated in mm^2. Chl – chloroform; EtAc – Ethyl acetate; MeOH – methanol. Data is means ± SD; n=3. Different letters indicate a statistically significant difference at the 95% confidence level.*

The chloroform and ethyl acetate solvent extracts of *Amycolatopsis* strain A37 had antimicrobial activity against *S. aureus*. The isolate did not have activity against any of the other pathogenic bacteria as observed with the overlay experiments. When strain A37 was grown in liquid broth, the viscosity increased which in turn limited aeration and oxygen availability. The morphology of strain A37 may be related to the production of antibiotics, as in a similar case observed with *Amycolatopsis mediterranei*, where production of rifamycin B was significantly affected by branching, colour, formation and size of the colonies (El-Tayeb *et al*., 2004). The *A. mediterranei* strain also had high viscosity growth in liquid culture that negatively affected the production of rifamycin B. The limited oxygen availability, may have influenced *Amycolatopsis* strain A37's ability to produce the range of potent antimicrobial compounds observed on the overlay plates. Optimising the media components, extracting solvents, and other environmental factors such as temperature and rotation speed will need to be investigated for optimal stimulation of antimicrobial compounds by *Amycolatopsis* strain A37.

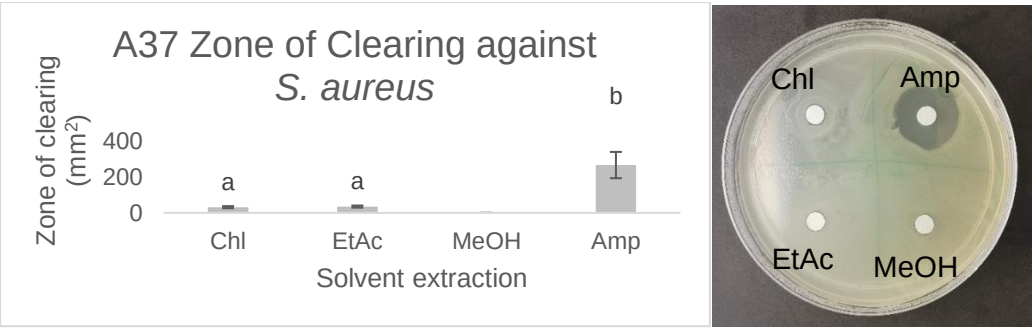


Figure 3.11: *The effect of different solvent extractions on the antimicrobial activity of* Amycolatopsis *strain A37 grown in ISP-1 media against* S. aureus*. Ampicillin (Amp) was used as a positive control. Zone size indicated in mm^2. Chl – chloroform; EtAc – Ethyl acetate; MeOH – methanol. Data is means ± SD; n=3. Different letters indicate a statistically significant difference at the 95% confidence level.*

When cultured in ISP-2, isolate A81 produced antimicrobial compounds that had antagonistic activity against *B. cereus* and *S. aureus*. Growth in ISP-1 also resulted in activity against *S. aureus*. Compounds produced in ISP-2 media had higher antimicrobial activity against *S. aureus* than when grown in ISP-1. The concentration of extract tested was similar and thus the differences may indicate the production of a different compound or lower quantities of the same compound with the production of other compounds, not necessarily related to antibiotics. Ethyl acetate ($p = 0.000231$) and methanol ($p = 0.000231$) extractions from ISP-2 had significantly higher antimicrobial activity against *B. cereus* than the positive control, whereas the chloroform ($p = 0.08484$) extraction had similar activity as the control (Figure 3.12). The inhibitory activities of these crude extracts against *B. cereus* are significant, due to the extracts not being pure. Further purifications coupled with delivery optimisation of the crude extracts may possibly lead to even higher inhibitory activities.

The extraction of the pinkish compound from ISP-2 media, is most likely griseorhodins. This antibiotic, also produced by its closest relative *Streptomyces californicus,* has both Gram-positive and -negative inhibitory activity. Optimal griseorhodin production was observed when a combination of soluble starch, yeast extract, peptone and sodium chloride was used. The griseorhodins were extracted from the mycelium with ethyl acetate. The extract was further purified and subjected to silica gel chromatography (Suetsuna and Osajima, 1989). In this study, the mycelium obtained from the culture broth were extracted using methanol and the culture broth was extracted with ethyl acetate and chloroform. Future studies can focus on improving extraction of the compound by using the same solvents previously used to extract griseorhodin-like compounds or to include a wider range of polar and non-polar solvents based on the compound's polarity. Extraction from the filtrated broth with ethyl acetate did result in higher antimicrobial activity against *B. cereus*. The extraction of the mycelium with ethyl acetate will also need to be performed to evaluate if higher inhibitory activity is observed.

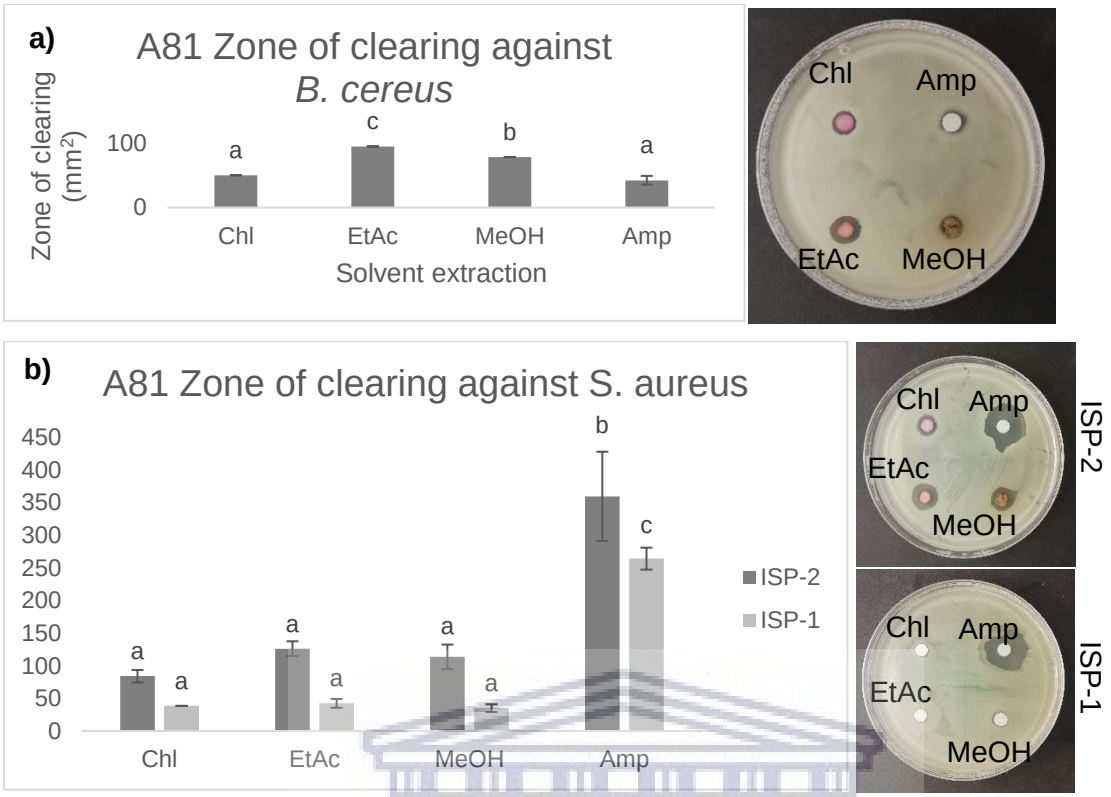


Figure 3.12: The effect of different solvent extractions on the antimicrobial activity of Streptomyces *strain A81 grown in a) ISP-2 media against* B. cereus *and b) ISP-2 and ISP-1 media against* S. aureus*. Ampicillin (Amp) was used as a positive control. Zone size indicated in mm^2. Chl – chloroform; EtAc – Ethyl acetate; MeOH – methanol. Data is means ± SD; n=3. Different letters indicate a statistically significant difference at the 95% confidence level.*

The solvent extractions from *Streptomyces* strain A159 had activities against *B. cereus*, *E. faecalis* and *S. aureus* when grown in ISP-2 and ISP-1 broth (Figure 3.13). Activity against *B. cereus* was similar to that of the ampicillin control, with the highest zone of clearing being 79 mm^2 from the methanol extract of cells grown in ISP-2. This is result is exceptional, since the extract tested is not pure and optimized for maximum uptake as with the positive ampicillin control. Through further purification and optimisation of the antimicrobial compound(s) it is possible that these compounds can be more antagonistic towards *B. cereus* than ampicillin. The activity of the extracts against *E. faecalis* and *S. aureus* was less than the positive control, with the extracts from ISP-2 being more antagonistic than the extracts from ISP-1 media. The extraction method from the *Streptomyces* grown in ISP-2 had no significant effect on the antagonistic activity of the compound. Thus, indicating that the compound is produced in the cell (methanol extraction) and excreted into the surrounding broth as well. The extraction of the pinkish compound from ISP-2 media, is also most likely griseorhodins. Optimising the media composition to stimulate the production of this compound should greatly increase the activity observed. Solvent extraction from the mycelium using ethyl acetate may furthermore

result in an increase in inhibition activity. Purification of the extracts with optimisation may potentially lead to an even greater observed inhibition activity.

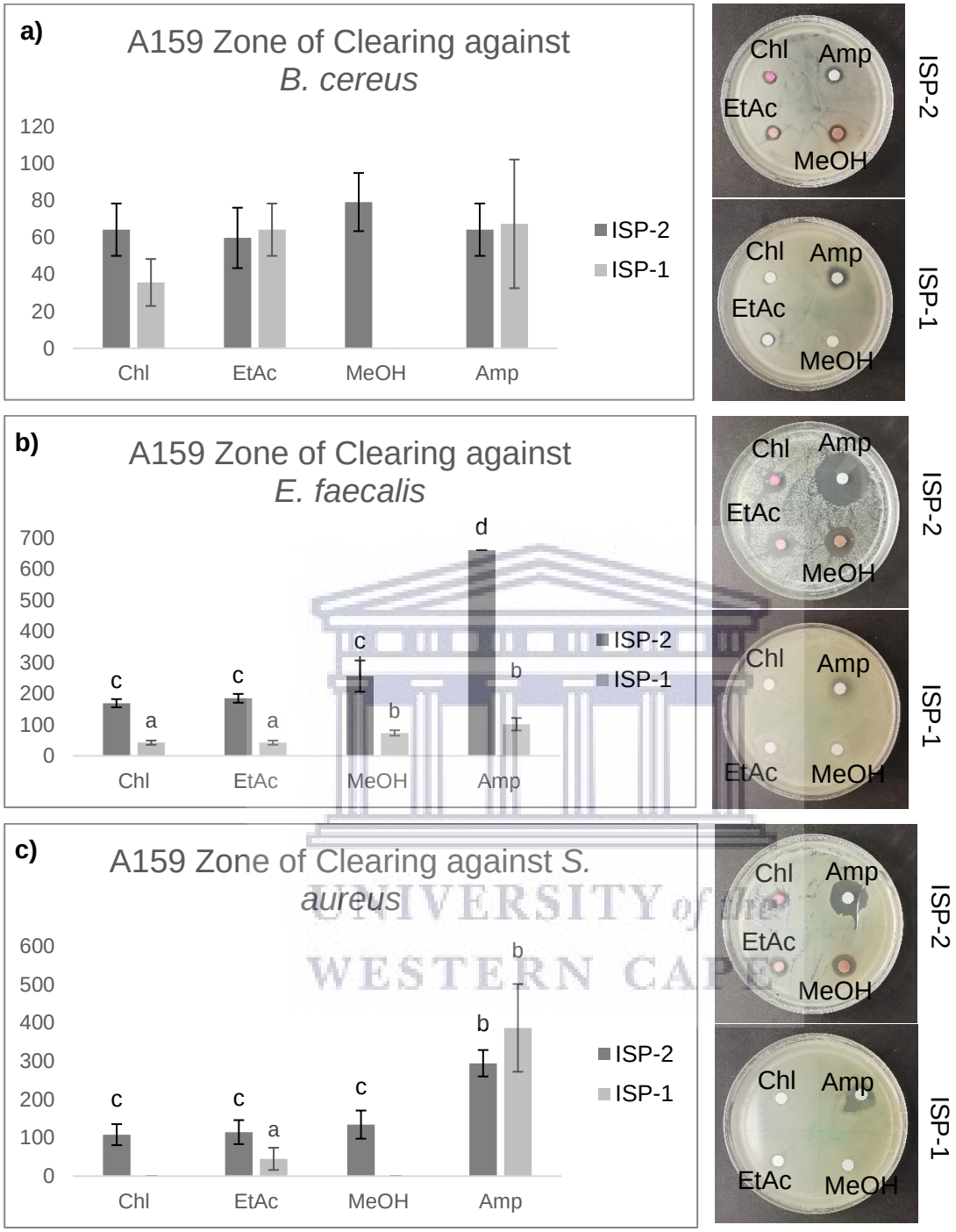


Figure 3.13: *The effect of different solvent extractions on the antimicrobial activity of* Streptomyces *strain A159 grown in ISP-2 and ISP-1 media against a)* B. cereus, *c) E. faecalis and c)* S. aureus*. Ampicillin (Amp) was used as a positive control. Zone size indicated in* mm^2*. Chl – chloroform; EtAc – Ethyl acetate; MeOH – methanol. Data is means ± SD; n=3. Different letters indicate a statistically significant difference at the 95% confidence level.*

Chloroform and ethyl acetate solvent extractions of *Streptomyces* strain S149 were active against *E. faecalis*, resulting in zones of clearing of 50 mm^2 (chloroform) and 38 mm^2 (ethyl acetate). No activity was observed from the methanol extraction from the cells. This indicates that the compound is either insoluble in methanol, therefore it is likely the compound is non polar, or that the compound is almost completely excreted into the broth with none of the compound left in the cells (Figure 3.14).

Streptomyces canus, closely related to *Streptomyces* strain S149, produces kanamycin and telomycin. Kanamycin was first isolated from *Streptomyces kanamyceticus*, using a cation exchange resin process and crystalized as a monosulfate (Umezawa, 1958). Kanamycin is soluble in water and insoluble in *n*-butanol, ethyl acetate, butyl acetate, ether, chloroform and benzene (Umezawa *et al*., 1960). Thus, it is difficult to extract using normal solvent extraction methods. Similarly, telomycin is a Gram-positive antibiotic, and extracted from the culture filtrate with XAD resin followed by a methanol wash (Fu *et al*., 2015). If *Streptomyces* strain S149 also produces kanamycin or telomycin, extraction using the solvents in this study, i.e. chloroform, ethyl acetate and methanol, would not work effectively. Extraction of kanamycin using methanol should have been possible since kanamycin is a polar compound. The active compound extracted from strain S149 was insoluble in methanol. It is thus highly unlikely that kanamycin or telomycin is produced and that another antimicrobial compound that is soluble in chloroform and ethyl acetate was extracted (Figure 3.14). Future work will need to be conducted to determine the types of antimicrobial compounds produced by this strain. Investigation into the whole genome sequence of this strain (Chapter 4) will give additional insights into possible antimicrobial targets for future investigation.

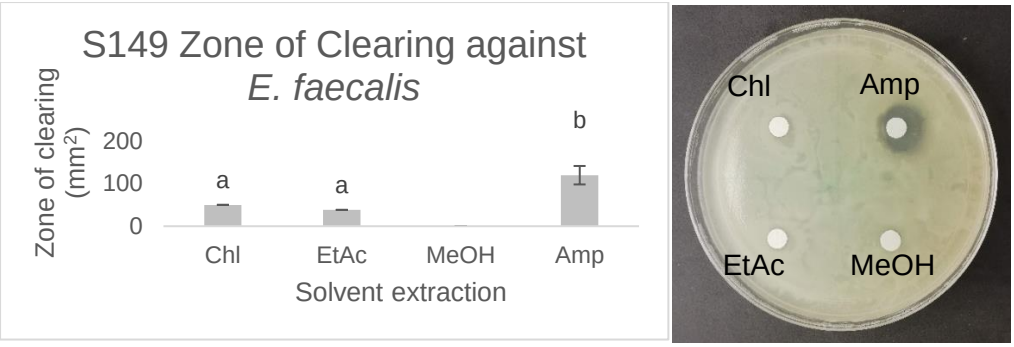


Figure 3.14: *The effect of different solvent extractions on the antimicrobial activity of* Streptomyces *strain S149 grown in ISP-1 media against E. faecalis. Ampicillin (Amp) was used as a positive control. Zone size indicated in mm^2. Chl – chloroform; EtAc – Ethyl acetate; MeOH – methanol. Data is means ± SD; n=3. Different letters indicate a statistically significant difference at the 95% confidence level.*

Methanol extracts from *Actinomadura* strains S173 fermented in ISP-2 media had antagonistic activity against *S. aureus* (310 mm^2) with similar activity (p = 0.957023) as the ampicillin control (340 mm^2, Figure 3.15a). The extraction of the compound was only achieved from the cells, and no activity was observed when solvent extraction was performed on the broth, indicating that the active compound is either not excreted into the surrounding environment or that the compound is highly polar and could not be extracted with ethyl acetate or chloroform. Solvent extractions from ISP-1 grown broth and cells had activity against *K. pneumoniae* similar to that of the positive control. The average zone of clearance was 35 mm^2 (methanol) and 52 mm^2 (chloroform and ethyl acetate). The extraction of the compound from the media indicates that the active compound is secreted into the media (Figure 3.15b).

As mentioned previously, there is little research into the bioactive metabolite production of *Actinomadura* species. One study isolated novel anticancer antibiotics, chandrananimycins A – C, from *Actinomadura* sp. M048. The researchers used ethyl acetate followed by methanol to extract the compounds from both the mycelium and the culture filtrate. The crude extracts were further purified via silica gel column chromatography (Maskey *et al*., 2003). A different study extracted the glycopeptide antibiotic A40926 from *Actinomadura* species ATCC 39727 by adjusting the pH of the fermented culture to more than pH 10.0. This caused the separation of the compound from the culture (Technikova-Dobrova *et al*., 2004). Extracting compounds from *Actinomadura* strain S173 will depend on the properties of the compound(s) produced. A range of techniques can be used as in the examples above to determine the optimal method for the extraction of the bioactive metabolites produced by the strain.

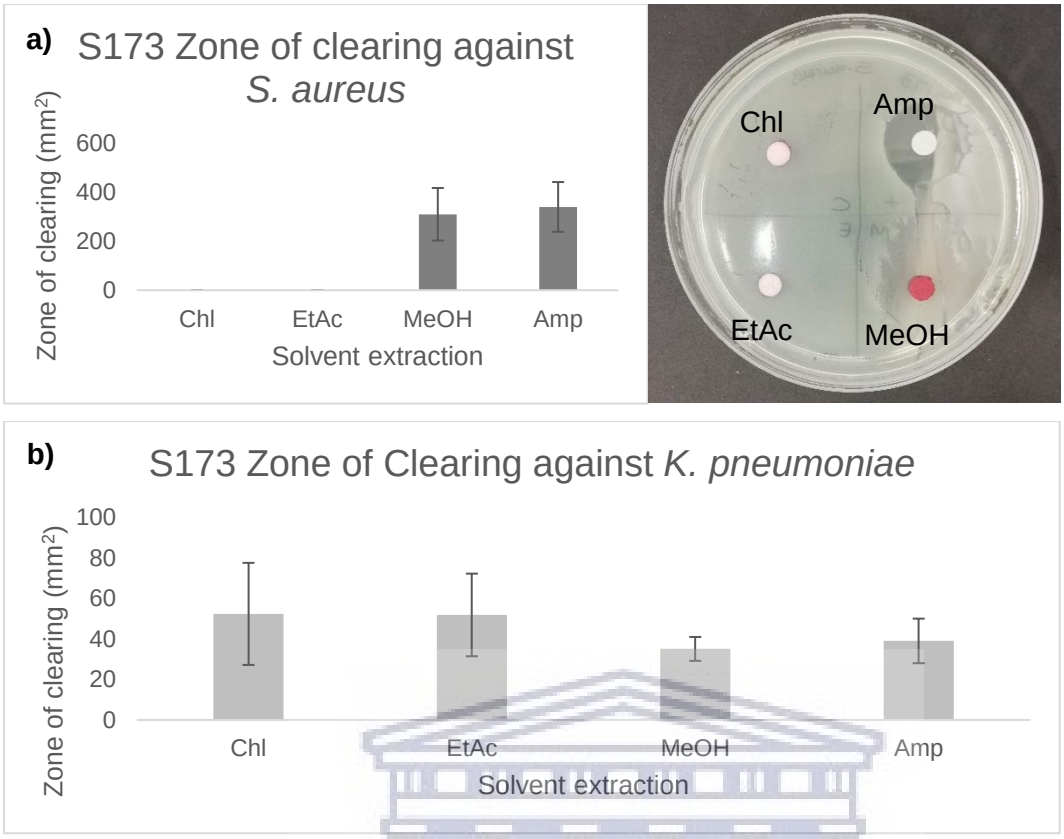


Figure 3.15: *The effect of different solvent extractions on the antimicrobial activity of* Actinomadura *strain S173 grown in a) ISP-2 broth against* S. aureus *and b) ISP-1 media against K. pneumoniae. Ampicillin (Amp) was used as a positive control. Zone size indicated in mm^2. Chl – chloroform; EtAc – Ethyl acetate; MeOH – methanol. Data is means ± SD; n=3. Different letters indicate a statistically significant difference at the 95% confidence level.*

In this study, a difference in the inhibitory activities of the isolates on solid media (overlay) versus liquid cultures (disc diffusion assays) was observed. This is possibly due to the growth in liquid culture, and constant shaking that breaks up the cells and does not allow the bacteria to sporulate. Sporulation of the bacteria can be linked to the production of antibiotics, and it is hypothesised that the production of antibiotics in liquid culture is limited due to the inability of the bacteria to sporulate. Another reason for the differences observed in activity is that the solvents used for the extraction of the compounds was not optimal for complete extraction of the compounds. Highly polar or non-polar compounds would not have been extracted successfully. Or that the compounds produced have unique structures and can only be extracted through cation resin exchange as is the case with kanamycin extraction or through adjusting the pH of the fermented culture. Additionally, the optimisation of the growth media and other environmental factors will further help to increase the number of compounds extracted. The extraction of the metabolites from each isolate needs to be optimised and different techniques and solvent systems will need to be evaluated to extract the compounds produced. The successful extraction of the compounds identified in this study, will need to be further subjected to purification using gel column chromatography techniques.

3.3.4 Bioautography

Bioautography was performed with solvent extracts of the isolates against the pathogenic test strains that showed positive activity from the Kirby-Bauer disk diffusion assay. The bioautography was performed to separate the compounds via TLC and determine the number and chemical properties of the antimicrobial compounds. In some cases, more than one compound had activity against the test pathogens. Bioautography helps to localize the antimicrobial activity of the extracts and helps to quickly search for new antimicrobial compounds. It is important to note that the resolution of the bioautography techniques may not be sufficient in all cases to attribute the activity of a single spot to a single compound. Thus, because crude extracts are being tested, the activity observed in a single spot may contain multiple compounds with the same R_f values.

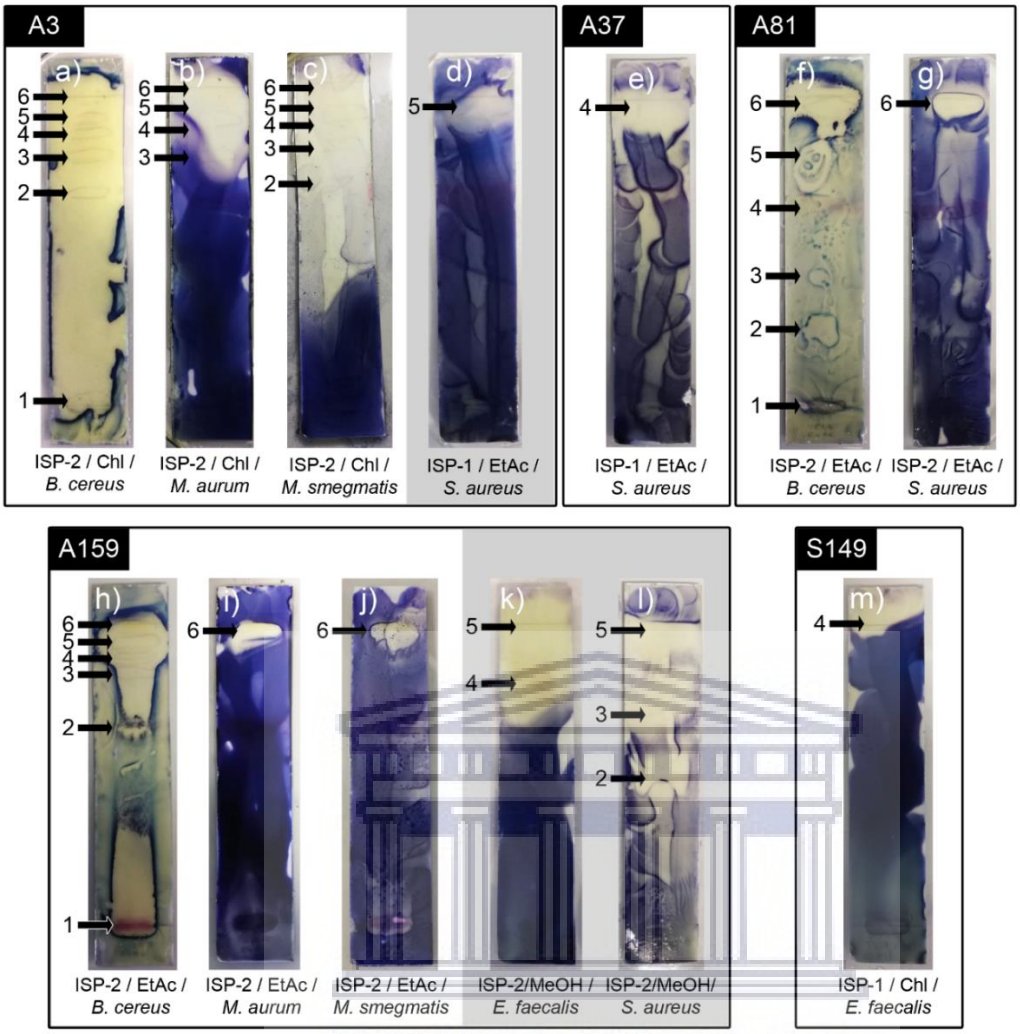


Figure 3.16: *Bioautography of isolates A3, A37, A81, A159, S149 and S173 against a range of pathogenic bacteria. A3 has activity against (a)* B. cereus*, (b)* M. aurum*, (c)* M. smegmatis *and (d)* S. aureus*. Isolate A37 had activity against (e)* S. aureus*. Strain A81 had activity against (f)* B. cereus *and (g)* S. aureus*. Isolate A159 had activity against (h)* B. cereus*, (i)* M. aurum*, (j)* M. smegmatis*, (k)* E. faecalis *and (l)* S. aureus*. Strain S149 had activity against (m)* E. faecalis*. Active compounds are indicated with numbers corresponding to the compounds in Table 3.2.*

Table 3.2: *R_f values of compounds separated on TLC plates that were subjected to bioautography.*

Isolate – Media – Solvent extraction	Inhibitory activity against pathogenic test strain	Compound (from bottom to top)	R_f
A3 - ISP-2 - Chl	*B. cereus*	Compound 1	0.025
	B. cereus, M. smegmatis	Compound 2	0.688
	B. cereus, M. aurum, M. smegmatis	Compound 3	0.838
	B. cereus, M. aurum, M. smegmatis	Compound 4	0.925
	B. cereus, M. aurum, M. smegmatis	Compound 5	0.963
	B. cereus, M. aurum, M. smegmatis	Compound 6	1.000
A3 - ISP-1 - Chl	-	Compound 1	0.025
	-	Compound 2	0.688
	-	Compound 3	0.838
	-	Compound 4	0.925
	S. aureus	Compound 5	0.963
A37 - ISP-1 - EtAc	-	Compound 1	0.013
	-	Compound 2	0.688
	-	Compound 3	0.838
	S. aureus	Compound 4	0.913
A81 - ISP-2 - EtAc	*B. cereus*	Compound 1	0.012
	B. cereus	Compound 2	0.610
	B. cereus	Compound 3	0.817
	B. cereus	Compound 4	0.854
	B. cereus	Compound 5	0.939
	B. cereus, S. aureus	Compound 6	0.988
A159 - ISP-2 - EtAc	*B. cereus*	Compound 1	0.012
	B. cereus	Compound 2	0.642
	B. cereus	Compound 3	0.827
	B. cereus	Compound 4	0.864
	B. cereus	Compound 5	0.926
	B. cereus, M. aurum, M. smegmatis	Compound 6	0.988
A159 - ISP-2 - MeOH	-	Compound 1	0.013
	S. aureus	Compound 2	0.481
	S. aureus	Compound 3	0.658
	E. faecalis, S. aureus	Compound 4	0.722
	E. faecalis	Compound 5	0.797
S149 - ISP-1 - Chl	-	Compound 1	0.025
	-	Compound 2	0.663
	-	Compound 3	0.975
	E. faecalis	Compound 4	0.950

Chloroform extractions from isolate A3 grown in ISP-2 media had multiple compounds with activity against *B. cereus* (compounds 1 – 6; Table 3.2), *M. aurum* (compounds 3 – 6; Table 3.2) and *M. smegmatis* (compounds 2 – 6; Table 3.2) (Figure 3.16 a, b and c). Multiple compounds with the same activity may represent isomers or derivatives of the same compound with different functional groups. ISP-1 chloroform extracts of isolate A3 had one

compound (compound 5, R_f = 0.963; Table 3.2) with activity against *S. aureus* (Figure 3.16 d). While the chloroform extractions from isolate A3 grown in both ISP-2 and ISP-1 produced compounds (compounds 1 – 5; Table 3.2) with the same Rf values, only compound 5 from ISP-1 extraction had activity. This may indicate that either the same compounds were extracted from both media but in lower quantities (thus lower activity seen) or that different compounds with the same R_f values were extracted. Using a different solvent system will help to resolve discrepancies between compounds with the same R_f values.

Ethyl acetate extraction from the ISP-1 broth of isolate A37, compound 4 (R_f = 0.913; Table 3.2) had activity against *S. aureus* (Figure 3.16 e). Isolate A81 grown in ISP-2 broth and extracted with ethyl acetate had multiple compounds that had inhibitory activity against *B. cereus* (compounds 1 – 6; Table 3.2) and one compound (compound 6, R_f = 0.988; Table 3.2) with activity against *S. aureus* (Figure 3.16 A81 a and b). Extraction with ethyl acetate from isolate A159 grown in ISP-2 broth had activity against *B. cereus*, *M. aurum* and *M. smegmatis* (Figure 3.16 h, i and j). Many of the separated compounds had activity against *B. cereus* (compounds 1 – 6, Table 3.2) with compound 6 (R_f = 0.988) having activity against *M. aurum* and *M. smegmatis*. Methanol extraction from ISP-2 broth of isolate A159 revealed multiple compounds with antagonistic activity against *E. faecalis* (compounds 4 and 5; Table 3.2; Figure 3.16 k) and *S. aureus* (compounds 2 – 4; Table 3.2; Figure 3.16 l). Chloroform extraction of isolate S149 grown in ISP-1 medium had compound 4 (R_f = 0.95; Table 3.2) with inhibitory activity against *E. faecalis* (Figure 3.16 m). Unfortunately, none of the solvent extractions from isolate S173 which displayed activity against *S. aureus* or *K. pneumoniae* tested during the Kirby-Bauer disk diffusion assay could be detected via TLC.

For the further evaluation of the identified compounds stated they will need to be purified and the activity of a single compound confirmed. One possible workflow would be to scale up the production, and perform a bulk extraction. These crude extracts can be separated via TLC and the band scraped off the TLC plate and dissolved in methanol for further analysis. Analysis would include well diffusion or disc diffusion assays of the single band. This confirms that the activity is due to the compound(s) from that particular spot. The compound(s) (if activity is still observed) can then be purified and identified using LC-MS or HPLC techniques.

3.3.5 Siderophore

Siderophore activity was measured using the CAS media assay. Iron chelators were produced by the six isolates tested. Activity was very high in isolate A37, with moderate activity observed

in the other isolates. Isolates A3 (711 mm^2, Figure 3.18 a), A81 (419 mm^2), A159 (668 mm^2), S149 (1206 mm^2) and S173 (469 mm^2, Figure 3.18 c) had moderate siderophore activity (Figure 3.17). Isolate A37 had a very high siderophore activity (5736 mm^2) and turned the blue CAS plate entirely orange (Figure 3.17 and Figure 3.18b).

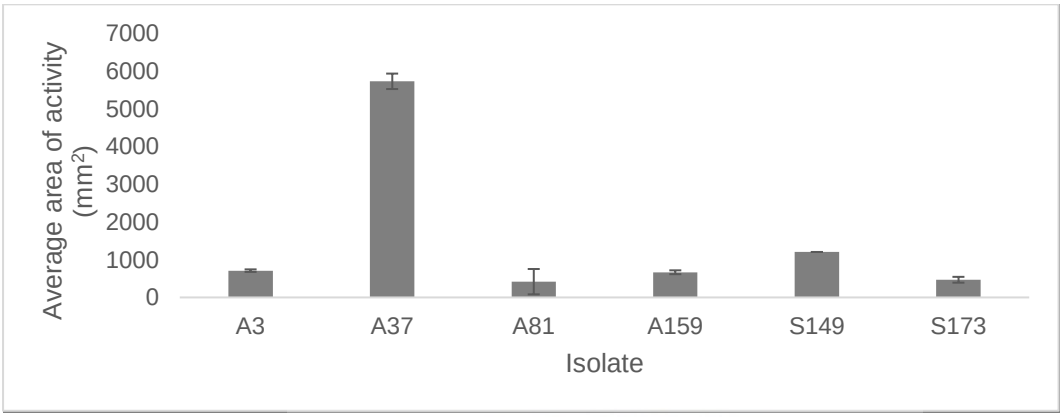


Figure 3.17: *Siderophore activity indicated by an orange zone (mm^2) of the isolates. Zone size indicated in mm^2. Data is means ± SD; n=3.*

Siderophores are produced in environments with limited available iron. These enzymes also have the ability to bind other metals in addition to iron and have become an interest for environmental researchers as they can function as biocontrols, bioremediation and chelation agents (Ahmed and Holmström, 2014). These small molecules have the potential to play an important role in applied environmental studies as well as in drug therapy as discussed previously (section 3.1). The CAS assay screen for all types of siderophores including catecholate, hydroxamate, carboxylate and mixed types. Investigation into the genomes (Chapter 4) and other assays need to be conducted to classify the type of siderophore being produced by the actinobacterial isolates. Previous studies have revealed that siderophore activity is a common feature of environmental isolates. Actinobacteria from Western Australian soil samples were tested for their ability to produce siderophores. Of the 112 actinobacteria isolated, 92 had activity when tested using the CAS assay. Thus, 82% of the actinobacteria were able to produce siderophores (Lee *et al*., 2012).

The iron chelating compound, ferroverdin A, was found in *Streptomyces lunaelactis* (Maciejewska *et al*., 2015), a closely related species (97.1% 16S rRNA similarity) to *Streptomyces* strain A3. The ferroverdin A was extracted as a green pigment. *Streptomyces* strain A3 does not produce a green pigment which may indicate that either very low amounts are produced or a different siderophore is produced.

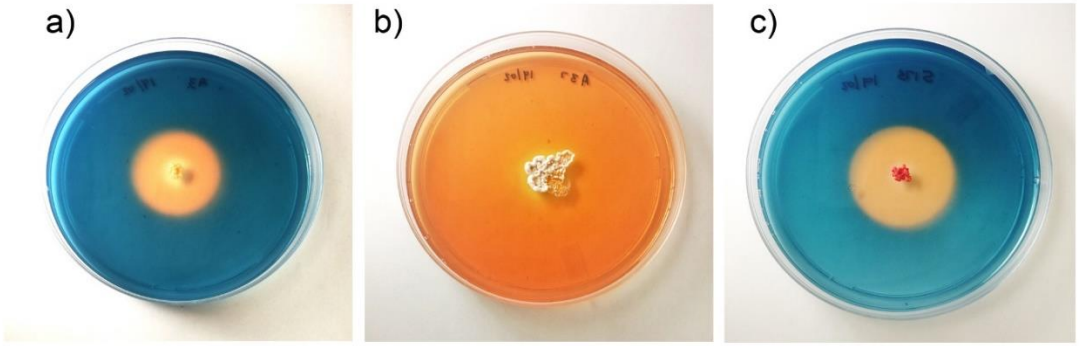


Figure 3.18: *Orange zone indicating siderophore activity of isolate A3 (a), A37 (b) and S173 (c) on blue CAS media plates after 14 days of growth.*

Exploring the activity of siderophores is important to establish their bioactive potential and to further evaluate their interaction with their host plants. Since these bacteria were isolated in close isolation to plants (roots, leaves or soil), it can be speculated that these bacteria also help to make iron available to their host plants. Siderophores have the potential to enhance plant growth. One such study evaluated a siderophore-producing streptomycete endophyte in rice plants. The siderophore-deficient (inactivation of *desD*) mutant was tested as well as the wild-type mutant for its plant growth promoting properties. It was found that the wild-type strain significantly increased root and shoot biomass and length (Rungin *et al*., 2012). Thus, indicating the importance of siderophore production in plant health and development.

3.3.6 Antioxidant activity

The antioxidant activity of the extracts was tested because actinobacteria have previously been shown to produce antioxidants (Bérdy, 2005; Manivasagan *et al*., 2013). Separation of the compounds was observed under UV light. The L-ascorbic acid is seen as a yellow spot on the TLC plate indicating quenching of the oxidative compound DPPH (Figure 3.19). However, no antioxidant activity was observed for the tested isolates. Antioxidant activity from *Streptomyces* strain Eri12, isolated from turmeric roots, was found only when the strain was grown on solid agar medium and not broth (Zhong *et al*., 2011). This may be an explanation as to why the extracts from this study did not show activity. The extraction of the spores of the isolates are needed to confirm whether or not the antioxidant compounds are produced on solid agar media. Due to the sensitivity of antioxidants to the environment, the possibility exists that the antioxidants in the extracts were quenched before testing. Thus, the extracts for testing need to be as fresh as possible. Future studies may need to extract the antioxidants from the spores the establish whether or not the isolates have antioxidant activities.

Figure 3.19: *Ethyl acetate extracts separated via TLC and a) viewed under 254 nm UV light and b) stained with DPPH solution to view antioxidant activity. L-Ascorbic acid was used as positive control.*

3.4 Conclusion

Actinobacteria have the ability to produce a large number of pharmaceutically important compounds. This study mainly focussed on one part of pharmaceutical products, antimicrobials. Actinobacterial metabolomes are big and thus even if a bacterium does not have antimicrobial activity, it can still have anticancer or other medicinal activity that was not screened for in this study. Thus, future screening of the metabolic potential of all isolates from this study will need to be conducted in case any significant bioactivities were missed. Insights from the whole genome sequences of the isolates will also help to give insights into what metabolites should be screened for (discussed further in chapter 4).

Screening the isolates for activity using the cross-streak method is a time efficient and low-cost method to screen large numbers of actinobacterial isolates against a variety of test pathogens. This method greatly helped to narrow down isolates that had antimicrobial activity for further screening. From experience it is suggested to test the antimicrobial activity against a range of pathogenic test strains. In this study the cross-streak assay used four pathogens, *B. cereus, E. coli, E. faecalis*, and *S. aureus*. More than 70% of the isolates tested had activity against one or more of these pathogens. This greatly helped to focus future assays, like the overlay assay, on only a few of the isolates and narrowing the more than 400 isolates down to only a couple of isolates. The activity of the isolates using the cross-streak method was similar to that of the overlay results. In some cases, activity was lower when the overlay method was used, but this can be due to either the age of the culture or that one of the pathogens on the cross-streak plate stimulates the production of an antibacterial compound that is active against the bacteria. For example, isolate S149 had activity against *B. cereus* during cross-streak testing but had no activity when tested using the overlay method. Future studies should include co-culturing of actinobacteria with other species of bacteria or fungi that can result in the production of new bioactive compounds. A marine *Streptomyces* strain JB5 produced a new piperazic acid-bearing cyclic peptide (Dentigerumycin E) when it was co-cultured with *Bacillus* strain GN1 (Shin *et al*., 2018). Likewise, *Streptomyces rochei* MB037 was co-cultured with the fungus *Rhinocladiella similis* 35 to stimulate the production of five metabolites, including four borrelidins (Yu *et al*., 2019). Future studies should include a variety of Gram-positive and Gram-negative bacteria when testing using the cross-streak method.

After the isolates were narrowed down for their antimicrobial potential, the activity was quantified using the overlay method. Four different types of media (ISP-2, ISP-1, Oats and SCN) were chosen to screen for activity and to evaluate the effect of the media to stimulate antimicrobial compound production. The type of media had a significant effect on the

antibacterial activity, and either increased or decreased the inhibitory activity towards the test pathogen. For example, the activity of *Streptomyces* strain A159 grown on ISP-1 or SCN media significantly increased its antagonistic activity against *M. aurum*, whereas Oats did not stimulate any activity. No significant effect of the growth media was also observed when this streptomycete was tested against *S. aureus* (Figure 3.6). Thus, utilising a variety of media can help to increase antimicrobial activity of actinobacterial isolates, due to the different compositions of the media. The actinobacteria are able to utilise these compounds to stimulate the production of secondary metabolites, some of which include antibiotic compounds. Modifying the types of media and the components in the media can help to upregulate the production of antibiotic compounds and should be considered in future studies. In this study, it was found that utilising different media can help to upregulate antimicrobial activities against pathogens. It is also important to weigh up the positives and negatives of the cross-streak vs overlay method. The cross-streak method is faster but might not detect activity, whereas the overlay method that takes longer but is able to detect more quantifiable activity.

To identify the compounds that have the antimicrobial activity, the extracts were separated on TLC plates and subsequently tested for antimicrobial activity against the test pathogen using bioautography. This technique helped to identify one or more compounds from the different actinobacterial isolates that had activity against the pathogenic test strains. In some cases, the same compound had antimicrobial activity against different pathogens. For example, compound 6 from *Streptomyces* strain A3 had activity against *B. cereus*, *M. aurum* and *M. smegmatis* (Figure 3.16). Using this technique helped to narrow down some interesting compounds that can be further purified and identified in future work using high-performance liquid-chromatography (HPLC) techniques.

General siderophore activity of the actinobacterial isolates was evaluated using the CAS assay. Siderophore activity may indicate the potential of the isolate to produce siderophore-antibiotic complexes (sideromycins), biocontrol activity, bioremediation activity and stimulate plant growth and development. Isolates A3, A37, A81, A159, S149 and S173 all had siderophore activity when using the CAS assay. Isolate A37, had the most notable siderophore activity and should be investigated in future studies. Siderophores have a wide range of applications, and actinobacteria are well known producers of these non-ribosomal peptides. Evaluating the presence of these compounds can prove valuable for future studies that identify the type of siderophore and its application in drug therapy and environmental studies.

Studying the bioactivity of actinobacteria is vast, since these bacteria species possess the potential to produce a large array of secondary metabolites that can have applications in

biotechnology, medicine, agriculture, textile industries, chemistry and many more. Thus, one organism has enormous metabolic potential. This study only focussed on finding the antimicrobial potential of the actinobacterial isolates and touched on siderophore and antioxidant activity. Future studies will need to dive deeper into the antimicrobial potential, but also further investigate the other pharmaceutical and agricultural potential of the isolates. This study is one of only a handful of studies that investigates the bioactive actinobacteria associated with South African medicinal plants.

This study narrowed down more than 400 actinobacterial isolates to only a few isolates using plate screening techniques. Most of the isolates showed promise in producing antimicrobial compounds, with some showing stronger antimicrobial activities than others. From these antimicrobial producing isolates, only three were selected for evaluating their genetic potential through the use of whole genome sequencing (discussed in chapter 4).

Chapter 4: Genetic Potential of Actinobacterial Isolates

4.1 Introduction

Since the early 1940s actinobacteria, especially *Streptomyces* species, have been well-studied due to their ability to produce potent secondary metabolites including antibiotics (Schatz *et al.,* 1944). The potential of actinobacteria to produce secondary metabolites was fully realised after the first whole genome sequence of the model streptomycete, *Streptomyces coelicolor* A3(2) in 2002 (Bentley *et al.,* 2002). Sequencing of the *S. coelicolor* genome revealed an unexpected potential of the bacteria to produce a range of previously unidentified and cryptic natural products that were not detected under laboratory conditions. The genome revealed that more than 20 biosynthetic gene clusters (BGCs) were present that have the potential to produce a range of secondary metabolites (Bentley *et al.,* 2002). One cryptic polyketide antibiotic from this species (coelimycin) was found to only be produced under certain conditions. Sequencing of other actinobacteria revealed that these organisms possess a much larger number of BGCs than was previously thought. This led to the sequencing and analysis of hundreds of actinobacterial genomes and their potential to produce a range of bioactive metabolites was revealed. Advances in next-generation sequencing (NGS) technologies have also resulted in the reduction of cost of whole genome sequencing, as well as a considerable increase in the quality of the data. Bioinformatic tools enable automated scanning and annotation of gene clusters, and include tools such as antiSMASH, CLUSEAN (Weber *et al.,* 2009), and PRISM. These tools enable continuous discovery and study of BGCs. Tools for the identification of specific classes of BGCs such as non-ribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) have also been developed and include NRPSPredictor and SEARCHPKS, respectively.

Sequencing of actinobacterial genomes are notoriously more difficult than other bacterial species. This is due to their high mol% G+C content that poses difficulties for both the sequencing technologies and the computing algorithms. Furthermore, the linear chromosomes and plasmids in many actinobacteria, like streptomycetes, have long terminal inverted repeats that can reach over one megabase pairs (Ohnishi *et al.,* 2008), making it very difficult to resolve using current sequencing technologies. Most of the natural products produced by actinobacteria belong to the polyketides and non-ribosomal peptides. These compounds are synthesised by large enzymes, PKSs and NRPSs, which consist of highly-

conserved modular enzymatic architecture. At nucleotide sequence level these enzymes have highly similar tandem repeats that frequently span over 700 base pairs. These repeats are in many cases longer than the read-length of NGS technologies, making it very difficult to correctly assemble these BGCs. The longer and more accurate the reads are, the more reliable the assembly can be computed. Thus, sequencing technologies that can offer long read length is vital for actinobacterial genome sequencing. The Illumina sequencing technologies offer high-accuracy read lengths of around 2 x 300 nt with the MiSeq machine, and can offer a maximum of 500 nt of contiguous reliable sequences (https://www.illumina.com/systems/sequencing-platforms/miseq.html, accessed on 9 September 2019). Unfortunately, this is still insufficient to resolve the highly repetitive PKS and NRPS genes. Newer technologies, like third-generation Single Molecule, Real-Time (SMRT) sequencing from Pacific Biosciences (PacBio) gives long, but less accurate, nucleotide-reads of *Streptomyces* DNA (Gomez-Escribano *et al.,* 2015; Castro *et al.,* 2018). If possible, combining these two technologies will give a more accurate and complete picture of the actinobacteria genome than using only one of these technologies.

After the sequencing of the bacterial genome, the reads need to be annotated i.e. the genes/reads need to be placed in the correct order. Annotation can be performed by utilising online services such as using the RAST server (Rapid Annotations using Subsystems Technology). This service identifies protein-encoding, rRNA and tRNA genes, gene functions, predicts subsystems, and uses this information to reconstruct the metabolic network (Aziz *et al.,* 2008). Once the genome has been annotated, further analysis can be done to determine the presence of bioactive compounds. One such tool, antiSMASH, mines the genome for antibiotics and secondary metabolites. This online or stand-alone tool identifies and analyses BGCs in bacterial genome sequences (Blin *et al.,* 2019). This software is easy to use, rapid and offers comprehensive analysis of the putative secondary metabolite genes. It detects all known classes of secondary metabolite BGCs, provides detailed NRPS/PKS functional annotation, and predicts the chemical structure of NRPS/PKS products (Medema *et al.,* 2011).

A variety of bioactive compounds including antioxidants, antibiotics, antifungals, antitumor agents, anthelmintic, and immunosuppressants are commonly synthesised in actinobacteria by the enzymes, polyketide synthases (PKSs) and nonribosomal peptide synthases (NRPSs). Both NRPS and PKS polypeptides are encoded by a variable number of modules with enzymatic activity. Each PKS module encodes for at least three domains involved in the selection and condensation of the correct extender unit, a ketosynthase (KS-domain), an acyltransferase (AT-domain), and an acyl carrier protein (ACP-domain). The PKS module may contain additional domains such as enoylreductase, dehydratase, and ketoreductase that are

involved in the reduction of the β-keto group formed during condensation. All of these domains are involved in the synthesis of the new polyketide chain (Ayuso-Sacido and Genilloud, 2004). Polyketide synthases are classified into three groups, type I, II and III. Type-1 PKSs are multifunctional enzymes that are organised into modules, having a set of distinct, non-iteratively acting activities responsible for the catalysis of one cycle of polyketide chain elongation. Type-2 PKSs are multienzyme complexes that harbour a single set of repetitive activities, and type-3 PKSs are homodimeric enzymes that are iterative condensing enzymes. Type-1 and -2 PKSs use ACP to activate the acyl CoA substrates and grow the polyketide intermediates, whereas type-3 acts directly on the acyl CoA substrates, regardless of ACP (Shen, 2003). Nonribosomal peptide synthetases (NRPS) are large multimodular biocatalysts that assemble structurally and functionally diverse peptides (Strieker *et al.,* 2010). The modules contain domains that are involved in condensation of amino acids (C-domains), adenylation of amino acids (A-domain), and the propagation of the growing peptide (thiolation or peptidyl carrier protein; PCP-domain) steps. Additional domains such as heterocyclase, N-methylase, epimerase, thioesterase, and reductase are also present (Ayuso-Sacido and Genilloud, 2004). The thieosterase (TE) domain is located in the termination module and catalyses the peptide release (Kopp and Marahiel, 2007).

Amplification of the PKS and NRPS genes can give insights into the genetic potential of actinobacterial isolates to produce valuable bioactive compounds. Analysis of these genes can help clarify the characteristics of the end product. For example, the PKS and NRPS genes from endophytic actinobacteria associated with Indian medicinal plants were evaluated. Four different genera, *Streptomyces, Brevibacterium, Microbacterium* and *Leifsonia*, showed high antimicrobial activity against human pathogenic strains. The biosynthetic potential of these isolates was evaluated by amplification of the biosynthetic genes, PKS-I and NRPS. Screening of these genes supported the antimicrobial activity that was observed, but in some cases, the biosynthetic genes were found in isolates that did not have antimicrobial activity (Passari *et al.,* 2015). This may indicate that the isolate produces a compound that either does not affect the pathogen tested (i.e. different bioactivity) or that it produces very low amounts of the compound or was not produced under the tested conditions. Thus, screening for these polypeptides is a quick, cost-effective and simple tool to evaluate the potential of the isolates to produce biosynthetic compounds. In another study, the biosynthetic potential of endophytic actinobacteria isolated from the Indian traditional medicinal plant, *Rhynochotoechum ellipticum*, was evaluated. The endophytes were screened via PCR-based detection for the presence of modular polyketide synthases (PKSI and II) and NRPS gene clusters. In the study, 25 isolates with PKS type-1, 41 isolates with PKS type-2 and 32 isolates with NRPS genes were detected. These isolates exhibited potent antimicrobial activities. Additionally, six

antibiotics (erythromycin, ketoconazole, fluconazole, chloramphenicol, rifampicin and miconazole) were detected, as well as the anticancer compound paclitaxel using UHPLC-QqQ_{LIT}-MS/MS. The researchers did not investigate whether or not the screened PKS and NRPS gene clusters correlated with the found bioactive compounds, and may be part of a future study. Nonetheless, the researchers do highlight the potential of endophytic actinobacteria as a source for bioactive natural products (Passari *et al.,* 2017).

Another class of pharmaceutically important compounds produced by actinobacteria are the ribosomally synthesized and post-translationally modified peptides (RiPPs). This structurally diverse class of natural products are produced by the ribosome and possess a wide range of bioactivities (Arnison *et al.,* 2013). Unlike non-ribosomal peptides (including PKSs and NRPSs) that require large multi-modular enzyme complexes to incorporate non-proteinogenic amino acids into a peptide, RiPPs are capable of achieving the same structural diversity through extensive post-translational modification of ribosomally synthesised precursor peptides consisting exclusively of the 20 standard amino acids (Ortega and Van der Donk, 2016). Advances in genome sequencing and mining has resulted in an increase in scientific research and discovery of new RiPPs. To date, 22 subfamilies of RiPPs have been characterised, including but not limited to, lanthipeptides, lasso peptides, thiopeptides, and bottromycins (Arnison *et al.,* 2013). The biosynthetic pathways of the subfamilies share common similarities. The biosynthesis of RiPPs starts with the ribosomal production of a precursor peptide that usually contains an N-terminal leader-peptide sequence and a C-terminal core region containing the different post-translational modification sites. The biosynthetic machinery recognises the leader peptide and installs different post-translational modifications in the core peptide. Finally, the leader peptide is removed to yield a mature active compound. In some cases, additional modifications may occur after the leader peptide has been removed (Arnison *et al.,* 2013, Figure 4.1).

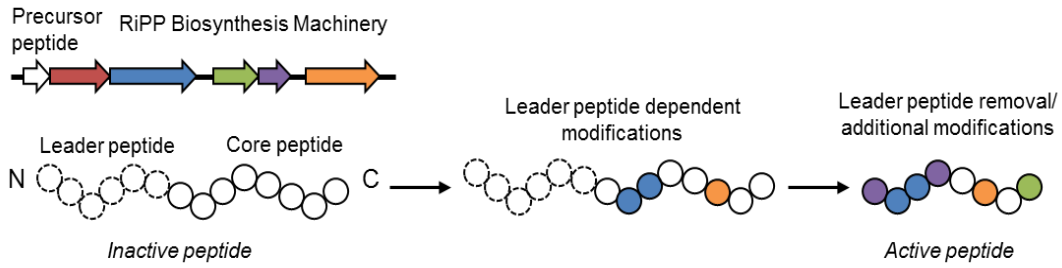


Figure 4.1: General RiPPs biosynthetic pathway. The genes encoding a biosynthetic enzyme within the RiPP gene cluster, is colour-coded by the modification it catalyses. Dotted circles represent amino acids within the leader peptide, whereas full circles represent amino acids within the core peptide. Adapted from Arnison et al., 2013.

Lanthipeptides are generated from a ribosomaly synthesised linear precursor peptide (generically termed LanA) and therefore belong to the RiPPs class of natural products. The peptides undergo post-translational modifications which give rise to rare amino acids such as lanthinionine (Lan), methyl-lanthionine (MeLan), dehydroalanine (Dha), and *Z*-dehydrobutyrine (Dhb) among others. Lan and MeLan possess internal rings formed by thioether bridges between Dha or Dhb, respectively, with a cysteine (Cys) residue. Thus, lanthipeptide modifications depend on the dehydration of serine (Ser) or threonine (Thr) to form Dha or Dhb, respectively, and the cyclisation for formation of Lan or MeLan, respectively. Lanthipeptides are categorised into four classes based on their biosynthetic pathways. For class I lanthipeptides, LanB dehydratases convert Ser and Thr present in precursor peptides to Dha and Dhb, respectively. Cysteine thiols are then added to Dha or Dhb, catalysed by LanC cyclases, to form lanthionine (from Ser) and methyllanthionine (from Thr). Class II lanthipeptides are produced by a single protein, the LanM modifying enzyme, responsible for both dehydration and cyclisation (Pag and Sahl, 2002; Goto *et al.,* 2010). Class III and IV lanthipeptides are also produced by a single protein that has lyase, kinase and cyclase domains but lacks the signature zinc-ligands in the cyclase domain (found in the other classes) and is carried out by LanKC and LanL enzymes, respectively (Van der Donk and Nair, 2014). The O-methyltransferases are associated proteins of class I lanthipeptides and have recently been found to catalyse the rearrangement of β-amino acids in lanthipeptides (Acedo *et al.,* 2019). A typical lanthipeptide operon will also contain genes encoding enzymes that carry out transport/processing (LanT), immunity (LanI and LanFEG), proteolytic processing (LanP) and a structural gene (LanA). Other enzymes responsible for the formation of less common residues may also be present (Field *et al.*, 2015). The products made by these four classes are not limited to lanthipeptides (Zhang *et al.,* 2015). Lanthipeptides that exhibit antimicrobial activity are called lantibiotics and their activity is related to the inhibition of cell wall biosynthesis by binding to lipid II. This prevents the incorporation of cell wall precursor units into the newly formed peptidoglycan and thus interferes with cellular membrane functions. Furthermore, lipid II is not the product of a single gene, but derived from multiple enzyme-catalysed reactions and thus the use of lantibiotics will have a low probability for resistance development (Gomes *et al.,* 2017).

Thiazole/oxazole-modified microcins (TOMMs) are ribosomally produced peptides with post-translationally installed heterocycles derived from cysteine, serine and threonine residues. The RiPPs subfamilies of linear azol(in)e-containing peptides (LAPs), cyanobactins, thiopeptides and battomycins are all characterised as TOMMs (Metelev and Ghilarov, 2014). The biosynthesis of TOMMs commences with the cyclohydration of amino acids with a beta-nucleophile (cysteine, serine, or threonine) by an ATP-dependent cyclodehydratase (C and D

proteins) into thiazoline or (methyl)oxaline rings. Additionally, the azoline rings can then be oxidized to azoles by a flavin mononucleotide (FMN)-dependent dehydrogenase (B protein) (Melby *et al.,* 2011; Burkhart *et al.,* 2015). Thiopeptides are produced by Gram-positive bacteria, with the majority of thiopeptide producers being actinobacteria (mostly *Streptomyces* species). A well-known thiopeptide produced by *Streptomyces azureus* (Donovick *et al.,* 1955) and *Streptomyces laurentii* (Trejo *et al.,* 1977), thiostrepton, is a promising antibiotic agent active against pathogenic Gram-positive bacteria including MRSA, vancomycin-resistant *Enterococcus* strains, penicillin-resistant *Streptococcus pneumoniae* strains and *Mycobacterium abscessus* (Baumann *et al.,* 2010; Kim *et al.,* 2019). The antibiotic also had anticancer activities and was shown to selectively induce cell cycle arrest and cell death in breast cancer cells through the down-regulating expression of forkhead box M1 (FOXM1) expression (Kwok *et al.,* 2008). Furthermore, thiostrepton also exhibit antimalarial and gametocytocidal activities (Aminake *et al.,* 2011).

To date, there are very few whole-genome sequences of actinobacteria isolated from medicinal plants (Golinska *et al.,* 2015). This leaves a big opening of opportunity for discovery of novel bioactive compounds, because the genome sequences of actinobacteria show promise in the discovery of new or more analogues of known bioactive compounds. Finding analogues of current drugs can help to facilitate drug discovery and help to discover drugs with less toxicity and more potent activities. One study focused on leinamycin, a promising anticancer drug lead that was discovered 30 years ago. Nonetheless, no analogue has been isolated. Using a discovery-based approach, leinamycin family of natural products were found by mining the genomes of actinobacterial species. Forty-nine potential producers were discovered that were grouped into 18 distinct clades based on the phylogenetic analysis of the DUF-SH didomains. These domains are made up of a domain of the unknown function (DUF) and cysteine lyase domain (SH). Discovery of the leinamycin domains using this approach is important in combinatorial biosynthesis for natural product discovery and can greatly facilitate drug discovery and development (Pan *et al.,* 2017). Novel compounds have also been discovered by using whole genome sequence analysis. Using microarray and bioinformatics analysis of the Δ*bldM Streptomyces venezuelae* mutant genome sequence, Thanapipatsiri and team (2016), were able to identify an unusual combination of modular type I and type III PKSs. This silent BGC from *S. venezuelae* was predicted to encode an unusual biaryl metabolite, venemycin, and was found to be expressed in the mutant and not the wild-type strain. The *vem* gene cluster could also be co-expressed in *S. coelicolor*, and production of venemycin was further stimulated by constitutive expression of *vemR* (encodes a putative LAL transcriptional activator) in both the mutant and *S. coelicolor*. In another study, combining both second- and third-generation sequencing, bioinformatic analysis of the whole genome

sequence of *Streptomyces leeuwenhoekii* revealed the presence of 35 BGCs with most of them completely novel and uncharacterised. Previously identified gene clusters for chaxamycin, chaxalactin, hygromycin A and desferrioxamine E were also found. The study found 31 putative gene clusters some of which encoding novel polyketides, non-ribosomal peptides and lasso peptides (Gomez-Escribano *et al.,* 2015).

Previously, the whole genome sequences of endophytic bacteria have been done. Krause *et al.* (2006) reported the first genome sequence of a bacterial endophyte, *Azoarcus* strain BH72, endophytic to rice and other grasses. This sequence provided valuable insight into the life of the endophyte and its interactions with the host plant. In a recent study, the genome sequence of endophytic *Sphingomonas* species LK11 revealed its potential in plant growth promotion. The strain was able to produce different type of gibberellins that significantly improved soybean plant growth. Genes encoding phosphate solubilisation and nitrate/nitrite ammonification that further stimulates plant growth promotion were found. Furthermore, genes involved in the synthesis of catalases, superoxide dismutase, and peroxidases were found that help protect plants against oxidative stress (Asaf *et al.,* 2018).

This chapter describes the genetic potential of actinobacteria isolated from South African medicinal plants, *Aloe ferox* and *Sutherlandia frutescens*. The modular polyketide synthase type I (PKS type I) genes were investigated using a PCR-based screen, as well as the whole genome sequence of three bioactive actinobacteria, of which *Streptomyces* strain S149 is an endophyte of *S. frutescens*. These actinobacteria have the genetic potential to produce a range of bioactive compounds including antibiotics, siderophores, phenolics, lanthipeptides, terpenes, lasso peptides and bacteriocins.

4.2 Materials and Methods

4.2.1 DNA extraction

Total genomic DNA was isolated from the actinobacterial strains as discussed previously (Section 2.2.5.1).

4.2.2 PKS-I gene amplification and sequencing

The KS domain of type I PKS gene was screened using the KS1F1 (5'-ATGGAYCCSCARCARCGBCT-3') and KS1R1 (5'-GCYTCGATSGGRTCNCCSA-3') primer pair (Russel Hill, unpublished). Each PCR reaction contained a final concentration of 1x buffer, 200 µM dNTPs, 0.5 µM of each primer, 0.1 mg/ml BSA, ± 5 ng/µL template DNA, 1.25 U of DreamTaq polymerase (ThermoFisher), and sterile MiliQ water to a final volume of 50 µL. A touchdown PCR was performed using the following cycling conditions. The reaction was initiated at 95 °C for 3 minutes followed by 10 cycles of denaturation at 95 °C for 30 s, annealing at 77.3 °C (-1 °C/cycle), and extension at 72 °C for 60 s. The 10 touchdown PCR cycles were followed by 20 cycles of denaturation at 95 °C for 30 s, annealing at 62.3 °C for 30 s, and extension at 72 °C for 50 s. Final extension was done for 5 minutes at 72 °C and the reaction was held at 10 °C. Amplification of this domain resulted in an amplicon of approximately 700 base pairs. Negative controls with no DNA template were included in all PCR reactions. The positive control was included as genomic DNA from *Streptomyces polyantibioticus* SPR^{T}. DNA sequencing was performed as discussed in Section 2.2.5.4 with the KS1F1 and KS1R1 primer set. Sequences were analysed and edited using the BioEdit version 7.2.6 software (Hall, 1999). The basic local alignment search tool (BLASTn) was used to determine sequence similarity and identity to known type strains in the GenBank database from the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov). The PKS sequences were submitted to the Natural Product Domain Seeker (NaPDoS) web-based tool to identify closely related KS-domains (Ziemert *et al.,* 2012).

4.2.3 Next generation sequencing

The genome of *Streptomyces* species A81, A159 and S149 was sequenced in-house on an Illumina MiSeq at the University of the Western Cape's Single Cell Genomics Platform. The library for each sample was prepared using a Nextera XT DNA Library Preparation Kit (Illumina), following the manufacturer's instructions (part #15031942; Rev C). 1ng of input

DNA was used per library and libraries were normalised using the standard bead-based reaction prior to pooling. The libraries were sequenced using a V3 kit (2 × 300-bp paired-end reads) and included a 10% phiX spike.

4.2.4 Pre-processing and Genome Assembly

To evaluate read quality before and after pre-processing, QC plots for sequenced reads were generated using the CLC Genomics Workbench (Version 7.5.1) (Qiagen). The quality of the reads was evaluated and expressed as PHRED-scores. Low quality reads were trimmed using a quality score limit of 0.05. Ambiguous nucleotides of five or more were also trimmed. The reads were filtered to remove ambiguous nucleotides and adapters. Quality control assessment plots were generated for sequenced reads to evaluate read quality before and after pre-processing. *De novo* assembly of the whole genome sequence was performed using the default parameters of the CLC Genomics Workbench software (Version 7.5.1). Paired-end reads were utilised in the assembly process and reads were mapped back to contigs.

4.2.5 Annotation and Biosynthetic Gene Cluster Identification

Annotation of the genomes was performed using the online RAST server (Rapid Annotation of Subsystem Technology, Version 2.0; Aziz *et al.,* 2008, Overbeek *et al.,* 2014, Brettin *et al.,* 2015) with the SEED database. The annotated genome sequences were submitted to the antiSMASH online server (version 5.0.0) for the genome-wide identification, annotation and analysis of secondary metabolite encoding gene clusters present within the bacterial genomes (Blin *et al.,* 2019). The average nucleotide identity (ANI) was evaluated of the whole genome sequence to the closest relative using the ANI online server developed by Kostas Lab (Rodriguez-R and Konstantinidis, 2016).

4.3 Results and Discussion

4.3.1 PKS-I gene amplification

The PKS-I specific PCR generated an amplicon of the expected size (approximately 700 bp) for five of the six isolates. The amplicons were sequenced and the translated sequences were imported to the NaPDoS database to search for closely related domains connected to the production of natural compounds. The NaPDoS bioinformatics tool is designed to detect and extract the C- and KS-domains from NRPS and PKS modules, respectively. These candidate domains are identified by sequence comparison to a broad set of reference genes from well-characterised chemical pathways. The candidate gene sequences are extracted, trimmed, translated and subjected to domain-specific phylogenetic clustering to predict what their assumed products might be. Furthermore, the sequences are used to determine whether the products are likely to produce compounds similar to or different from previously known biosynthetic pathways. Hits with more than 85% identity at the amino acid level indicate that the query domains may be associated with the production of the same or similar compound as those produced by the reference pathway. Lower than 85% identity may indicate that the gene has most likely not been characterised and that it is possible that the encoded compound is novel or has not been identified within a chemical pathway.

Table 4.1: *NaPDoS results indicating the match, percentage identity, align length, e-value, pathway product and the PKS domain class.*

Isolate	Database match ID	Percent identity	Align length (bp)	e-value	Pathway product	Domain class
A3	MerB_ABJ97437_2KSB	80	83	2E-37	Meridamycin	Modular
A37	NysB_Q9L4W4_2mod	74	176	3E-62	Nystatin	Modular
A81	AveA3_Q9S0R4_2mod	88	127	9E-62	Avermectin	Modular
S149	JamP_AAS98787_H	61	172	1E-56	Jamaicamide	Hybrid KS
S173	ChlB1_AAZ77673_i	61	145	9E-47	Chlorothricin	Iterative

The PKS domain of *Streptomyces* strain A3 has an 80% similarity to that of *merB* that forms part of the pathway that synthesises meridamycin (Table 4.1). Six genes encode for the construction of meridamycin, namely *merP* (encodes a non-ribosomal peptide synthase for pipecolate-incorporation), *merA-D* (PKS genes encoding one module and 14 extension modules), and *merE* (encodes a cytochrome P450 monooxygenase) (He *et al.,* 2006). Meridamycin is a non-immunosuppressive neuroprotectant isolated from *Streptomyces*

hygroscopicus (Salituro *et al.,* 1995). Although only one of the PKS genes was identified in this amplification, it is possible that the other genes are also present in strain A3. The neuroprotective properties of *Streptomyces* strain A3 was not investigated in this study, but may be of interest to investigate in future studies.

Amycolatopsis strain A37 had a PKS domain similar to the *nysB* gene involved in the synthesis of nystatin (Table 4.1). Nystatin is produced by *Streptomyces noursei* and was discovered by Brown and Hazen in 1957. Nystatin is a polyene macrolide with potent antifungal activity with little or no affect to other microorganisms and animal cells (Lampen *et al.,* 1959; Brown and Hazen, 1957). Today, nystatin has been developed into various topical antifungal ointments as well as a liposomal, intravenous formulation known as Nyotran. The antifungal agent is active against strains of *Aspergillus* and *Candida* (Arikan and Rex, 2001). An mucoadhesive tablet containing nystatin was developed for the treatment of oral candidosis (Llabot *et al.,* 2002). The potential of strain A37 to produce nystatin is of great pharmaceutical importance. Furthermore, there are no reports showing the production of nystatin by an *Amycolatopsis* species. Thus, this strain may have the ability to produce an analogue of nystatin. Antifungal activity of this strain will also need to be evaluated to conclude production of antifungal compounds by this strain.

Streptomyces strain A81 had high similarity (88%) to the *aveA3* domain involved in the synthesis of the anthelmintic avermectin (Table 4.1). Investigation of the avermectin gene cluster of *Streptomyces avermitilis* revealed that the PKS genes responsible for avermectin biosynthesis together encode 12 homologous sets of enzyme activities, each catalysing a specific round of polyketide chain elongation. The clustered genes encoding the PKSs are organized as two sets of six modular repeats, *aveA1-aveA2* and *aveA3-aveA4*, which are convergently transcribed (Ikeda *et al.,* 1999). Avermectins are used to treat parasitic worms and insect pests. The production of this compound by soil *Streptomyces* may play a role in protecting the host plant from insect predation. The potential of *Streptomyces* strain A81 to produce avermectin or similar compounds with anthelmintic and insecticidal activity will need to be investigated in future studies.

Streptomyces strain S149 had 61% similarity to the KS domain of *jamP* which is involved in the production of jamaicamide, a hybrid PKS (Table 4.1). Jamaicamides are sodium channel blockers derived from the cyanobacterium *Lyngbya majuscule* (Edwards *et al.,* 2004; Graf *et al.,* 2009). No production of jamaicamide has been reported in any other microbe including *Streptomyces* species. Due to this domain having relatively low similarity to jamaicamide, it is probable that this domain in *Streptomyces* strain S149 is not involved in the production of

jamaicamide, but a different hybrid PKS pathway. Sodium channel blockers can be used as antiepileptic drugs to inhibit the high-frequency repetitive spike firing during a seizure activity without affecting ordinary ongoing neural activity. The inhibitory neurotransmitter, gamma aminobutyric acid (GABA) is produced by *Sutherlandia frutescens*. Since *Streptomyces* strain S149 was isolated as an endophyte of *S. frutescens*, it is possible that the strain is producing a different compound involved in neurotransmission. GABA has been previously isolated from *Streptomyces lincolnensis* (Haak and Reineke, 1981). Thus, this strain may also produce a compound similar to GABA or a compound that may have inhibitory neurotransmission activity.

The KS domain of *Actinomadura* strain S173 had 61% similarity to *chlB1* domain of chlorothricin (Table 4.1), a spirotetronate macrolide antibiotic isolated from *Streptomyces antibioticus* Tü99 (Keller-Schierlein *et al.,* 1969). Spirotetronate polyketides are a family of microbial metabolites with potent antitumor antibiotic properties. With their impressive chemical structure, and pharmacological potential, these metabolites are of great interest as leads in drug discovery (Lacoske and Theodorakis, 2015). Chlorothricin has not been reported to be produced by *Actinomadura*, but other spirotetronate antibiotics have been isolated from this genus. Nomimcin was isolated from *Actinomadura* sp. TP-A0878 with antimicrobial activity against *Micrococcus luteus*, *Candida albincans* and *Kluyveromyces fragilis* (Igarashi *et al.,* 2012). The kijanimicin antibiotic produced by the actinomycetia *Actinomadura kijaniata* was isolated and had a broad spectrum of antimicrobial activities against Gram-positive bacteria, anaerobes and the malaria parasite *Plasmodium falciparum* as well as antitumor activity (Zhang *et al.,* 2007). Other spirotetronate antibiotics isolated include MM46115 from *Actinomadura pelletieri,* IP/729.63, BE-45722A, BE-45722B and BE-45722C from *Actinomadura* sp. 2EPS (Luk and Readshaw, 1991; Euanorasetr *et al.,* 2015). Previously (Section 3.3.2), experiments confirmed that *Actinomadura* strain S173 had antimicrobial activity against Gram-positive bacteria, including *B. cereus*, *E. faecalis* and *S. aureus*. The strain also had antimicrobial activity against the Gram-negative bacteria, *K. pneumoniae*. It is thus possible that this strain is producing a spirotetronate antibiotic with antagonistic activity against Gram-positive and -negative bacteria. Further investigation into the antitumor potential of this strain is highly recommended as this strain was isolated from the seeds of *Sutherlandia frutescens*. This plant is commonly known as cancer bush and traditionally used to relieve and treat internal cancers. Studies on *Sutherlandia* extracts have shown antiproliferative effects on human breast and leukemia tumour cell lines (Tai *et al.,* 2004), esophageal cancer cell lines (Skerman *et al.,* 2011), and tumorigenic breast adenocarcinoma cells (Stander *et al.,* 2009). The close association of this strain with the plant may have resulted in the strain producing compounds with similar activities as observed within the plant species.

The PCR-amplification of the type-1 PKS gene helped to elucidate the genetic potential of the isolated actinobacteria. The production of these potential compounds needs to be further confirmed. The identification of these KS-domains is the first step to evaluate the potential of the isolates to produce bioactive compounds. The identification and evaluation of other KS-domains from type-2 and -3, as well as the C-domains from NRPS genes, will help to clarify the genetic potential of the isolates. Future studies will need to evaluate these genes as well. It is also important to note, that in a previous study, the presence of these genes did not always correlate to the antibiotic activity seen. In some cases, the PKS or NRPS genes were amplified, but the tested isolate had no observed antimicrobial activity (Gohain *et al.,* 2019). This indicates that the gene is either silent or that it is not producing a metabolite that has the activity tested for.

4.3.2 Sequencing and Assembly of Actinobacterial genomes

Three strains were selected for whole genome sequencing. *Streptomyces* strains A81 and A159 were selected based on their antimicrobial activity and *Streptomyces* strain S149 was selected because it was isolated as a leaf endophyte of *S. frutescens*. Next-generation sequencing consists of a series of steps which all contribute to the final quality of the data. Evaluating the quality of these steps can provide important information of each step in the process, including library preparation, base calling, read alignment and variant calling. The PHRED quality score (Q score) is used to evaluate the base calling accuracy and is the most commonly used metric to assess the accuracy of the sequence. The score indicates the probability that the given base is called incorrectly by the sequencer. To calculate the PHRED quality score, the Q score is defined as a property that is logarithmically related to the base calling error probabilities (P), written in the following formula:

$$Q = -10 \log_{10} (P)$$

For example, a Q score of 30 is equivalent to the probability of an incorrect base call is 1 in 1000 times, i.e. the base call accuracy is 99.9%. A PHRED Q score of 20 has the probability of an incorrect base call of 1 in 100 times, i.e. a base call accuracy of 99%. Thus, high quality values correspond to low error probabilities (Ewing and Green, 1998). The PHRED scores higher than 20 are considered to be good quality, with scores more than 30, of high quality. Reads were trimmed on quality and ambiguity to ensure all reads have a PHRED score of 18 and higher to ensure good quality for *de novo* assembly.

Sequencing of *Streptomyces* strain A81 resulted in 1 263 968 reads with an average length of 259.9 bp. After trimming the number of reads for *Streptomyces* strain A81 were 1 267 922 with an average length of 188.9 bp. *Streptomyces* strain A159 had an average length read of 238.7 bp and 301 566 reads before trimming. After the trim, there were 300 423 reads with an average length of 181.3 bp. *Streptomyces* strain S149 had 1 984 886 reads with an average length of 215.7 bp which was trimmed to 1 978 762 reads with an average length of 171.9 bp (Table 4.2 and Figure 4.2)

Table 4.2: *Read information of paired-end reads for Streptomyces species A81, A159 and S149 before and after processing.*

Strain	Number of Reads	Average Length (bp)	Number of Reads after Trim	Average Length after Trim (bp)
A81	1 263 968	259.9	1 261 922	188.9
A159	301 566	238.7	300 423	181.3
S149	1 984 886	215.7	1 978 762	171.9

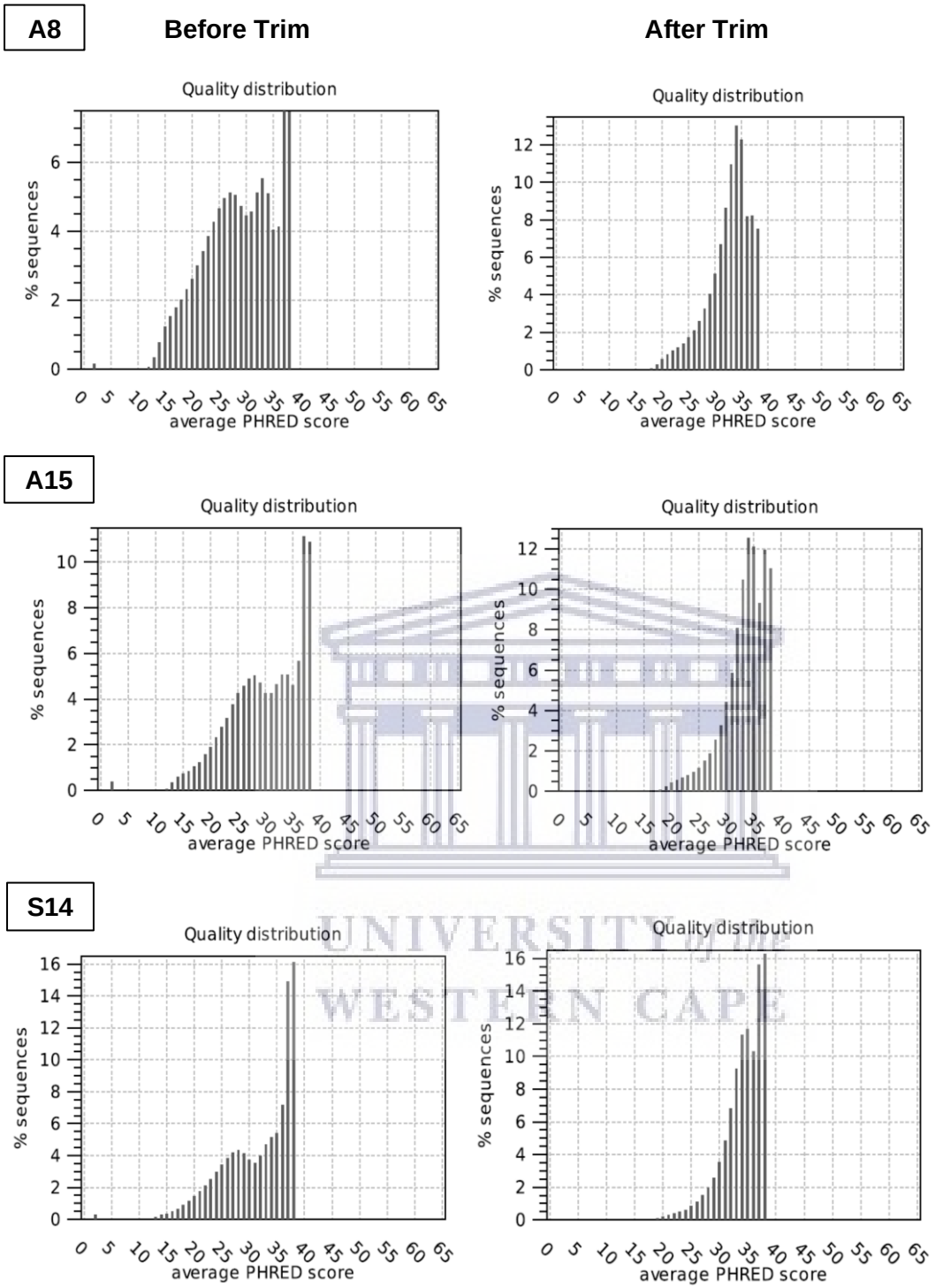


Figure 4.2: *Quality of reads of* Streptomyces *strains A81, A159 and S149 before and after processing expressed as PHRED score. The quality of the data for strain A81 improved significantly after processing; ~48% of sequences had a Q score of 30 or over before and ~80% after processing. ~50% of sequences of strain A159 had a Q score of 30 or more before an ~85% after processing. Strain S149 had the best quality data with ~70% of the sequences having a Q score of 30 or more before processing and ~90% after processing.*

After the paired reads were trimmed, *de novo* assembly of the sequences were preformed to assemble the reads into contigs and scaffolds. The lengths of the contigs are indicated in table 4.3. The N50 value indicates the length of the shortest contig needed to cover 50% of the

genome. Thus, a larger N50 number will indicate fewer contigs needed to cover 50% of the genome and may indicate better assembly quality. *De novo* assembly of *Streptomyces* strain A81, yielded a total of 1 267 contigs with scaffolds. The longest contig was 63 679 bp in length and the shortest being 987 bp. The N50 value was 8 255 bp and the whole genome size being 7 284 493 bp. *Streptomyces* strain A159 had 1670 contigs with an average length of 2 377 bp. The N50 value was 2 687 bp with a genome size of 3 968 996 bp. *Streptomyces* strain S149 had a genome size of 8 291 870 bp consisting of 704 contigs with an average size of 1 001bp and an N50 value of 19 020 bp (Table 4.3). Thus, based on the N50 values, strain S149 had the best assembly quality followed by strain A81, with strain A159 not having good assembly quality. Given the low number of reads obtained from strain A159, this was to be expected.

Table 4.3: *Contig measurements including scaffolded regions after de novo assembly of* Streptomyces *strain A81, A159 and S149:*

Contig measurements	**A81**	**A159**	**S149**
N50 (bp)	8 255	2 687	19 020
Minimum length (bp)	987	873	1 001
Maximum length (bp)	63 679	15 420	78 619
Average length (bp)	5 749	2 377	11 778
Number of contigs	1 267	1 670	704
Total genome size (bp)	7 284 493	3 968 996	8 291 870

Typically, the *Streptomyces* species are expected to have large genomes, with sizes ranging between 8.7 Mbp and 11.9 Mbp (Zhou *et al.,* 2012). However, genome sizes much smaller have been reported in recent years. The genome size of *Streptomyces albus* J1074 is only 6.8 Mb (Zaburannyi *et al.,* 2014), with the genome size of *Streptomyces violaceusniger* strain SP6 being even smaller at just 6.4 Mb (Chen *et al.,* 2013). The smallest streptomyces genome to date is from the pathogenic *Streptomyces somaliensis* strain DSM 40738 with a genome of only 5.18 Mbp in length (Kirby *et al.,* 2012). A recent study also found that streptomycete strains isolated from marine environments had smaller genome sizes with *Streptomyces* species CNS606 having a genome size of 5.93 Mbp (Tian *et al.,* 2016). Despite these findings, we can assume that the genomes assembled in this study are only partial and not fully complete because the genome size of the sequenced genomes is smaller than the typical genome size of *Streptomyces* species. The assembly quality of strain S149 resulted in fewer contigs with longer lengths and a larger genome size. Nevertheless, a genome size of 8.2 Mbp for an endophyte may be near to complete as endophytic actinobacteria sometimes have smaller genomes sizes (de Oliveira *et al.,* 2014). Similarly, the genome assembly of

Streptomyces strain A81 had an average assembly quality with relatively long contigs and was made up of more contigs than strain S149. It is clear that the genome assembly from strain A159 only represents a small part of the genome and had a low assembly quality made up of numerous shorter contigs. Resequencing of the genomes will need to be performed to fully complete the genome assemblies of these isolates.

The quality and quantity of the reads depends on the sequencing library. The first step in the Illumina library preparation is the tagmentation reaction, where the transposon cleaves and tags the double-stranded DNA with a universal overhang. The success of the library preparation is determined by how well the sample was tagmented. The Illumina kit used in this study is optimised for 1 ng of dsDNA and using more can lead to undertagmentation and using less can lead to overtagmentation of the sample. The high %G+C content of actinobacteria can also lead to tagmentation problems in that the transposon has difficulty to cleave and tag the DNA. Overtagmented samples can lead to reduced library yield and can result in coverage dropout. Causes of overtagmentation include inaccurate quantification of DNA, using degraded DNA samples and use of smaller sized amplicons. Transposons require at least 300 bp of genomic space to attach to the DNA. The quality of the DNA was confirmed on an agarose gel to confirm that the DNA was not degraded before sequencing. Thus, the lower library yield of strain A159 is most likely due to inaccurate quantification of the DNA. The sequencing biases due to the high %G+C content and the long terminal inverted repeats (reaching over 1 Mb; Weaver et al, 2004) of the linear chromosomes and plasmids of streptomycetes is very difficult to resolve with the current sequencing technologies. And may be another reason for the low-quality data obtained from the genome assembly of strain A159.

Sequencing of actinobacterial genomes are notoriously difficult due to their high %GC content and the long terminal inverted repeats within their chromosomes and plasmids. The sequencing of the strains resulted in a large number of short reads. In the case of *Streptomyces* strain A159, the quality and quantity of the reads were also affected. This was most likely due to the amount of input DNA used that may have been quantified incorrectly. The incorrect quantification resulted in poorly tagmented fragments and thus resulting in poor and low number of reads. Recently, the Nextera DNA Library Prep Kit (used in this study) has been discontinued by Illumina and replaced by the Nextera DNA Flex Library Prep Kit. This newer kit offers a faster workflow, a broad DNA input range (1 – 500ng), and on-bead tagmentation that ensures more consistent fragmentation (Ilumina DNA Flex Library Prep Kit; https://emea.illumina.com/products/by-type/sequencing-kits/library-prep-kits/nextera-dna-flex.html, accessed 14-02-2020) that will help to decrease errors and increase the quality and quantity of the reads from a variety of species.

4.3.2 General characteristics of the genomes

Annotation using the RAST software was performed. The software annotates microbial genomes by projecting manually curated gene annotations from the SEED database onto the newly submitted genomes. The annotation data curated in the SEED database is organised into subsystems (Brettin *et al.,* 2015). A subsystem represents a set of logically related functional roles that together implement a specific biological process or structural complex. Annotations using subsystems produces more accurate annotations and is more straightforward and less error prone than annotating the genes one-by-one within a single organism (Overbeek *et al.,* 2005).

The annotations of the draft genomes resulted in genome sizes of 7.28 Mbp, 3.97Mbp and 8.29 Mbp for *Streptomyces* strains A81, A159 and S149, respectively (Table 4.3). The %G+C content was above 71% which is normal for *Streptomyces* species (Table 4.3). *Streptomyces* strains A81 and S149 have near to complete draft genomes as the size is almost the same as their closest relatives, as well as the %G+C content is within the expected range. The assembly of strains A81 and S149 represent 7 363 and 8 193 coding sequences, and 59 and 60 RNAs, respectively (Table 4.3). Whereas the assembly of strain A159 does not represent a complete genome. The size of the genome is far too small with only 4 976 coding sequences and 30 RNAs, almost half of the amount the other assemblies had (Table 4.3). The high %G+C content indicate that the strain is possibly a *Streptomyces*. The missing sequencing data resulted in only a partial assembly. Future sequencing of the genome will need to be performed for a draft genome assembly of this strain.

Table 4.3: *Overview of the genomes of* Streptomyces *strains A81, A159 and S149.*

Annotation Overview	**A81**	**A159**	**S149**
Size (bp)	7 284 493	3 968 996	8 291 870
G+C content (%)	72.1	71.4	71.5
N50	8 255	2 687	19 020
L50	257	476	126
Number of contigs (with protein encoding genes)	1 267	1 670	704
Number of subsystems	305	230	336
Number of coding sequences	7 363	4 976	8 193
Number of RNAs	59	30	60

Annotation of the draft genome of *Streptomyces* strain A81 indicated that the strain had an approximate genome size of over 7.28 Mbp with 72.1 % G+C content. The genome was made

up of 1 267 contigs with 305 subsystems, 7 363 coding sequences and 59 RNAs (Table 4.3). The strain had similar properties when compared to *S. californicus* strain NRRL B-2895 (closely related to *Streptomyces* strain A81), which was assembled at a contig level and had a genome size of 8.1 Mbp, 72.5 % G+C content, 7 584 coding sequences and 77 RNAs (https://www.ncbi.nlm.nih.gov/genome/32370?genome_assembly_id=203964 accessed on 3-06-2020).

The assembled genome of *Streptomyces* strain A81 had 18% of the protein encoding sequences that could be classified into subsystems (Figure 4.3 a). Investigation into the subsystems revealed a high number of genes involved in various cellular processes (Figure 4.3 b). The majority of the genes were involved in the metabolism of amino acids and derivatives (398 genes) followed by carbohydrate metabolism (319 genes).

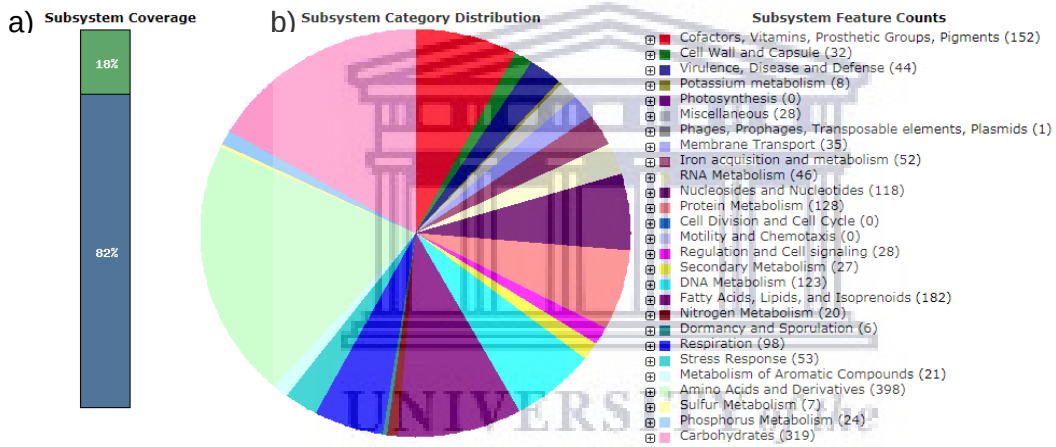


Figure 4.3: *Subsystem information of* Streptomyces *strain A81 obtained from the SEED database. a) The bar graph represents the percentage of protein encoding genes that are present in at least one subsystem (green bar). b) The pie chart and accompanying hierarchical tree, colour coded to match the pie chart, organises the subsystems (green bar) of the genome assembly by cellular process. The number of protein encoding genes in each category is listed in parentheses.*

Genes involved in secondary metabolism was identified and included 17 sequences for lanthionine synthethases (involved in lanthipeptide synthesis) and 10 sequences for thiazole-oxazole-modified microcin (TOMM) synthesis (Table 4.4). The lanthionine synthetases from *Streptomyces* strain A81 included: 1 *lanA* (lanthionine precursor peptide), 2 *lanB*, (lanthionine biosynthesis protein), 2 *lanC* (lanthionine biosynthesis cyclase), 1 O-methyltransferase clustered with lanBC, 5 Protein-L-isoaspartate O-methyltransferases, 3 *lanL* (lanthionine biosynthesis protein), 2 *lanM* (lanthionine biosynthesis protein), and 1 *tsrD* family dihydropyridine synthase. The presence of these lanthionine enzymes indicate the potential for this strain to produce a wide range of lanthipeptides, including class I, II, and IV; and

potentially other natural products other than lanthipeptides. A recent study mined over 100 000 bacterial and archaeal genomes targeting precursor peptides and found that the majority of lanthipeptide enzymes were found in *Actinobacteria* with class I, II, III and IV precursor enzymes found in various *Streptomyces* species (Walker *et al.,* 2020). Class I lantibiotics produced by actinobacteria include microbisporicin, produced by *Microbispora* sp. ATCC PTA-5024, which has potent antimicrobial activity against aerobic and anaerobic Gram-positive pathogens, such as *S. aureus*, vancomycin-resistant *Enterococcus* and *Streptococcus pneumoniae*. Activity against *Clostridia* and *Propionibacteria* was also observed (Castiglione *et al.,* 2008). To date, the products of *Streptomyces* class I lanthipeptide BGCs have not been identified. As mentioned previously, the product of the lanthipeptide BGC may be involved in producing compounds other than lanthipeptides. Genome mining of *Streptomyces* species in recent years has aided in the discovery of new lanthipeptides. A putative class I lanthipeptide BGC was found in *Streptomyces yeochonensis* CN732, the product of which is still unidentified (Malik *et al.,* 2020). An avermipeptin analogue (class III lanthipeptide) from *Streptomyces actuosus* ATCC 25421 was found following a genome mining approach (Liu *et al.,* 2018). Genome mining of *Streptomyces venezuelae* enabled the discovery of the first class IV lanthipeptide, termed venezuelin. The lanthipeptide could only be obtained via *in vitro* modification of the VenA precursor peptide by VenL followed by the proteolytic removal of the leader peptide (Goto *et al.,* 2010). Another class IV lanthipeptide, streptocollin was identified from *Streptomyces collinus* Tü 365, also using a genome mining approach (Iftime *et al.,* 2015). Analysis of lanthipeptide-like gene clusters in actinobacteria led to the further identification of venezuelin-like lanthipeptide gene clusters in three *Streptomyces* strains (*S. katrae* ISP5550, *S. lavendulae* subsp. *lavendulae* NRRL B-2508, and *Streptomyces* sp. strain NRRL B-2375) that produced venezuelins in detectable amounts. Venezuelins of different lengths were detected and is due to the presence or absence of Phe28 and Ala29 in the VenA precursor peptide (Zhang *et al.,* 2015). The genome mining study that investigated over 100 000 bacterial and archaeal genomes for the presence of lanthipeptide-like BGCs (Walker *et al.,* 2020) found a large number of lanthipeptide-gene clusters present in actinobacteria, but so far only a few of these gene clusters and their products have been identified. Analysis of the draft genome sequence of *Streptomyces* strain A81 via antiSMASH identified two unknown lanthipeptide BGCs (Figure 4.8). Future investigation will need to be conducted to determine the lanthipeptide products and their functions.

The following coding sequences involved in TOMM biosynthesis from *Streptomyces* strain A81 were identified: 1 precursor peptide (protein A), 3 cyclodehydratases (protein C), 3 docking proteins (protein D), 1 zinc protease, 1 TOMM export ABC transporter (permease protein) and 1 hypothetical protein (FIG013786). Thiopeptide biosynthesis is relatively

straightforward, with a precursor peptide consisting of an N-terminal leader and a C-terminal core peptide, encoded by a single gene, that is synthesised and undergoes post-translational modifications. Thiopeptide BGCs furthermore contain at least five additional genes involved in heterocyclization, dehydration, and the formation of the central six-membered nitrogen heterocycle (Schneider *et al.,* 2018). As discussed previously, TOMM biosynthesis starts with the cyclohydration of amino acids with a beta-nucleophile (cysteine, serine, or threonine) into thiazoline or (methyl)oxaline rings by an ATP-dependent cyclodehydratase (C and D proteins). Additionally, the azoline rings can then be oxidized to azoles by a flavin mononucleotide (FMN)-dependent dehydrogenase (B protein) (Melby *et al.,* 2011; Burkhart *et al.,* 2015). *Streptomyces* strain A81 has the genes encoding the C and D proteins with no genes encoding for the B protein. Thus, it can be assumed that the thiazoline or (methyl)oxaline rings does not undergo further oxidization. Additionally, there is only one gene encoding for a precursor peptide and thus likely only one thiopeptide is synthesised from the available TOMM machinery. Future work will need to be conducted to determine the thiopeptide synthesised by these genes. In depth genome mining and heterologous expression may reveal the thiopeptide product. A similar approach aided in the discovery of the new thiopeptide, geninthiocin B produced by *Streptomyces* sp. YIM 130001 (Schneider *et al.,* 2018).

The strain also contained 31 coding sequences that give resistance to antibiotics and toxic compounds which included two coding sequences each that give resistance to streptothricin and fluoroquinolone class of antibiotics (Table 4.4). The presence of antibiotic-resistance genes in antibiotic-producing strains, helps protect the strain against these toxic metabolites it is producing, i.e. self-resistance. An example of this self-resistance was observed with actinorhodin production from the model actinomycetia, *S. coelicolor*. The actinorhodin BGC was studied and it was found that the cluster contains genes encoding for repressor/efflux pumps that thus confer resistance to the antibiotic being produced. The expression of these genes is activated by the mature antibiotic, as well as intermediates in the pathway. The export of the antibiotic(s) is thus coupled to the biosynthesis of the antibiotic. This type of coupling may be a common feature in other bioactive actinomycetia (Tahlan *et al.,* 2007). Thus, the presence of streptothricin- and fluoroquinolone-resistance genes may indicate the production of these antibiotic classes by *Streptomyces* strain A81. While investigation of the BGCs (via antiSMASH; Figure 4.8) did not indicate the presence of known antibiotics in either of these antibiotic classes, investigating the unknown/unidentified BGCs may reveal if the gene clusters for these antibiotic classes are in fact present.

Streptomyces species are well-known siderophore producers with strain A81 having 52 coding sequences involved in iron acquisition and metabolism, including genes for the siderophores,

enterobactin and desferrioxamine E (Table 4.4). As investigated previously, *Streptomyces* strain A81 had moderate siderophore activity (Section 3.3.5) on CAS media plates. Moreover, the RAST and antiSMASH results both indicated the presence of siderophore genes. The one enterobactin gene identified with RAST may be more likely one of the genes involved in griseobactin biosynthesis as enterobactin is a siderophore produced mainly by Gram-negative bacteria, whereas griseorbactin is produced by *Streptomyces* species. Furthermore, two griseobactin BGCs was identified via antiSMASH (Figure 4.8).

Table 4.4: Number of protein encoding genes within highlighted subsystem and their subcategory from Streptomyces *strain A81*

Subsystems and subcategories	**Number of coding sequences**
Virulence, Disease and Defense	**44**
Resistance to antibiotics and toxic compounds	*31*
Streptothricin resistance	2
Copper homeostasis	10
Cobalt-zinc-cadmium resistance	13
Resistance to fluoroquinolones	2
Copper homeostasis: copper tolerance	3
Mercuric reductase	1
Invasion and intracellular resistance	*13*
Mycobacterium virulence operon involved in protein synthesis (SSU ribosomal proteins)	5
Mycobacterium virulence operon involved in DNA transcription	2
Mycobacterium virulence operon possibly involved in quinolinate biosynthesis	3
Mycobacterium virulence operon involved in protein synthesis (LSU ribosomal proteins)	3
Secondary Metabolism	**27**
Thiazole- oxazole-modified microcin (TOMM) synthesis	*10*
Lanthionine Synthetases	*17*
Iron acquisition and metabolism	**52**
Siderophores	*45*
Siderophore Enterobactin	1
Siderophore assembly kit	40
Siderophore Desferrioxamine E	4
Iron acquisition and metabolism - no subcategory	*7*
Heme, hemin uptake and utilization systems in GramPositives	6
Encapsulating protein for DyP-type peroxidase and ferritin-like protein oligomers	1

The average nucleotide identity of the genome sequences of *Streptomyces* strain A81 and *S. californicus* strain NRRL B-2895 was determined to be 97.99% (Figure 4.4). The ANI metric is used as a robust similarity metric to resolve inter- and intra-strain relatedness. The ANI value of 95% and above between two genomes has been typically used to indicate the same species, with lower values indicating different species (Jain *et al.,* 2018). This high ANI value indicates that despite the phenotypic differences strain A81 is in fact a strain of *S. californicus*.

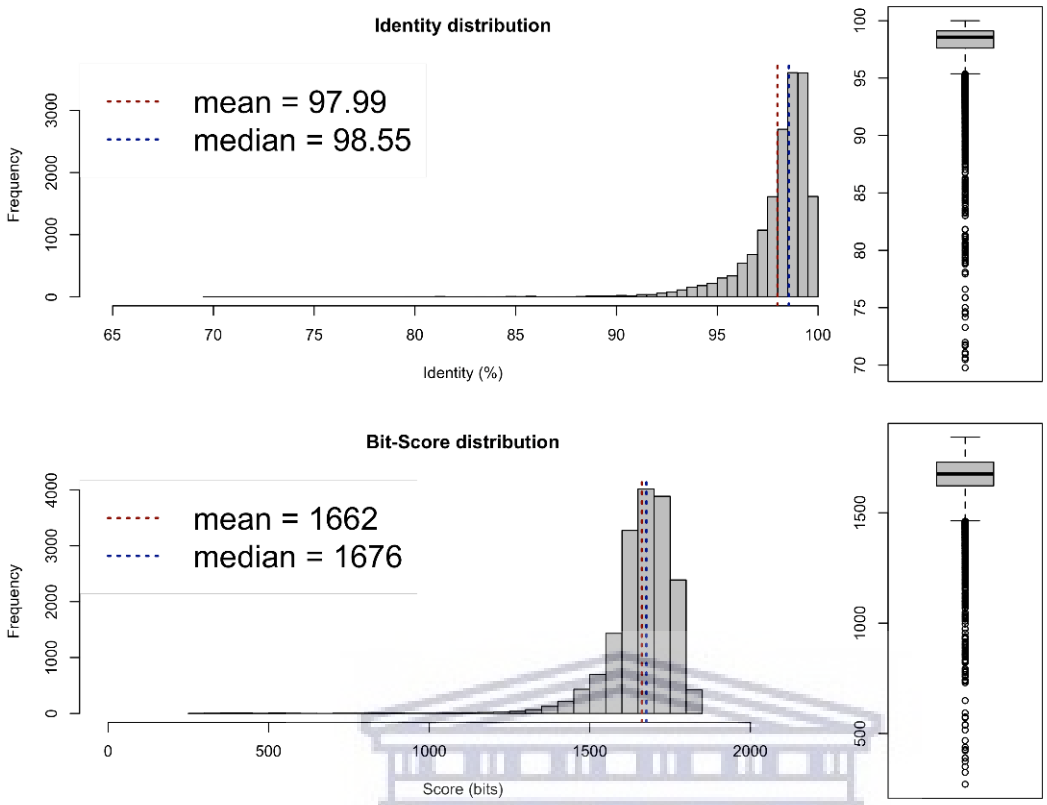


Figure 4.4: *Average nucleotide identity between* Streptomyces *strain A81 and* Streptomyces californicus *strain NRRL B-2895. Obtained from the online ANI calculator from Kostas Lab.*

The assembly of *Streptomyces* strain A159 represented 3.97 Mb of the genomic data and a %G+C content of 71.4%. Even though the genome assembly was only partial, some genetic data could still be evaluated. The bacterium's genome contained 230 subsystems (Table 4.3), with 18% of the coding regions found within the subsystems. The majority of the coding sequences were found within the amino acids and derivatives subsystem (244), carbohydrates subsystems (190) and the fatty acids, lipids and isoprenoids subsystem (103) (Figure 4.5).

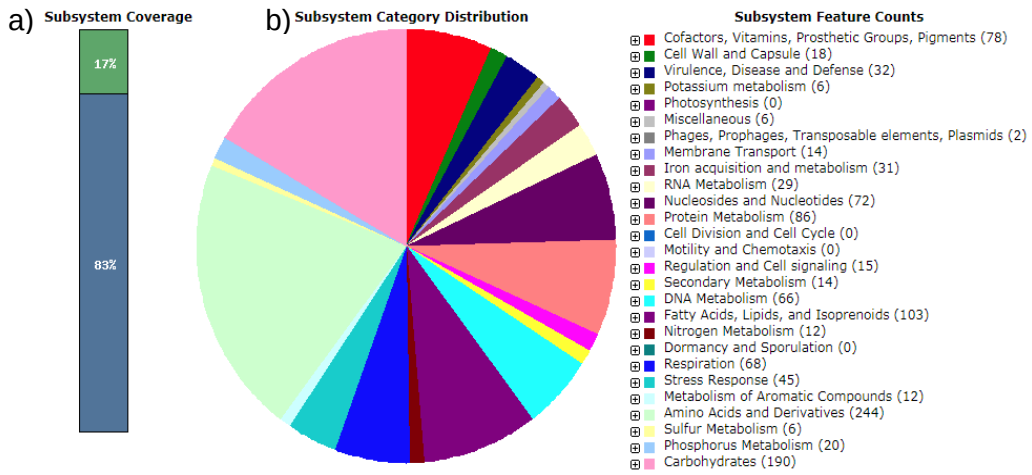


Figure 4.5: *Subsystem information of* Streptomyces *strain A159 obtained from the SEED database. a) The bar graph represents the percentage of protein encoding genes that are present in at least one subsystem (green bar). b) The pie chart and accompanying hierarchical tree, colour coded to match the pie chart, organises the subsystems (green bar) of the genome assembly by cellular process. The number of protein encoding genes in each category is listed in parentheses.*

Fourteen genes were found within the secondary metabolism subsystem. These genes were involved in TOMM and lanthionine biosynthesis (Table 4.5). Nine genes were involved in TOMM biosynthesis and included: 2 protein B genes (dehydrogenase), 2 protein C genes (cyclodehydratase), 3 protein D genes (docking scaffold) and 2 hypothetical proteins (FIG013786). As discussed previously, the C and D proteins are responsible for the cyclohydration of amino acids with a beta-nucleophile (cysteine, serine, or threonine) into thiazoline or (methyl)oxaline rings. These azoline rings can then be oxidized to azoles by the B protein (Melby *et al.,* 2011; Burkhart *et al.,* 2015). Since all of these coding sequences are present, we can assume that the final TOMM that is produced has azoles within its structure. Further investigation will need to be done to confirm the type of TOMM that is being produced by *Streptomyces* strain A159.

Five genes involved in lanthionine synthesis were identified. These genes included: 1 *lanB*, 2 *lanL*, 1 *lanM* and 1 protein-L-isoaspartate O-methyltransferase. The presence of these coding sequences indicate that the strain has the potential to produce class I, II and IV lanthiopeptides. Identification of new lanthiopeptides are of great pharmaceutical potential to treat antibiotic resistant pathogens, as resistance against lanthiopeptide antibiotics is very low. Thus, further investigation into these peptides will be of great interest. The coding sequences from this partial assembly only give a hint of the metabolic potential of the strain and future studies will need to be conducted to confirm the metabolic potential of the strain.

Twenty coding sequences were involved in resistance to antibiotics and toxic compounds including sequences that are involved in the resistance to the antibiotics streptothricin and fluoroquinolone. As discussed previously, these resistances may indicate the production of these antibiotic classes. The siderophore subsystem contained 30 coding sequences (Table 4.5) with siderophore production confirmed in section 3.3.5. The coding sequences does not give an indication of the type of siderophore that is being produced by this strain. Due to the only partial assembly of the strain, the ANI could not be determined. In addition, investigation of the secondary metabolite BGCs was also not conducted due to this partial assembly.

Table 4.5: Number of protein encoding genes within highlighted subsystem and their subcategory from Streptomyces *strain A159*

Subsystems and subcategories	**Number of coding sequences**
Virulence, Disease and Defense	**32**
Resistance to antibiotics and toxic compounds	*20*
Streptothricin resistance	2
Copper homeostasis	4
Cobalt-zinc-cadmium resistance	8
Resistance to fluoroquinolones	2
Copper homeostasis: copper tolerance	2
Mercuric reductase	2
Invasion and intracellular resistance	*12*
Mycobacterium virulence operon involved in protein synthesis (SSU ribosomal proteins)	5
Mycobacterium virulence operon involved in DNA transcription	3
Mycobacterium virulence operon involved in protein synthesis (LSU ribosomal proteins)	4
Secondary Metabolism	**14**
Thiazole- oxazole-modified microcin (TOMM) synthesis	*9*
Lanthionine Synthetases	*5*
Iron acquisition and metabolism	**31**
Siderophores	*30*
Siderophore assembly kit	30
Iron acquisition and metabolism - no subcategory	*1*
Encapsulating protein for DyP-type peroxidase and ferritin-like protein oligomers	1

The genome size of different strains of *Streptomyces californicus*, the closest relative to strains A81 and A159, is between 7.8 Mbp and 8.4 Mbp with a %GC content between 69.7 and 72.7% (https://www.ncbi.nlm.nih.gov/genome/genomes/32370; NCBI, accessed 14-02-2020). *Streptomyces* strain A81 has a genome size that is slightly smaller than one of the submitted strains with a similar %GC content.

Streptomyces strain S149 had a genome size of 8.29 Mbp with a 71.5% GC content (Table 4.3). The coding sequences were represented in 19% of the subsystem with majority of the genes represented by the following subsystems: carbohydrates (426 genes), the amino acids and derivatives (423 genes) and protein metabolism (227 genes) (Figure 4.6).

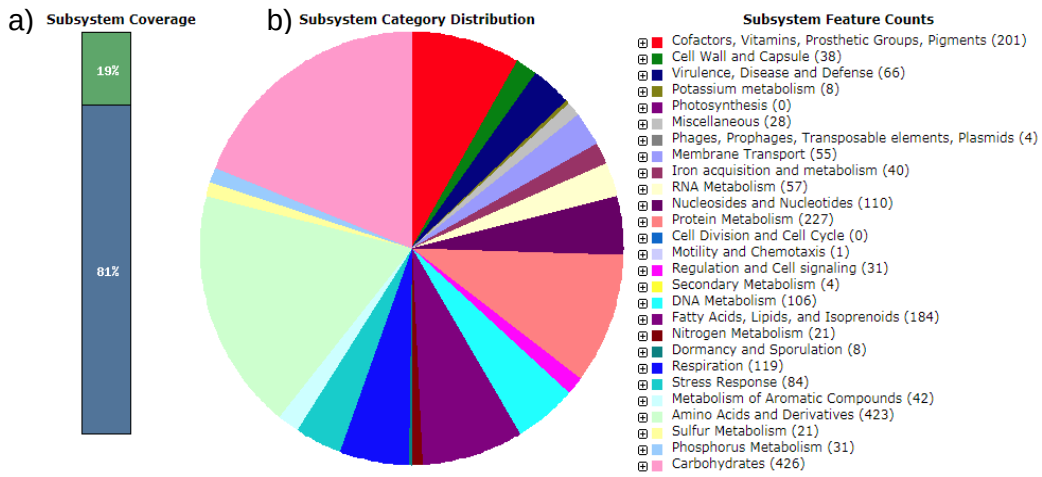


Figure 4.6: *Subsystem information of* Streptomyces *strain S149 obtained from the SEED database. a) The bar graph represents the percentage of protein encoding genes that are present in at least one subsystem (green bar). b) The pie chart and accompanying hierarchical tree, colour coded to match the pie chart, organises the subsystems (green bar) of the genome assembly by cellular process. The number of protein encoding genes in each category is listed in parentheses.*

Four genes from the secondary metabolism subsystem were identified. These genes are involved in auxin biosynthesis (Table 4.6). Auxin is a plant hormone derived from the amino acid tryptophan and is involved in plant growth and development. The auxins are involved in shoot elongation and root development. Various pathways for auxin biosynthesis exist in bacteria and a few use tryptophan as a precursor. The genes identified from *Streptomyces* strain S149 included three encoded protein sequences included in tryptophan biosynthesis; *trpD* (anthranilate phosphoribosyltransferase), *trpA* (tryptophan synthase alpha chain), *trpB* (tryptophan synthase beta chain) and one gene from the tryptamine pathway, monoamine oxidase gene. *Streptomyces* strain S149 was isolated from the leaves of *S. frutescens* and thus may stimulate plant growth. Auxin producing bacteria are known to stimulate seed germination, root formation and root proliferation (Ahemad and Kibret, 2014). Future investigation of *Streptomyces* strain S149 should evaluate the strains potential to stimulate plant growth promotion of not only *S. frutescens* but also other important crop species, especially legumes, as *S. frutescens* is a type of legume.

A recent study evaluated the genome sequences of sixteen plant growth promoting *Streptomyces* strains and found that 15 of the strains had the ability to produce auxin. Furthermore, 14 of the strains had genes involved in siderophore production (Subramaniam *et al.,* 2020). The presence of 35 siderophore coding sequences supports that *Streptomyces* strain S149 may stimulate plant growth promotion. Siderophore production of the strain was previously confirmed (Section 3.3.5). Six genes were found to be involved in the production of

the siderophore desferrioxamine E and four genes from aerobactin production (Table 4.6). Two main pathways for siderophore biosynthesis exists in microorganisms. One pathway involves NRPS multienzymes while the other pathway is NRPS-independent. Desferrioxamines are produced NRPS-independently and are a structurally related family of tris-hydroxamate siderophores that form strong hexadentate complexes with ferric iron. Additionally, desferrioxamine E is the main siderophore produced by *Streptomyces coelicolor* M145. A cluster of four genes (*desA – D*) that directs desferrioxamine biosynthesis was previously identified (Barona-Gómez *et al.,* 2004), and these four genes were also identified in *Streptomyces* strain S149 with two additional genes: a putative desferrioxamine E transporter and a hypothetical protein associated with desferrioxamine E biosynthesis. Biosynthetic gene clusters were also identified with high similarities to desferrioxamine genes via antiSMASH (Section 4.3.3.2). Aerobactin is also synthesised NRPS-independently and aerobactin-like gene clusters have been identified in various *Streptomyces* species (Doroghazi and Metcalf, 2013). Strain S149 did not contain any aerobactin-like BGCs. The genes identified via RAST included genes not involved in the main biosynthesis of aerobactin, and instead encoded for ferric hydroxamate ABC transporter proteins FhuD – B. These transport proteins are not exclusively involved in aerobactin synthesis but can also mediate the general uptake of iron, siderophore, heme and vitamin B12 (Köster, 2001). As strain S149 most likely does not produce aerobactin, the *fhuD – B* gene products may rather be involved in other iron-related roles.

Both genes involved in marinocine synthesis, *lodA* and *lodB*, were identified in *Streptomyces* strain S149 (Table 4.6). Marinocine, isolated from *Marinomonas mediterranea,* has activity against both Gram-positive and -negative bacteria (Lucas-Elio *et al.,* 2005). The marinocine (LodA) has lysine-epsilon oxidase activity. A second gene, *lodB*, is located adjacent *lodA*, with these genes constituting an operon. LodA is secreted to the external medium when the culture reaches stationary phase, whereas LodB has only been detected inside the cells. The production of marinocine in *M. mediterranea* requires functional copies of both genes (Gómez *et al.,* 2010). Since both these genes were found in *Streptomyces* strain S149, this bacteriocin may be produced by the strain. The strain had antibacterial activities against Gram-positive bacteria, *B. cereus*, *E. faecalis* and *M. aurum* (Section 3.3.2). Optimising the growth conditions of the strain may result in increased production of bacteriocins like marinocine. As indicated earlier, antibiotic resistance genes may also indicate the production of the antibiotic. Investigation of the BGCs (via antiSMASH; Figure 4.13) did not indicate the presence of known antibiotics in either of these antibiotic classes. Investigating the unknown/unidentified BGCs may reveal if gene clusters for these antibiotic classes are present.

Table 4.6: Number of protein encoding genes within highlighted subsystem and their subcategory from Streptomyces *strain S149*

Subsystems and subcategories	**Number of coding sequences**
Secondary Metabolism	**4**
Plant Hormones	*4*
Auxin biosynthesis	4
Virulence, Disease and Defense	**66**
Bacteriocins, ribosomally synthesized antibacterial peptides	*3*
Marinocine, a broad-spectrum antibacterial protein	2
Tolerance to colicin E2	1
Resistance to antibiotics and toxic compounds	*50*
Copper homeostasis	15
Cobalt-zinc-cadmium resistance	15
Mercuric reductase	1
Tetracycline resistance, ribosome protection type	2
Aminoglycoside adenylyltransferases	1
Arsenic resistance	8
Copper homeostasis: copper tolerance	3
Resistance to fluoroquinolones	2
Tetracycline resistance, ribosome protection type, too	2
Beta-lactamase	1
Iron acquisition and metabolism	**40**
Siderophores	*35*
Siderophore assembly kit	25
Siderophore Desferrioxamine E	6
Siderophore Aerobactin	4
Iron acquisition and metabolism - no subcategory	*5*
Heme, hemin uptake and utilization systems in Gram Positives	2
Ferrous iron transporter EfeUOB, low-pH-induced	3

Streptomyces strain S149 is closely related to *Streptomyces canus.* The genome sizes of *S. canus* strains are between 7.79 Mbp and 11.57 Mbp with a %GC content ranging from 70.2 to 72% (https://www.ncbi.nlm.nih.gov/genome/genomes/16476; NCBI, accessed 14-02-2020). *Streptomyces* strain S149 has a genome size and %GC content that was comparable to *S. canus* strains. The average nucleotide identity of the genome sequences of *Streptomyces* strain S149 and *S. canus* strain C-509 was compared and found to be 84.56% (Figure 4.7). The low ANI value indicates that strain S149 is not the same species as *S. canus* and should be classified as a different species. An ANI value of 95% or higher has been shown to correspond to the DNA-DNA hybridisation value of 70%. Thus, an ANI value lower than 95% indicates non-related species. Further research, including chemotaxonomy and better

genome sequencing, of *Streptomyces* S149 need to be performed to confirm that this strain is a novel isolate within the class of *Streptomyces*.

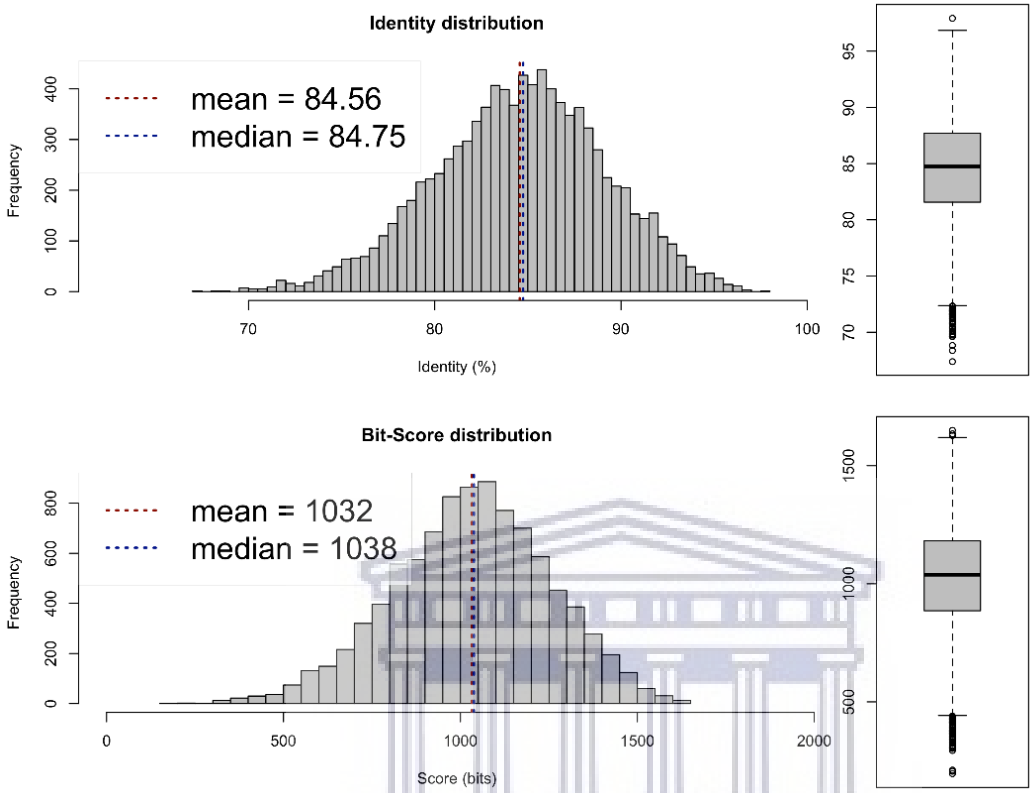


Figure 4.7: *Average nucleotide identity between* Streptomyces *strain S149 and* Streptomyces canus *strain C-509. Obtained from the online ANI calculator from Kostas Lab.*

The general characteristics of the genomes of the strains corresponded to the general characteristics of *Streptomyces* species. The genomes had %GC content of more than 70% and had genome sizes bigger than 5.18 Mbp (Kirby *et al.,* 2012), although the genome size for A159 could not be determined. This partial genome assembly may be a result of poor sequence quality and needs to be repeated for better results as discussed previously. The genomes of the strains also revealed machinery involved in spore production, antibiotic resistance and siderophore synthesis. All the strains produced spores and had positive siderophore activity. Antibiotic resistance of the strains has not been tested.

4.3.3 Biosynthetic gene clusters

Secondary metabolites produced by bacteria are an important source of antimicrobial and other bioactive compounds with potential application in a variety of industries including pharmaceutical, food, agriculture and more. In recent years, genome mining of biosynthetic gene clusters has become important in identifying and characterising new compounds and assisted in metabolic engineering.

Biosynthetic gene clusters from the three assembled genomes were evaluated using antiSMASH software. The software identifies BGCs and determines which known cluster is the most similar to the query sequence (Blin *et al.,* 2019). Unknown gene clusters can thus not be identified and thus low similarities may indicate new gene clusters involved in the synthesis of novel compounds.

4.3.3.1 Streptomyces strain A81:

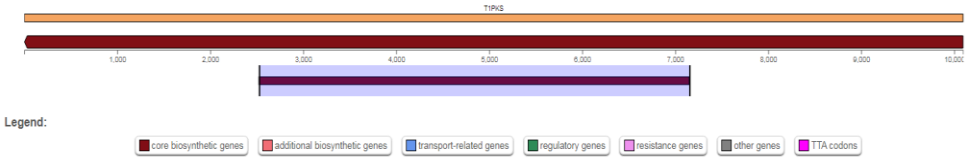
Streptomyces strain A81 contained 35 putative biosynthetic gene clusters of which the following were involved in the biosynthesis of polyketide synthase (PKS) compounds: six type-1 PKS, one type-2 PKS and two type-3 PKS biosynthesis clusters. Eight biosynthetic gene clusters were identified in the synthesis of non-ribosomal peptide synthetases (NRPSs). Furthermore, two BGCs were found for NRPS-independent siderophores, two for lanthipeptides, one for bacteriocin and three for terpenes. Hybrid clusters were also detected and included a type-1 PKS/NRPS, a ladderane/arylpolyene/NRPS and a NRPS/linaridin cluster (Figure 4.7).

Identified secondary metabolite regions

Region	Type	From	To	Most similar known cluster		Similarity
Region 4.1	terpene	1	7,063	Steffimycin	t2pks-saccharide	16%
Region 12.1	lanthipeptide	1	12,349			
Region 20.1	T1PKS, NRPS-like	1	7,469			
Region 51.1	T1PKS	1	5,862			
Region 52.1	T1PKS	1	10,094			
Region 54.1	ectoine	15,188	21,577	Ectoine	other	100%
Region 66.1	NRPS	1	16,845	Coelichelin	NRPS	63%
Region 71.1	NRPS	1	15,924	Griseobactin	NRPS	35%
Region 73.1	NRPS	1	10,605	Meridamycin	nrps-t1pks	7%
Region 100.1	terpene	1	17,670	Geosmin	terpene	100%
Region 118.1	bacteriocin	1	7,321			
Region 120.1	ectoine	1	7,405	Showdomycin	other	35%
Region 123.1	siderophore	9,033	20,331	Desferrioxamine B	other	80%
Region 125.1	ladderane, arylpolyene, NRPS-like	1	32,399	Skyllamycin	NRPS	26%
Region 130.1	T1PKS	1	6,206			
Region 134.1	T1PKS	1	5,825	Micromonolactam	t1pks	100%
Region 144.1	NRPS, linaridin	1	44,177	Pentostatine / vidarabine	other	12%
Region 161.1	butyrolactone	567	8,418	Coelimycin	t1pks	12%
Region 180.1	terpene	1	26,538	Hopene	terpene	69%
Region 217.1	T3PKS	1	8,569	Alkylresorcinol	t3pks	100%
Region 235.1	butyrolactone	1	5,648	Showdomycin	other	11%
Region 237.1	NRPS-like	1	11,210	Paromomycin	saccharide	7%
Region 241.1	T1PKS	1	5,442	Mediomycin A	polyketide	28%
Region 264.1	melanin	2,883	11,031	Melanin	other	100%
Region 289.1	NRPS	1	14,956	Griseobactin	NRPS	47%
Region 291.1	lanthipeptide	1	23,916			
Region 295.1	NRPS	1	33,221	Viomycin	NRPS	28%
Region 318.1	NRPS	1	23,857	Sporolide	nrps-t1pks	6%
Region 328.1	T3PKS	1	22,592	Naringenin	terpene	100%
Region 343.1	siderophore	1	9,324	Ficellomycin	NRPS	3%
Region 352.1	other	1	9,072	Kanamycin	saccharide	1%
Region 401.1	NRPS	1	6,626			
Region 457.1	melanin	1	6,752	Melanin	other	100%
Region 468.1	T1PKS	1	9,234	Cremimycin	t1pks	30%
Region 499.1	T2PKS	1	20,301	Griseorhodin	t2pks	42%

Figure 4.8: *Putative biosynthetic gene clusters of* Streptomyces *strain A81 predicted 35 secondary metabolite regions via the antiSMASH software.*

a



b

c

KS domain 1:

Database match id	percent identity	align length (bp)	e-value	pathway product	domain class
AveA3_Q9S0R4_3 mod	74	427	9e-177	avermectin	modular
AveA3_Q9S0R4_2 mod	73	427	2e-175	avermectin	modular
AveA1_Q9S0R8_2 mod	73	426	8e-174	avermectin	modular

KS domain 2:

Database match id	percent identity	align length (bp)	e-value	pathway product	domain class
AveA3_Q9S0R4_3 mod	75	428	1e-166	avermectin	modular
AveA2_Q9S0R7_4 mod	74	430	6e-162	avermectin	modular
AveA3_Q9S0R4_2 mod	73	428	3e-161	avermectin	modular

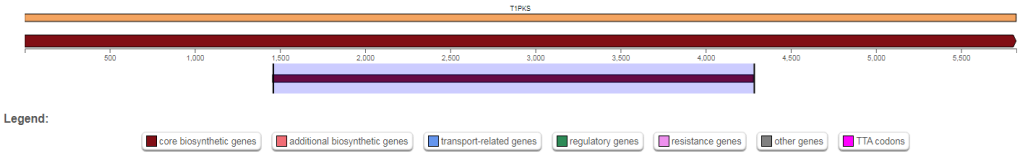
Figure 4.9: *a) Region 52.1 showed sequence similarity to an unknown type 1 PKS BGC. b) The region also had 100% similarity to sequences from various* Streptomyces *species,* Micromonospora sp Rc5 *and* Microtetraspora niveoalba*. c) Both KS domains showed sequence similarity to domains involved in the production of avermectin. Results obtained from antiSMASH software.*

Region 52.1 has high sequence similarity to an unknown type-1 PKS gene cluster (Figure 4.9 a) and 100% similarity to various *Streptomyces* species genes (Figure 4.9 b). Evaluation of

the KS domains of this cluster via NaPDoS revealed that the domains had 73% or higher sequence similarity to domains involved in the production of avermectin (Figure 4.8 c). Avermectins are drugs used for the treatment of parasitic worms and insect pests (Ikeda *et al.,* 1999). The KS domain of *Streptomyces* strain A81 was further evaluated via PCR-amplification and Sanger sequencing and showed an even higher similarity (88%) to the AveA3 domain (Section 4.3.1, Table 4.1). The aligned size from the PCR-amplified product was smaller than the size from the whole genome sequence and thus will influence the similarity percentage. Further investigation into this BGC is needed to identify whether the domain is involved in the production of avermectin or if it is involved in the production of a new bioactive compound.

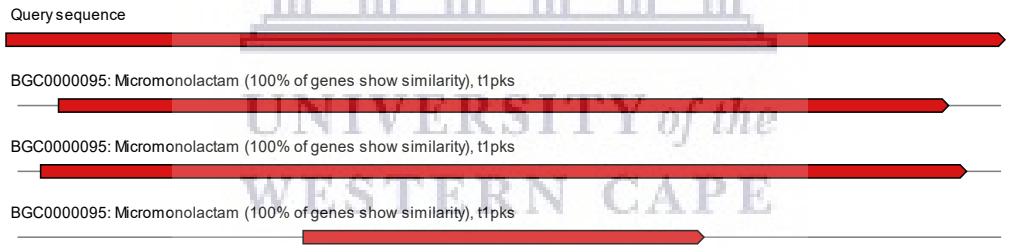
Likewise, the KS-domain from region 134.1 (also identified as a type-1 PKS; Figure 4.10 a) has 74% similarity to the domain involved in avermectin biosynthesis (Figure 4.10 d). The biosynthetic cluster had 100% similarity to genes from various actinobacteria species including, *Streptomyces leeuwenhoekii*, *Streptacidiphilus neutrinimicus*, *Kitasatospora aureofaciens* and *Actinomadura kijaniata* (Figure 4.10 b). The cluster genes were 100% similar to those involved in micromonolactam synthesis (Figure 4.10 c). Micromonolactam is a polyene macrocyclic lactam and is only produced by *Micromonospora* species (Skellam *et al.,* 2013). *Streptomyces* species produce a range of polyene macrolactams including, vicenistatin (Otsuka *et al.,* 2000), niizalactam (Hoshino *et al.,* 2015), cyclamenol (Shen *et al.,* 2020), heronamides (Raju *et al.,* 2010), ciromicin (Hoshino *et al.,* 2018), sceliphrolactam (Low *et al.,* 2018), and more. Macrolactams have antifungal, antibiotic and antitumor activities and discovery of new macrolactams are of pharmaceutical importance. Due to the large amount of macrolactams produced by *Streptomyces* species, it is possible that the macrolactam produced by strain A81 is either a known macrolactam or possibly a novel macrolactam. It is highly unlikely that it is producing micromonolactam due to it only being produced by *Micromonospora* species. If it is indeed micromnolactam, this would be the first report of micromonolactam production by a *Streptomyces* species.

a)

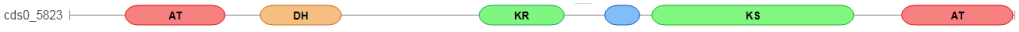


b

c



d)



Database match id	percent identity	align length (bp)	e-value	pathway product	domain class
AveA3_Q9S0R4_3mod	74	428	0.0	avermectin	modular
AveA3_Q9S0R4_2mod	73	428	2e-177	avermectin	modular
NysK_Q9L4X1_1mod	74	424	1e-175	nystatin	modular

Figure 4.10: *a) Region 134.1 has 100% similarity to type 1 PKS genes from b) various Actinobacterial species. c) the genes show 100% similarity to genes involved in micromonolactam biosynthesis. d) Evaluation of the KS-domain revealed similarities to domains involved in avermectin and nystatin production. Results obtained from antiSMASH software.*

A total of five siderophore BGCs were identified including NRPS and NRPS-independent clusters. Region 66.1 was found to be similar to BGCs involved in NRPS-dependent siderophore synthesis and was 45% similar to the NRPS gene cluster from *Streptomyces* sp. NRRL F-3218. The query gene clusters were 63% similar to the genes involved in the synthesis of coelichelin and 66% similar to the genes involved in the synthesis of exochelin (Supplementary Figure D1.1). Coelichelin is a tripeptide siderophore, first discovered through genome mining of *Streptomyces coelicolor* (Challis and Ravel, 2000). Exochelin is a pentapeptide siderophore and is produced by *Mycobacterium smegmatis* and is essential for biofilm formation under iron-limiting conditions (Ojha and Hatfull, 2007). Exochelin has only been reported to be produced in *Mycobacterium* species, whereas coelichelin is reported to be produced in *Streptomyces* species, and is thus highly likely that *Streptomyces* strain A81 produces coelichelin or an analogue of it. Another NRPS-dependent siderophore gene cluster was predicted on region 71.1 that had 26% similarity to *Streptomyces* species NRRL F-3218. The BGCs had 35% similarity to genes involved in griseobactin synthesis (Supplementary Figure D1.2). Griseobactin is a catechol-peptide siderophore produced by several *Streptomyces griseus* strains as well as *Streptomyces* sp. ATCC 700974 (Patzer and Braun, 2010). The NRPS cluster of region 289.1 also had similarities to griseobactin (47%, Supplementary Figure D1.3). An NRPS-independent siderophore cluster similar to desferrioxamine was identified. This siderophore is the typical siderophore produced by *Streptomyces* species (Imbert *et al.,* 1995). This BGC from region 123.1 is 100% similar to genes involved in siderophore production in various *Streptomyces* species (Figure 4.11 a, b) with 80% similarity to genes involved in the synthesis of desferrioxamine B (Figure 4.11 c). Siderophore production was confirmed for this strain on CAS media plates and moderate activity was observed, with a zone size of 419 mm^2 (Section 3.3.5). The strain has the metabolic potential to produce various siderophores. Multiple siderophore gene clusters may give an indication of the strain to play a direct or indirect role in plant growth and development by supplying the plant with soluble iron or protecting the plant against pathogens.

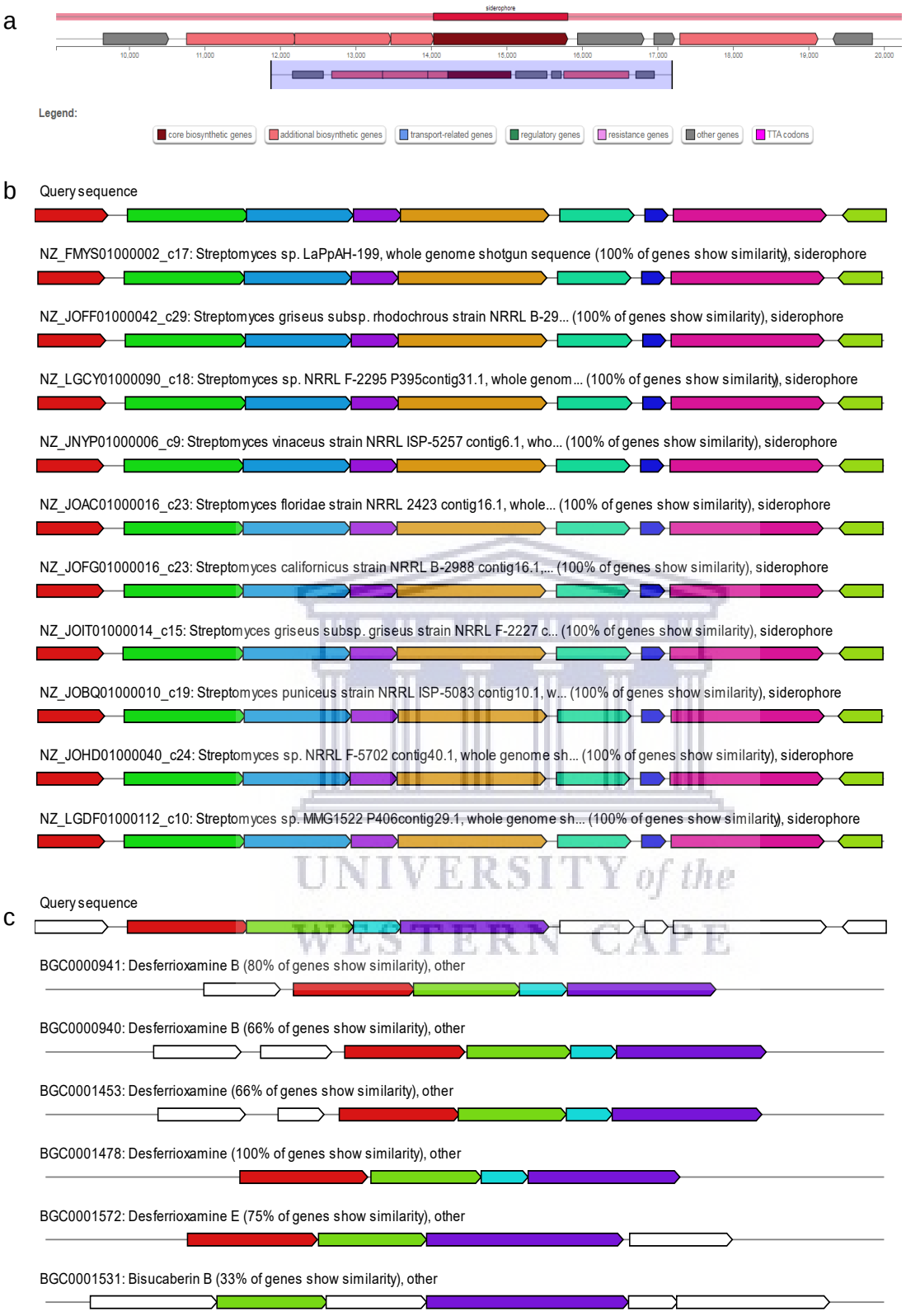


Figure 4.11: *a) Region 123.1 showed high similarity to biosynthetic genes of siderophores from b) various* Streptomyces *species. c) 80% of genes had similarity to genes involved in the synthesis of desferrioxamine B. Results obtained from antiSMASH software.*

Type-3 PKS clusters were identified in regions 217.1 (Figure 4.12) and 328.1 (Supplementary Figure D1.4) which both shared sequence homology to conventional plant metabolites. Region

217.1 shared 21% of the biosynthetic genes with *Streptomyces californicus* strain NRRL B-2988 (Figure 4.12 b). The genes in this region were 100% similar to biosynthetic genes involved in the synthesis of alkylresorcinol. This metabolite is a phenolic lipid which is mainly found in plants, but have been identified in bacteria and fungi as well (Kozubek and Tyman, 1999). Previously, an alkylresorcinol-type antibiotic, DB-2073, was isolated from *Pseudomonas* species B-9004 (Kitahara and Kanda, 1975). Phenolic lipid synthesis is regulated by the srs operon identified in *Streptomyces griseus* and consists of three genes *srsA*, *srsB* and *srsC*, which encode a type-3 PKS, a methyltransferase and a flavoprotein hydroxylase, respectively (Funabashi *et al.,* 2008; Nakano *et al.,* 2012). The type-3 PKS is responsible for the synthesis of the phenolic lipids, alkylresorcinols and alkylpyrones. A *srsA*-mutant, which produced no phenolic compounds, was highly sensitive to β-lactam antibiotics and thus suggests that the phenolic lipids confer β-lactam antibiotic resistance to the bacteria (Funabashi *et al.,* 2008). This srs operon has only been reported in *S. griseus* and thus discovery of this operon in other *Streptomyces* may be of importance, as alkylresorcinol-type antibiotics have been reported from other bacterial families. *Streptomyces* strain A81 was isolated from soil samples associated with *Aloe ferox*, which produces a 5-alkylresorcinol, orcinol (Salehi *et al.,* 2018). This further supports that strain A81 may produce an alkylresorcinol-type antibiotic and that it may have obtained the genes involved from the host plants. Additionally, another biosynthetic gene cluster associated with a typical plant metabolite was found in the genome of *Streptomyces* strain A81. The cluster is located on region 328.1 and had 59% similarity to genes from *Streptomyces* sp. NRRL F-5702 (Supplementary Figure D1.4). The genes corresponded to a type-3 PKS and had 100% similarity to genes involved in the synthesis of naringenin. Naringenin is a typical plant flavone, with *Streptomyces clavilugerus* being the first microorganism discovered to produce the compound naturally (Álvarez-Álvarez *et al.,* 2015). This is another example of a plant associated microbe that has the potential to produce so-called plant metabolites. Naringenin is one of the main flavonoids produced by *Aloe* species (Dagne *et al.,* 2000; Salehi *et al.,* 2018) and this may give an indication that the genes identified in *Streptomyces* strain A81 for naringenin production may have been obtained from the associated *A. ferox* plant. The presence of these two type-3 PKS clusters that are both involved in the production of plant metabolites (also found within its host *A. ferox*) shows the potential for strain A81 to produce these typical plant metabolites, possibly due to the close association it has with the plant. This may further support the strains potential to influence plant growth and development and that the microorganism may even play an important role in the plant's metabolite biosynthesis by providing important precursors that the plant may use and vice versa.

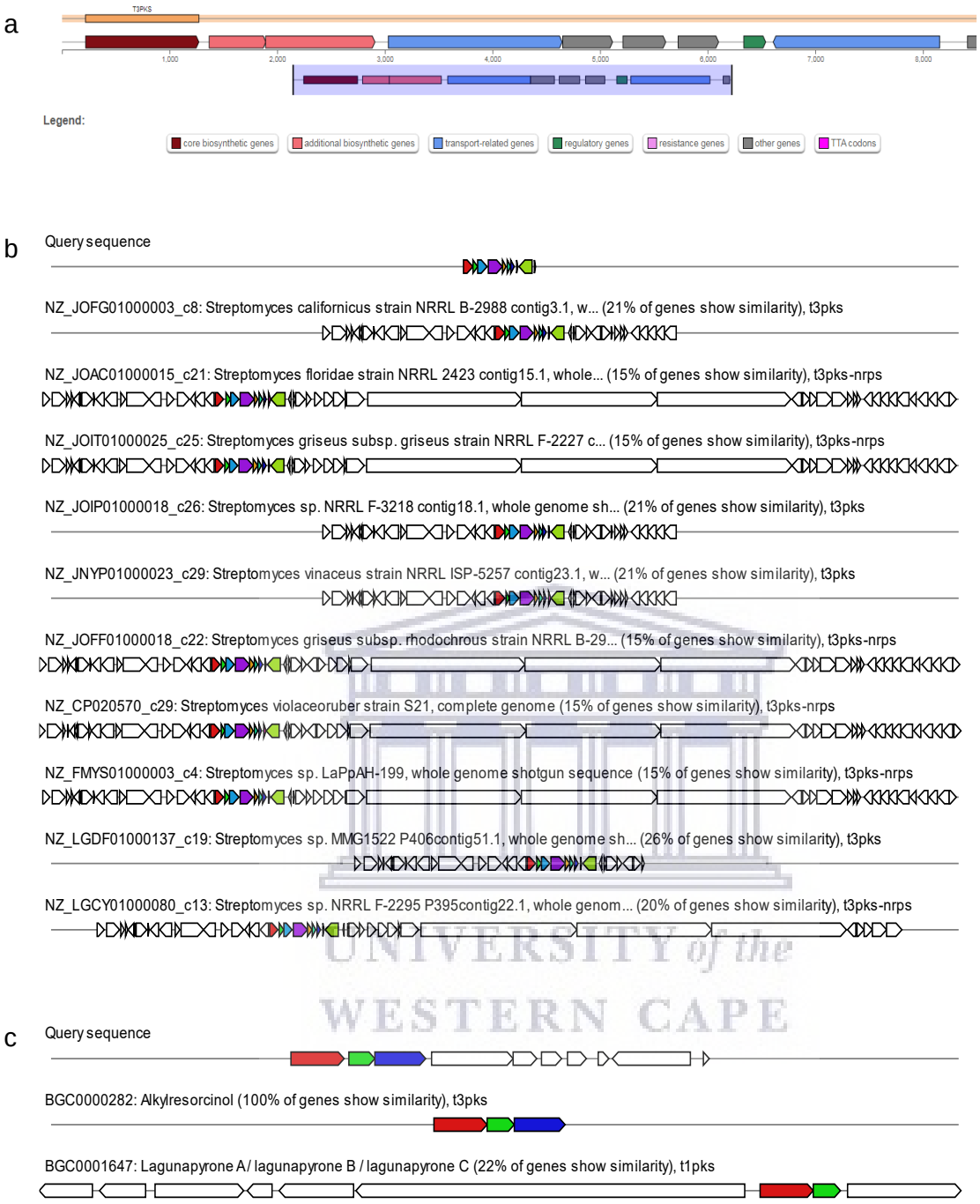


Figure 4.12: *a) Region 217.1 showed similarity to type-3 PKS genes from b)* Streptomyces *species. c) The biosynthetic genes were 100% similar to the genes involved in the production of alkylresorcinol. Results obtained from antiSMASH software.*

A type-2 PKS BGC with similarity to the antibiotic, griseorhodin was identified (Figure 4.13). Griseorhodins are hydroxyquinone antibiotics first isolated from a red substance produced by *Streptomyces californicus* (Eckardt *et al.,* 1978). Griseorhodins are a family of modified aromatic polyketides and have great structural diversity which is largely introduced by enzymatic oxidation. This significantly influences bioactivity, with these compounds exhibiting activities such as the inhibition of HIV reverse transcriptase and human telomerase. New

neuroactive griseorhodins D-F were isolated from the marine bacteria, *Streptomyces* sp. CN48+ (Lin *et al.,* 2014). The unique metabolism of this organism allowed for the production of these griseorhodins through enzymatic oxidation. Rubromycin, like griseorhodin, was also first isolated from a red substance produced by *Streptomyces collinus* (Brasholz *et al.,* 2007) with these two compounds being structurally related (Puder *et al.,* 2000). Rubromycins are antitumor antibiotics with a complex molecular architecture having a central spiroketal unit as the key feature. The biosynthetic genes located on region 499.1 of *Streptomyces* strain A81 was 42% similar to that of the genes involved in the synthesis of griseorhodin and 56% to the genes involved in rubromycin production (Figure 4.13). Solvent extractions of *Streptomyces* strain A81 are bright pink-red in colour and one of its closest relatives is *S. californicus* (Section 2.3.2.4), and thus, there is a high probability that *Streptomyces* strain A81 produces one of these complex aromatic polyketide-type antibiotics. Investigating these compounds from *Streptomyces* strain A81 would be of interest since the strain was isolated from a unique environment and may also possess enzymes that can possibly modify the end product of the antibiotic compounds, with the potential of discovering compounds that are structurally similar to griseorhodins and rubromycins.

Streptomyces strain A81, had antibacterial activities against *B. cereus*, *E. faecalis*, *M. aurum*, *M. smegmatis*, *P. aeruginosa* and *S. aureus*. The strain has 35 putative secondary biosynthetic gene clusters and includes BGCs for a variety of antibiotic compounds. The KS-domain for avermectin, an anthelmintic drug, was present in the genome of strain A81. The ability of strain A81 to inhibit the growth of parasitic worms was not investigated and should be included in a future study after avermectin production by the strain has been confirmed. The BGC for a macrolactam-type antibiotic was also found. This type of antibiotic has a broad range of functions including antifungal, antibacterial and antitumor activities. The type of macrolactam produced by the strain needs to be characterised and the activity tested.

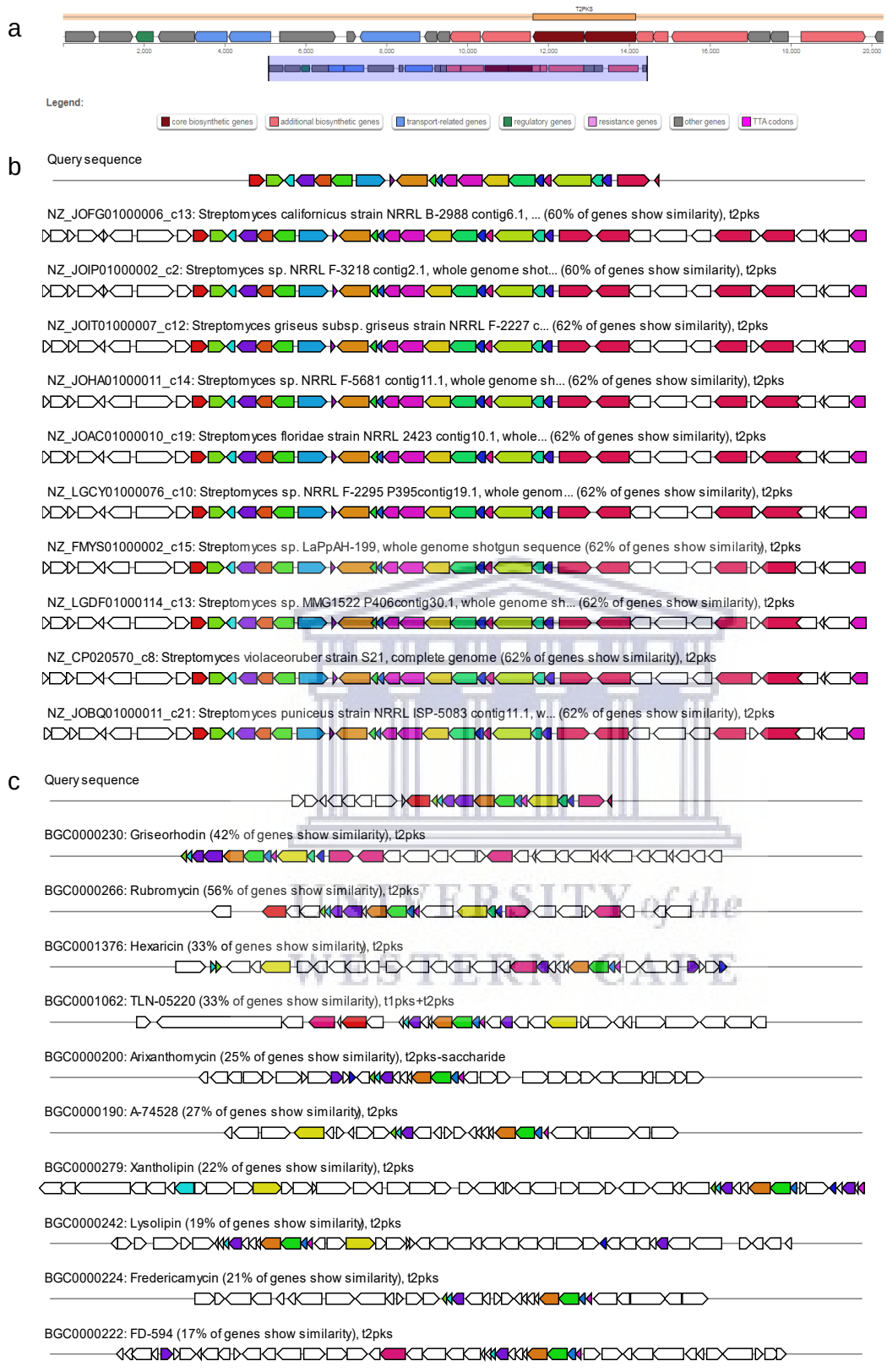


Figure 4.13: *a) Region 499.1 showed similarities to type-2 PKS biosynthetic genes and b) had 60% and higher similarities to genes from various* Streptomyces *species. c) The biosynthetic genes showed 42% similarity to griseorhodin and 56% similarity to rubromycin biosynthetic genes. Results obtained from antiSMASH software.*

4.3.3.2 Streptomyces strain S149:

The antiSMASH software predicted 39 biosynthetic gene clusters in *Streptomyces* strain S149. The putative BGCs included five type-1 PKSs, one type-2 PKS, one type-3 PKS, seven NRPSs, three lanthipeptides, three siderophores, five terpenes, two bacteriocins, one lasso peptide, and one butyrolactone. Hybrid BGCs was also predicted and included four hybrid type-1 PKS-NRPSs, and a type-3 PKS-indole (Figure 4.13).

Identified secondary metabolite regions

Region	Type	From	To	Most similar known cluster		Similarity
Region 14.1	butyrolactone	8,749	17,430	Scleric acid	NRPS	11%
Region 15.1	lanthipeptide	1	10,215			
Region 42.1	T3PKS	14,238	45,513	Herboxidiene	t1pks+t3pks	7%
Region 109.1	melanin	1	9,927	Melanin	other	60%
Region 120.1	T3PKS, indole	1	21,079	7-prenylisatin	other	60%
Region 127.1	siderophore	5,417	17,186	Desferrioxamine B	other	83%
Region 128.1	terpene	4,157	25,242	Albaflavenone	terpene	100%
Region 171.1	T1PKS	1	10,300	Oligomycin	t1pks	33%
Region 200.1	NRPS	4,629	29,884	Allylmalonyl-CoA	other	20%
Region 203.1	NRPS	1	10,330			
Region 211.1	terpene	1	16,492	Isorenieratene	terpene	100%
Region 212.1	lanthipeptide	1	11,507	Informatipeptin	lanthipeptide	28%
Region 225.1	NRPS, T1PKS	1	22,446	Phthoxazolin	nrps-t1pks	6%
Region 227.1	T1PKS	1	14,997	Lasalocid	t1pks	27%
Region 239.1	NRPS, T1PKS	6,304	50,771	Rakicidin A / rakicidin B	nrps-t1pks	22%
Region 262.1	T1PKS	1	12,640	Amycomicin	t1pks	25%
Region 270.1	melanin	3,014	9,425	Melanin	other	57%
Region 300.1	bacteriocin	1	5,789			
Region 306.1	NRPS	1	19,510	Coelichelin	NRPS	72%
Region 309.1	terpene	1	5,582			
Region 334.1	NRPS-like	1	11,500	Ikarugamycin	nrps-t1pks	12%
Region 357.1	T1PKS	1	31,262	Maduropeptin	t1pks+t1pks	3%
Region 358.1	bacteriocin	5,008	15,223	Informatipeptin	lanthipeptide	42%
Region 359.1	terpene	33,703	44,098	Ebelactone	polyketide	5%
Region 363.1	siderophore	4,396	11,857			
Region 369.1	T1PKS	1	9,145			
Region 372.1	NRPS	1	6,074	Coelichelin	NRPS	18%
Region 376.1	T1PKS	1,597	32,209	Oligomycin	t1pks	44%
Region 385.1	NRPS	1	6,571	WAP-8294A2 (lotilibcin)	NRPS	30%
Region 390.1	terpene	1	18,579	Hopene	terpene	92%
Region 395.1	NRPS, T1PKS	1	42,683			
Region 419.1	ectoine	16,740	27,138	Ectoine	other	100%
Region 439.1	siderophore	450	8,483			
Region 446.1	lanthipeptide	1	5,771	Catenulipeptin	lanthipeptide	40%
Region 447.1	NRPS, T1PKS	1	22,086	Guadinomine	nrps-t1pks	19%
Region 450.1	NRPS	1	9,711			
Region 462.1	terpene	1	10,534	Geosmin	terpene	100%
Region 476.1	T2PKS	1	25,686	Spore pigment	t2pks	83%
Region 480.1	lassopeptide	1	12,620	Citrulassin D	ripp	100%

Figure 4.13: *Putative biosynthetic gene clusters of* Streptomyces *strain S149 predicted 39 secondary metabolite regions via the antiSMASH software.*

Region 120.1 was similar to a hybrid type-3 PKS-indole BGC. The genes were 100% similar to the gene cluster from *Streptomyces* species NRRL S-475 (Supplementary Figure D2.1 a, b). The indole genes were 60% similar to the genes involved in 7-prenylisatin biosynthesis (Supplementary Figure D2.1 c). This prenylated isatin-type antibiotic was first isolated from *Sterptomyces* species MBT28 using NMR-based metabolomics. The compound was characterised by NMR-guided tracking of the target proton signal. After which metabolite-guided genome mining combined with proteomics was used to identify the gene cluster with an indole prenyltransferase that catalyses the conversion of tryptophan into 7-prenylisatin. The newly found antibiotic additionally had antimicrobial activity against *Bacillus subtilis* (Wu *et al.,* 2015). The type-3 PKS genes in this region were also 100% similar to genes of germicidin (Supplementary Figure D2.1 c). Germicidins are compounds that inhibit spore germination and hyphal elongation of *Streptomyces* species (Petersen and Zähner, 1993; Aoki *et al.,* 2011). Germicidin synthase (Gcs) has broad substrate flexibility for acyl groups linked through a thioester bond to either coenzyme A (CoA) or acyl carrier protein (ACP) (Chemler *et al.,* 2012).

The gene clusters from region 262.1 was identified as a type-1 PKS and had 25% similarity to the genes of *Steptomyces* species NRRL S-475. The genes had 100% similarity to micromonolactam and 25% similarity to amycomicin biosynthetic genes (Figure 4.14). As discussed previously, micromonolactam is a polyene macrocyclic lactam that is only produced by *Micromonospora* species (Skellam *et al.,* 2013). Even though it has 100% similarity to the biosynthetic genes of this cluster, it is possible that a different type of macrocyclic lactam is being produced. Skellam *et al.,* (2013) also observed that the biosynthetic gene cluster for micromonolactam contained 11 polyketide modules that was found on various contigs. The gene cluster found in this study on region 262.1 only contained two polyketide modules and thus the other modules for the synthesis of a macrolactam may be on other contigs. Macrolactams are PKS/NRPS hybrids and thus the other modules involved in the synthesis may be found within other yet unidentified NRPS modules. Amycomicin was recently discovered by co-culturing of two actinobacteria. *Streptomyces coelicolor* M145 stimulated the production of amycomicin in *Amycolatopsis* species AA4. This highly modified fatty acid contained an epoxide isonitrile and had potent inhibition activity against the Gram-positive pathogen, *Staphylococcus aureus* (Pishchany *et al.,* 2018). An epoxide isonitrile-type antibiotic was first isolated in 1988 by Parker *et al.,* and was named aerocyanidin, produced by *Chromobacterium violaceum* ATCC 53434. Another isonitrile-type antibiotic, YM-47515, was produced by the actinobacteria, *Micromonospora echinospora* subspecies *echinospora* (Sugawara *et al.,* 1997). To date, no synthesis of an isonitrile-type antibiotic has been reported to be produced by *Streptomyces* species. *Streptomyces* strain S149, did have antimicrobial

activity against the Gram-positive *B. cereus, M. aurum* and *E. faecalis* (Section 3.3.2 – 3.3.4) and may indicate the production of an isonitrile-type antibiotic similar to amycomicin.

Region 446.1 was similar to a type-2 PKS, with 63% similarity to genes of *Streptomyces* species NRRL S-475 (Supplementary Figure D2.2). The genes had 100% similarity to genes involved in curamycin biosynthesis. Curamycin, a chlorine-containing glycosidic antibiotic, was isolated from *Streptomyces curacoi* (Galmarini and Deulofeu, 1961). Few genetic studies have been conducted on the genes involved in curamycin biosynthesis. In 1992, Bergh and Uhlén described the cloning of the PKS cluster from *S. curacoi*. The researchers concluded that the cluster was either involved in the biosynthesis of curamycin or a spore pigment. From the whole genome sequence of *Streptomyces* strain S149, high similarities to curamycin (100%) and spore pigment (83%) genes are observed (Supplementary Figure D2.2 c).

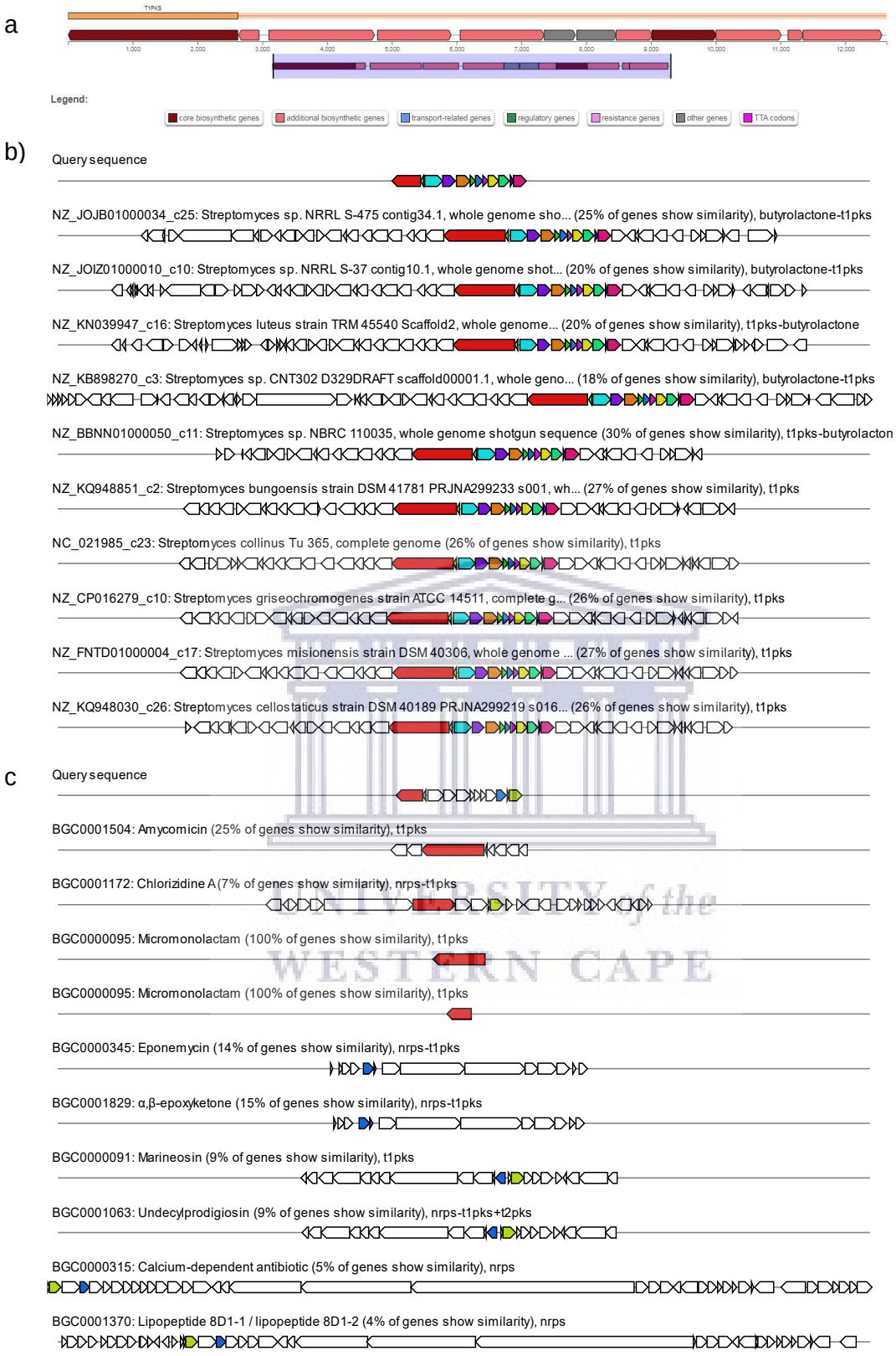


Figure 4.15: *a) Region 262.1 showed similarities to type-1 PKS biosynthetic genes and b) had similarities to genes from various* Streptomyces *species. c) The biosynthetic genes showed 25% similarity to amycomicin and 100% similarity to micromonolactam biosynthetic genes. Results obtained from antiSMASH software.*

An NRPS gene cluster found on region 306.1 form *Streptomyces* strain S149 had 38% similarity to *Streptomyces* species NRRL S-475 genes. The biosynthetic genes of this region had 72% similarity to genes involved in coelichelin synthesis (Supplementary Figure D2.3). As discussed previously, coelichelin is a tripeptide siderophore, first discovered through genome mining of *Streptomyces coelicolor* (Challis and Ravel, 2000). Desferrioxamine-type siderophores are the main siderophore produced by *Streptomyces* species (Imbert *et al.,* 1995), with genes for the production of this siderophore found on region 127.1 (Figure 4.4.16a). The genes on this region were 100% similar to genes from various *Streptomyces* species (Figure 4.16b) and was 75% and more similar to genes involved in desferrioxamine production (Figure 4.16c). Siderophore activity of the isolate was measured previously using a plate CAS-assay and the strain had a zone size of 1206 mm^2 (Section 3.3.5). Biosynthetic gene clusters for ectoine biosynthesis were also found. This compound helps protect the bacterial cell against high temperatures and salt conditions (Bursy *et al*., 2008). The strain was able to tolerate temperatures of up to 37 °C and NaCl salt concentrations of up to 8% (w/v).

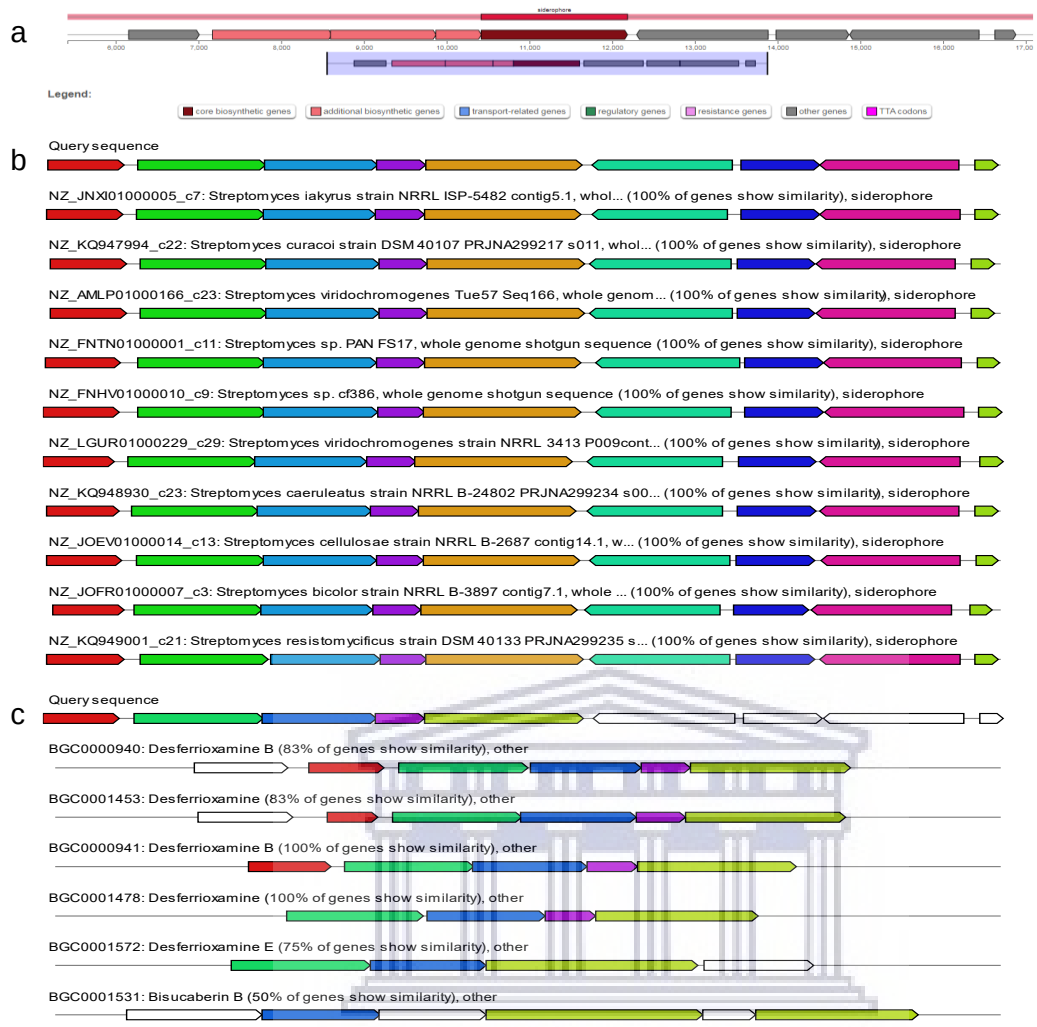


Figure 4.16: *a) Region 127.1 showed similarities to siderophore biosynthetic genes and b) had 100% similarity to genes from various* Streptomyces *species. c) The biosynthetic genes showed 83% similarity to desferrioxamine B biosynthetic genes. Results obtained from antiSMASH software.*

Five terpene gene clusters were found in *Streptomyces* strain S149 (Figure 4.13). One of the clusters was found on region 128.1 had 100% similarity to genes of *Streptomyces* species NRRL S-475. This terpene was 100% similar to genes involved in albaflavenone production (Figure 4.17). Albaflavenone is a sesquiterpene ketone antibiotic isolated from a highly odorous *Streptomyces albidoflavus* with antibacterial activity against *B. subtilis* (Gürtler *et al.,* 1994). The biosynthetic pathway of albaflavenone was elucidated in *Streptomyces coelicolor* A3(2). The biosynthesis requires the expression of a two-gene operon encoding a sesquiterpene cyclase (*sco5222*) and a cytochrome P450 (*sco5223*) (Zhao *et al.,* 2008). Interestingly, this albaflavenone biosynthetic pathway is the most highly conserved antibiotic pathway in *Streptomyces* species (Moody *et al.,* 2012). This antibiotic is most likely produced by *Streptomyces* strain S149, because it has high similarity to the compound, it is a highly conserved pathway in *Streptomyces* species, and the confirmed antibiotic activity against

Gram-positive bacteria including *B. cereus*. Furthermore, albaflavenone is a terpenoid, with its host plant, *S. frutescens*, producing an array of unique terpenoid compounds known as sutherlandiosides (Albrecht *et al.,* 2012). It is possible that *Streptomyces* strain S149 may be producing a terpenoid compound similar to albeflavanone or a different terpenoid using resources from the plant. Another terpene gene cluster was found on region 211.1 shared 54% similarity to *Streptomyces* species NRRL S-475, with 100% similarity to the gene cluster involved in the synthesis of isorenieratene and 87% of carotenoid genes (Supplementary Figure D2.4). Isorenieratene is a carotenoid compound with aromatic end groups and is generally produced by green photosynthetic bacteria and only a few actinomycetia (Krügel *et al.,* 1999). Carotenoids are colourful terpenoids found within all photosynthetic organisms and some non-phototrophic organisms. They are potent antioxidants and have applications as food colourants, nutraceuticals and pharmaceuticals (Rao and Rao, 2007). Many streptomycetes contain the biosynthetic gene cluster for isorenieratene but the pathways are usually silent. Only a few studies have reported the expression of this silent BGC (Krügel *et al.,* 1999; Iftime *et al.,* 2015; Myronovskyi *et al.,* 2013; Takano *et al.,* 2005; Becerril *et al.,* 2018). Antioxidant activity was not observed for the strain (Section 3.3.6) and thus may also indicate that the gene cluster is silent within the strain. The biosynthetic genes for this colourful carotenoid are usually silent, and this may explain the beige phenotype of the strain (Section 2.3.2.5) and also why this antioxidant did not show any activity when tested (Section 3.3.6).

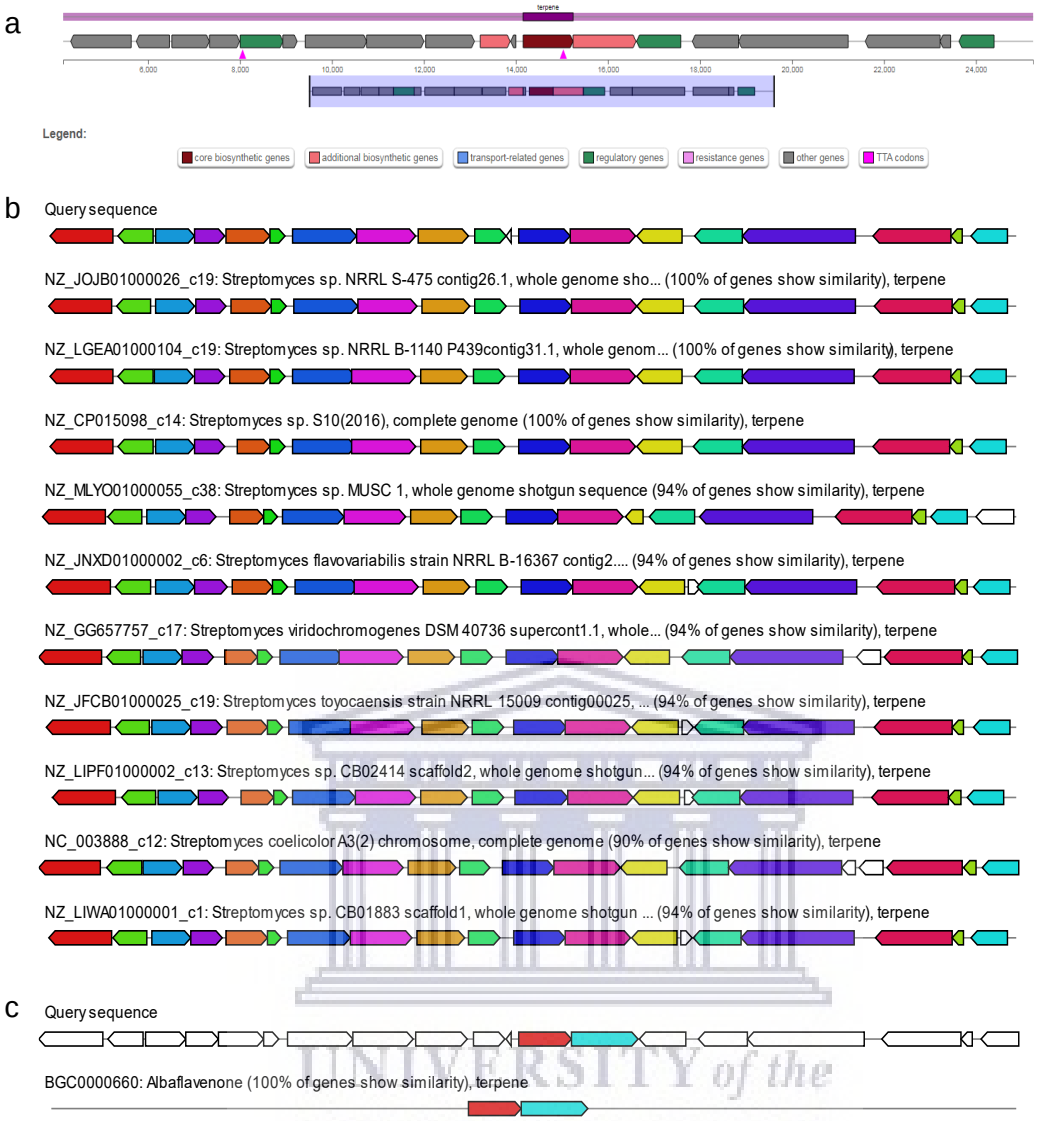


Figure 4.17: *a) Region 128.1 showed similarities to terpene biosynthetic genes and b) had 100% and lower similarity to genes from various* Streptomyces *species. c) The biosynthetic genes showed 100% similarity to the terpene antibiotic biosynthetic genes of albaflavenone. Results obtained from antiSMASH software.*

Six RiPPs clusters were identified in *Streptomyces* strain S149 (Figure 4.13) and included lanthipeptides, bacteriocins and a lassopeptide cluster(s). Regions 212.1 (Figure 4.18) and 446.1 (Figure 4.19) had similarities to lanthipeptides. Lanthipeptides are an important group of peptides with applications in the pharmaceutical and food industries. These peptides contain *meso*-lanthionine (Lan) and 3-methyllanthionine (MeLan) residues. As discussed previously, lanthipeptides are classified into four different classes depending on the biosynthetic enzymes that insert the Lan and MeLan motifs (Arnison *et al.,* 2013; Willey and Van der Donk, 2007). The BGC from region 212.1 had 46% similarity to genes from *Streptomyces* species NRRL S-475 with 28% of the genes corresponding to genes involved in the synthesis of informatipeptin and 57% of genes similar to caprazamycin biosynthetic genes (Figure 4.25). Informatipeptin is a class III lanthipeptide, that was discovered from *Streptomyces viridochromogenes* DSM 40736 via mass spectrometry-based genome mining (Mohimani *et al.,* 2014). Recently, a new avermipeptin analogue was discovered through genome-mining. The lanthipeptide gene cluster from the study had 85% similarity to the informatipeptin BGC, that indicated that the putative gene cluster had the ability to produce an informatipeptin analogue. The new avermipeptin B was characterised through heterologous expression and high-resolution mass spectrometry (ESI-MS/MS). The lanthipeptide also had strong Gram-positive antibacterial activity (Liu *et al.,* 2018). Thus, investigation into putative lanthipeptides, even with high similarities, may lead to the discovery of lanthipeptide analogues. Caprazamycins are lipo-nucleoside antibiotics isolated from *Streptomyces* species MK730-62F2 with activity against acid-fast bacteria, including *Mycobacterium tuberculosis* (Igarashi *et al.,* 2003; Takahashi *et al.,* 2013). The biosynthetic genes of this region need to be investigated further, as this cluster may produce a new antibiotic as it contains genes similar to lanthipeptide and nucleoside antibiotic synthesis. The interactions of these genes need to be evaluated as the strain also had antimicrobial activity against *M. aurum*.

The genes from region 446.1 is 50% similar to genes involved in sapB synthesis and 40% similar to biosynthetic genes of cantenulipeptin and SAL-2242 synthesis (Figure 4.19). Commonly, lanthipeptides have antimicrobial activities, but sometimes display other biological activities. SapB and SapT are morphogenic peptides (class III lanthipeptides) that function as biosurfactants and are essential for aerial hyphae formation (Willey and Gaskell, 2011; Kodani *et al.,* 2004; Kodani *et al.,* 2005; Repka *et al.,* 2017). Catenulipeptin, a class III lanthipeptide, was discovered from *Catenulispora acidiphila* DSM 44928. Catenulipeptin does not have antimicrobial activity, but can partially restore aerial hyphae growth of surfactin-treated *S. coelicolor* (Wang and Van der Donk, 2012). SAL-2242 was characterised through mass spectrometry-based genome mining from *Streptomyces albus* J1074 as a class III

lanthipeptide (Kersten *et al.,* 2011). No further information regarding bioactivity is available on this lanthipeptide.

The genes from region 358.1 was 100% similar to genes from various *Streptomyces* species and was identified as a bacteriocin. Bacteriocins are RiPPs and are classified into two groups, post translationally modified bacteriocins and unmodified or cyclic bacteriocins (Cotter *et al.,* 2013). Lanthipeptides are modified bacteriocins and this may be the reason why the biosynthetic genes were 42% similar to the lanthipeptide informatipeptin (Supplementary Figure D2.5).

A lasso peptide gene cluster was found on region 480.1 that was 45% similar to genes of *Streptomyces* NRRL S-475. The BGC was 100% similar to citrulassin D, B, F and E biosynthetic genes (Supplementray Figure D2.6). Lasso peptides are also a class of RiPPs and have a diverse set of pharmacological activities, including inhibition of bacterial growth, receptor antagonism, and enzyme inhibition (Maksimov *et al.,* 2012). Citrulassin family of lasso peptides was recently discovered, and with lagmycin, are the first examples of lasso peptides with a Leu N-terminal. Citrulassin A had no bacterial growth inhibition activity when it was tested (Tietz *et al.,* 2017).

Phenotypically, strain S149 only had slight inhibitory activity against *B. cereus*, *E. faecalis* and *M. aurum* (Section 3.3.1 and Section 3.3.2). Genetically, strain S149 has the potential to produce 39 secondary metabolites including a range of antibiotics, such as 7-prenylisatin and albaflavenone, that have inhibitory activity against *B. subtilis*. There is a possibility that one or both of these types of antibiotics are produced by strain S149, since the strain did have activity against *B. cereus*, a close relative to *B. subtilis*. The strain also potentially produces the broad-spectrum antibiotic, curamycin; a macrolactam-type antibiotic with antifungal, antitumor and antibacterial activity; as well as the mycobacterium antibiotic, caprazamycin. These antibiotics may be potentially produced by *Streptomyces* strain S149 and further investigation needs to be conducted. When antibacterial activity of the strain was tested, the strain displayed weak activity, and thus activation of these BGCs should be conducted. This can be achieved via heterologous expression of the BGC or stimulating the BGC natively by changing the environmental conditions, or through co-culturing. The genome also revealed BGCs for various RiPPs including lanthipeptides (informatipeptin, sapB and catnenulipeptin), bacteriocins, and lasso peptides (citrulassins). None of these identified RiPPs had published antimicrobial activities. The sapB and catnenulipeptin RiPPs have roles in sporulation and aerial hyphae formation.

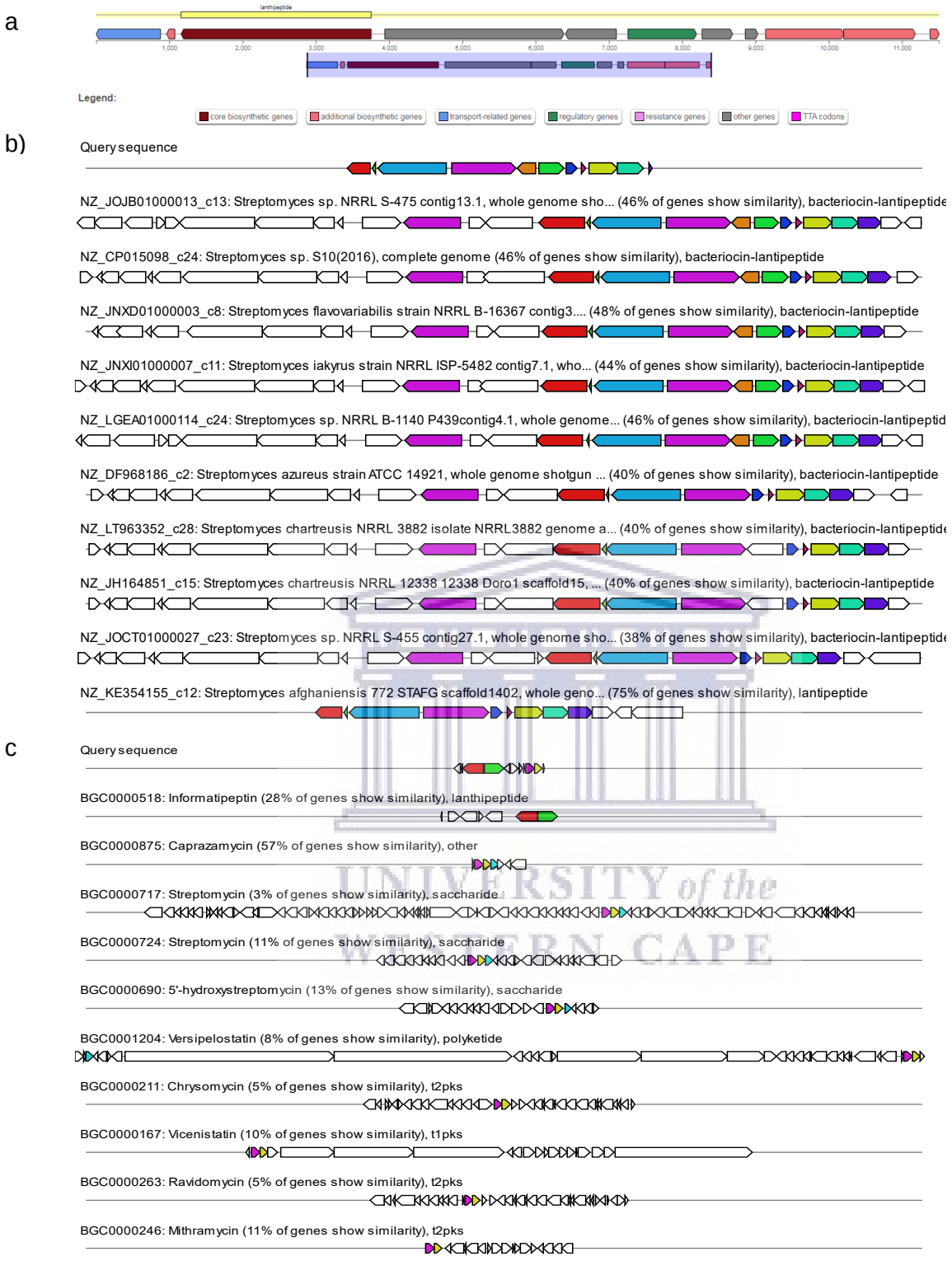


Figure 4.18: *a) Region 212.1 showed similarities to lanthipeptide biosynthetic genes and b) had similarities to genes from various* Streptomyces *species. c) The biosynthetic genes showed 28% similarity to the lanthipeptide biosynthetic genes of informatipeptin and 57% similarity to the anti-tuberculosis antibiotic biosynthetic genes of caprazamycin. Results obtained from antiSMASH software.*

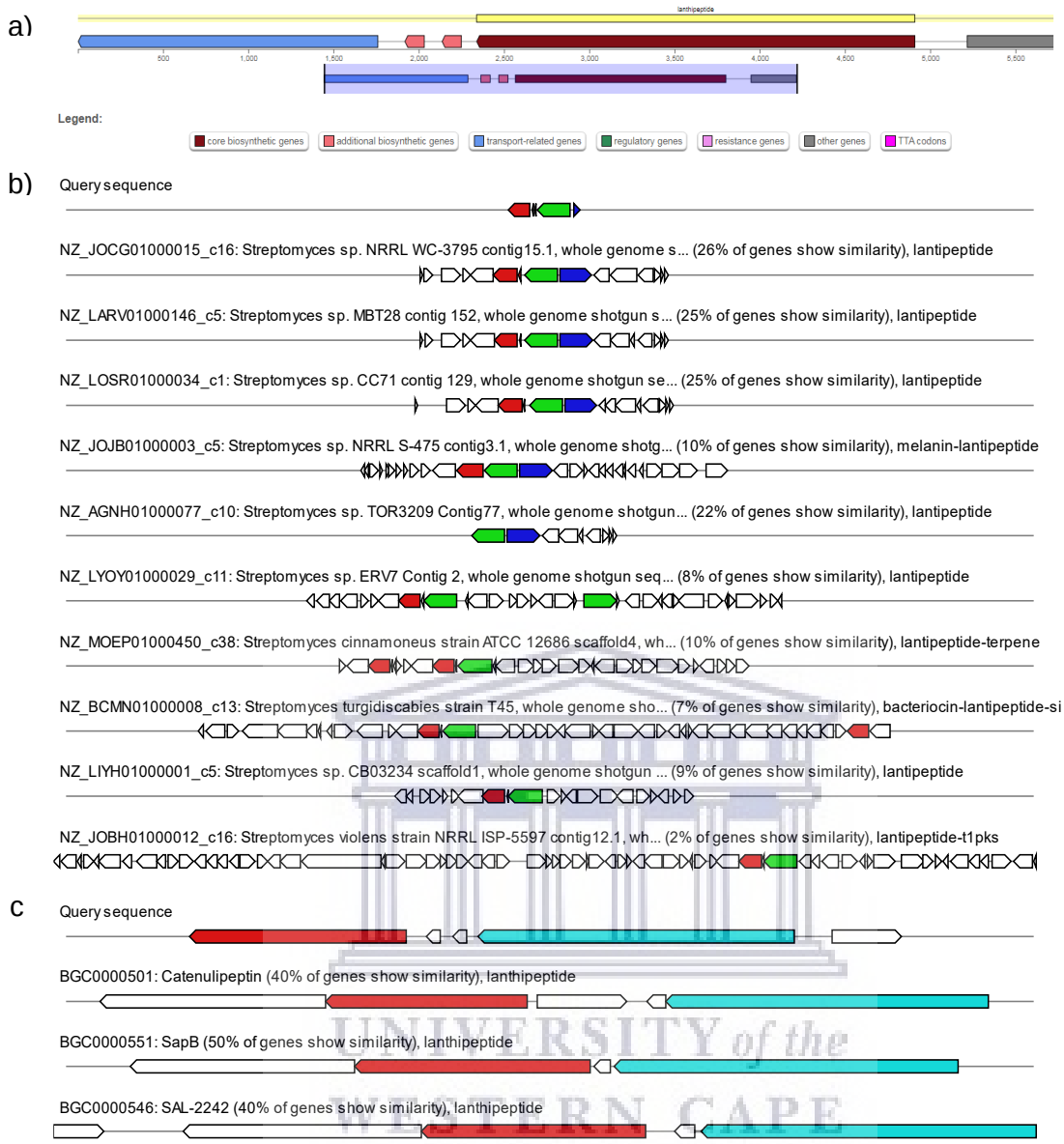


Figure 4.19: *a) Region 446.1 showed similarities to lanthipeptide biosynthetic genes and b) had low similarities to genes from various* Streptomyces *species. c) The biosynthetic genes showed 50% similarity to the biosynthetic genes of sapB and 40% similarity to both catenulipeptin and SAL-2242 genes. Results obtained from antiSMASH software.*

4.4 Conclusion

Genetic analysis of the actinobacteria associated with medicinal plants in this study showed noteworthy potential for the development of the bioactive compounds into pharmaceutically important products. Isolation and discovery of novel bioactive compounds from actinobacteria associated with medicinal plants have grown in the past few decades (Qin *et al.,* 2011). This study contributes to this pool of knowledge and broadens the understanding of bioactive compounds produced by plant-associated bacteria.

Investigation of the putative secondary gene clusters of *Streptomyces* strain A81 and S149 revealed that these strains have great pharmaceutical potential. Both strains had BGCs for PKS, NRPS, siderophores, terpenes and other secondary metabolites. These secondary metabolites have a range of functions, some of which may be silent or dormant within the strain.

This study shows the vast genetic potential of these actinobacteria associated with *A. ferox* and *S. frutescens* to produce secondary metabolites with the potential use as pharmaceutical compounds. As mentioned previously in chapter 3, this study focussed mainly on the antimicrobial potential of the actinobacterial isolates. The evaluation of the genes and genomes of these isolates did indeed show a variety of antibiotic compounds, but also revealed the potential of these isolates to produce anthelmintic, antifungal, antitumor, anti-HIV, neuroprotective and plant growth promotion compounds. Thus, future studies should not only focus on the antibacterial activity of these isolates, but also focus on all of these different activities mentioned. Furthermore, the first genome sequence of an endophytic actinobacteria, *Streptomyces* strain S149, was performed in this study and the strain was shown to have great genetic potential to produce bioactive compounds. This study confirms that actinobacteria associated with medicinal plants have the potential to produce bioactive compounds and that bacteria associated with medicinal plants from unique environments should be investigated for the discovery of novel compounds and strains of bacteria.

Chapter 5: Conclusion and Future Perspectives

Antimicrobial resistance (AMR) has been observed since the first antibiotics were discovered, and is currently on the rise due to the misuse and abuse of antimicrobials. It is estimated that without appropriate and immediate action the death toll from AMR infections could increase up to 10 million per year by 2050 (O'Neill, 2014). The World Health Organisation (WHO) emphasised the importance of searching for solutions to AMR, amidst the current coronavirus outbreak with the Director-General of WHO, Dr Tedros Adhanom Ghebreyesus saying, "Never has the threat of antimicrobial resistance been more immediate and the need for solutions more urgent." (WHO, Geneva, 17 January 2020). The lack of new antibacterial products in development was also highlighted, with only 32 antibiotics in the pipeline that target the WHO's priority pathogens (World Health Organization, 2019). Mortalities from viral infections are mostly caused by comorbidities such as autoimmune diseases and secondary infections. Viral infections, like the 2019 novel coronavirus (2019-nCoV), weakens your immune system, making secondary infections by bacteria more prevalent. Moreover, infections by AMR bacteria will make treatment even more difficult.

Discovery and development of new antimicrobials is needed to combat AMR infections. More importantly, using antibiotics correctly and only when necessary will further help to decrease AMR. Investigating bioactive compounds from natural resources can help to not only uncover new antimicrobials, but also new antitumor, anti-inflammatory, neuroprotective and many other bioactive compounds. Natural products can be used as the inspiration for synthetic chemists to develop new drugs with increased potency and less toxicity. Finding new NPs have great potential to be developed into new drug leads.

Unique environments were identified by Strobel *et al.* (2004) as a source to find novel microbes that potentially produce original bioactive metabolites. Based on this assumption, we have identified the Fynbos region as a unique environment that fits the criteria outlined previously (Chapter 1, Section 1.6). Utilising these novel environments can help increase the discovery of new metabolites and microbes. The investigation of actinobacteria associated with two medicinal plants, *Aloe ferox* and *Sutherlandia frutescens*, was undertaken in this study and these plants were found to host a range of actinobacterial species. The genera associated with these plants included, *Actinomadura*, *Alloactinosynnema, Amycolatopsis*, *Micromonospora*, and *Streptomyces* (Chapter 2, Section 2.3.2).

The actinobacterial isolates that were investigated were all related to actinobacteria previously isolated from soil or rock samples, making these isolates unique in that they form relationships with medicinal plants growing in the soil, leaves and seeds of the plants. *Streptomyces* strains A81, A159 and S149, and *Actinomadura* strain S173 are related to antimicrobial producing actinobacteria, whereas *Streptomyces* strain A3 and *Amycolatopsis* strain A37 was not related to antibiotic-producing actinobacteria, therefore there is a potential that these strains are producing novel compounds. All of the isolates had inhibitory activities against a range of pathogenic bacteria and thus are likely to produce several antibiotic compounds. Isolates A3, A37, S149 and S173 was phenotypically and genetically distinct from their closest relatives and are likely to be the type strains representing novel species.

Actinobacterial isolates were screened for antimicrobial activity against a range of Gram-positive and -negative human bacterial pathogens including *Bacillus cereus, Staphylococcus aureus, Mycobacterium aurum, Mycobacterium smegmatis, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The WHO has listed pathogenic strains according to priority for research and development of new antibiotics due to the pathogen's resistance to drugs. Of the pathogens tested in this study, *P. aeruginosa* is classified as a critical priority *S. aureus* is also listed as a high priority organism, and in recent years, *Mycobacterium tuberculosis* (MTB) has also been classified as high priority due to an increase in multi-drug resistant strains (World Health Organization, 2017). This is why screening against *M. aurum* and *M. smegmatis* was also included as a preliminary test for mycobacterium inhibition. The other pathogens that were tested cause known human infections, with a rise in antimicrobial resistance of these organisms. Future research will include testing against other critical and high priority pathogens. These inhibition tests require a biosafety level 3 laboratory and will be performed at a collaborator's laboratory.

Streptomyces strain A3 has an unusual phenotype for a streptomycete in that it did not produce aerial mycelia. The strain was isolated from soil associated with *A. ferox* and had relatively low 16S rRNA gene similarity to its closest relative, *Streptomyces lunaelactis* (97.1%) that did not exhibit any reported antimicrobial activity. Whereas, *Streptomyces* strain A3 had inhibitory activity towards *B. cereus*, *E. coli*, *M. aurum* and *S. aureus*. Potent inhibition activity against *M. aurum* was observed (zone of inhibition = 6062.49 mm^2) indicating that this strain may have the potential to produce an antibiotic affective against MTB. Further activity screening of this strain needs to be done to confirm activity against MTB and to characterise the compound. Crude solvent extractions, followed by bioautography, revealed four possible compounds with inhibition activity towards *M. aurum*. Five compounds were also identified with antimicrobial activity against *M. smegmatis* (Section 3.3.4). The anti-

mycobacterium compound(s) can be extracted from the broth of the isolate using chloroform. Chloroform is a non-polar solvent and thus the extracted active compound(s) are also non-polar in nature. Additionally, the produced compound is exported out of the cell and into the broth. This can help to scale up the production of the compound in future studies. Further investigation into this anti-mycobacterium compound(s) needs to be performed to characterise them using chromatography techniques. Additionally, activity testing of the purified compound(s) against *M. tuberculosis* will need to be conducted. Due to its low genetic similarity to its closest relatives, phenotypic and chemotaxonomic differences, it is suggested that strain A3 be classified as a type strain of a novel species within the genus of *Streptomyces* as *Streptomyces albertinaensis*^{T}.

Amycolatopsis strain A37 was isolated from soil associated with *A. ferox* roots and had very low 16S rRNA gene similarity to its closest relative, *Amycolatopsis albidoflavus* (95.99%). *Amycolatopsis* strain A37 was genetically and phenotypically distinct from its closest relatives and it is suggested that strain A37 be added to the genus of *Amycolatopsis* as a novel type species named *Amycolatopsis maiziuminfarum*^{T} (Chapter 2; Section 2.3.2.3). This strain also had antimicrobial activity, which was not reported for its closest relatives. Strain A37 had antibiotic activity against Gram-positive bacteria, including *B. cereus*, *E. faecalis*, *M. aurum*, *M. smegmatis* and *S. aureus*. This strain had potent activities with a zone clearing sizes of 4818.2 mm^2 and higher (Chapter 3; Section 3.3.2; Figure 3.4). Unfortunately, the potent bioactive compound was difficult to extract from the broth and solid media. Only one bioactive compound with activity against *S. aureus* could be extracted using ethyl acetate (Chapter 3; Section 3.3.4). Thus, further experimentation needs to be done to extract the potent compounds produced by this strain. The bioactive compound produced may be more or less polar than the solvents used in this study (methanol, ethyl acetate and chloroform) and it is suggested that different extraction solvents and techniques be tested for metabolite extraction. Additionally, the production of the bioactive compounds in the absence of pathogenic bacteria will need to be confirmed. The biosynthesis of the antibacterial compounds may only be activated in the presence of competing bacteria. Thus, a co-culturing approach may need to be applied to produce the bioactive compounds.

Actinomadura strain S173 was isolated as a seed epiphyte from *S. frutescens* plants, and forms bright pink and white colonies when cultured. The strain had 98.21% 16S rRNA similarity to its closest relative, *Actinomadura bangladeshensis* and differed from its closest relatives phenotypically and genetically. It is suggested that strain S173 be added as a novel type strain within the genus *Actinomadura* as *Actinomadura sutherlandiae*^{T}. The strain had antimicrobial activity against *B. cereus, E. faecalis, K. pneumoniae* and *S. aureus*. The crude solvent

extracts successfully extracted compounds with activities against *S. aureus* and *K. pneumonia*. Unfortunately, when the solvent extracts were separated via TLC and tested for antimicrobial activity using bioautography, none of the compounds showed inhibition activity against these pathogens. It is possible that the bioactive compounds are produced in a very low quantity. Also, multiple compounds can be responsible for the antibacterial activity seen, and separating these compounds via TLC decreases their effectiveness. Upscaling the growth of this strain may help to isolate larger quantities of the compound(s) to use for future testing and screening.

Streptomyces strains A81 and A159 was isolated from soil associated with *A. ferox* roots and was genetically similar to each other based on 16S rRNA analysis (99.12%). These strains were closely related to strains within the *Streptomyces puniceus* clade. This clade also includes *Streptomyces californicus* and *Streptomyces floridae,* and is known for the production of potent metabolites, including griseorhodins, viomycin, dinactin and other antimicrobial and antitumor compounds. *Streptomyces californicus* and *S. floridae* was previously suggested to be classified as type strains of *S. puniceus*. Genetically these strains are similar, but metabolically they produce different compounds. Thus, even though the strains isolated from this study is genetically and phenotypically similar to *S. puniceus*, metabolically they may produce novel compounds. The isolates had antibiotic activities against *B. cereus, E. faecalis, M. aurum, M. smegmatis, P. aeruginosa* and *S. aureus*. Replication of the tests showed varying activity against *P. aeruginosa* indicating that the bioactive compounds are produced under very specific conditions. The conditions for the activation of production of the antibiotic compounds need to be further investigated and determine how to stimulate production. A variety of solvents, growth conditions and techniques need to be tested for the extraction of these compounds. Since drug development against *P. aeruginosa* is of critical priority, investigating these compounds are of great importance.

Genomic analysis of *Streptomyces* strain A81 revealed 35 putative biosynthetic gene clusters. Interestingly, one of these BGCs had 100% similarity to micromonolactam, a macrolactam solely produced by *Micromonospora* species. Macrolactams are of pharmaceutical importance due to their antifungal, antibiotic and antitumor activities. Discovering new macrolactams, especially one produced by a non-*Micromonospora* species, is a very exciting discovery and has the potential to help with drug development. *Streptomyces* species produce a variety of macrolactams and strain A81 may produce a novel macrolactam similar to micromonolactam. One BGC had similarities to the genes involved in griseorhodin and rubromycin production. Griseorhodins have antibiotic activities against both Gram-positive and -negative bacteria (Suetsuna and Osajima, 1989). Griseorhodin furthermore inhibits HIV reverse transcriptase

and human telomerase, with some analogues having neuroactivities. Rubromycins have antitumor properties. Both griseorhodin and rubromycin was extracted as a red compound and may be similar to the coloured compound extracted from strain A81. The genetic potential for the strain to produce antibiotics is supported by the antibacterial activity observed when the isolate was tested in this study. Other activities such as neuroactivity, antitumor, inhibition of HIV reverse transcriptase and human telomerase needs to be evaluated. The strain also contained two BGCs related to typical plant metabolites, alkylresorcinol-type antibiotic and naringenin. These two metabolites are also found within its host plant, *A. ferox*, and this may indicate the potential of strain A81 to produce plant metabolites similar to its host plant. Other plant metabolites similar to those from *A. ferox* may also be produced by strain A81. This is an exciting discovery, as important medicinal compounds natively produced in *A. ferox* may also be produced by the bacterium which has potential applications for mass production of these medicinal compounds in bacteria.

Streptomyces strain S149 was isolated as an endophyte from *S. frutescens* leaves. This isolate formed an abundance of white aerial mycelia. Based on 16S rRNA gene analysis, the strain was closely related to *Streptomyces canus* (99.73%). The *gyrB* sequence analysis between its closest relatives were lower and indicated that the strain may be different from its closest match. Comparing the average nucleotides of the genomes of strain S149 to *S. canus*, revealed a low average nucleotide identity (ANI) of 84.56%. An ANI value lower than 95% indicates different species (Jain *et al.,* 2018). This low value thus indicates that *Streptomyces* strain S149 and *S. canus* is not the same species. It is suggested from this data that strain S149 should be classified as a novel type strain of *Streptomyces* and named *Streptomyces frutescens*^{T} (named after *S. frutescens* from which the strain was isolated). Additionally, antibiotic activity of *Streptomyces* strain S149 was observed against *B. cereus, E. faecalis* and *M. aurum*. The bioactive compounds active against *E. faecalis* was successfully partially purified using chloroform. The compounds that had inhibitory activity against *B. cereus* and *M. aurum* could not be extracted. Thus, stimulation of the expression of these metabolites need to be investigated.

Genome analysis of *Streptomyces* strain S149 revealed great potential for the production of bioactive compounds. The strain had 39 putative secondary metabolite biosynthetic gene clusters. BGCs similar to 7-prenylisatin genes were identified. This antibiotic had activity against *B. subtilis* and may be produced by strain S149 that had activity against *B. cereus*. Further investigation will need to be done to confirm the production of this or a similar compound. The strain also had a BGC 100% similar to micromonolactam. As discussed, this compound is solely produced by *Micromonospora*, and a different macrolactam may be

produced by this strain. The strain had BGCs that were 100% similar to genes involved in curamycin production, a broad-spectrum antibiotic. One gene cluster was 57% similar to genes of the anti-tuberculosis antibiotic, caprazamycin. *Streptomyces* strain S149 had antimycobacterial activity against *M. aurum* when tested via overlay. This may be due to the production of a caprazamycin-like compound. Further investigation into the production of these antibiotics by *Streptomyces* strain S149 needs to be done. The strain had low antibiotic activity, and these gene clusters may be silenced or only activated under certain environmental conditions. Thus, the stimulation and upregulation of these gene clusters need to be conducted to characterise the compounds.

Streptomyces strain S149 also possess machinery for the production of the plant hormone, auxin. Since the strain was isolated from the leaves of the plant, it may indicate a synergistic relationship. In plants, auxins are normally produced in the plant leaves and transported to the roots where it stimulates root growth. Increased root growth helps the plant take up more nutrients. The strain may have other plant growth promoting properties that would be worth investigating.

Future work should focus on purifying and characterising bioactive compounds. Interesting bioactive compounds from *Streptomyces* strain A81 that should be focussed on first is the macrolactams and the griseorhodins. Macrolactams are potent antimicrobial and anticancer compounds used as pharmaceutical products. Since high similarity to micromonolactam was detected via antiSMASH, there is a high probability that a macrolactam compound is being produced by *Streptomyces* strain A81. The second bioactive compound that should be investigated are griseorhodins. These compounds appear as a red/pink substance, first isolated from *Streptomyces californicus* (Eckardt *et al.,* 1978), a close relative of *Streptomyces* strain A81, with strain A81 also producing a red/pink substance. Griseorhodins are a family of aromatic polyketides with great structural diversity. Due to this structural diversity that is a result of enzymatic oxidation, the bioactivity of this family of compounds are also diverse. These compounds have activities that inhibit the HIV reverse transcriptase and human telomerase, and also had neuroactivity. The neuroactive compounds were modified due to enzymatic oxidisation and were discovered from a marine streptomycete that had a unique metabolism (Lin *et al.,* 2014). Since *Streptomyces* strain A81 was isolated from a niche environment and may also have a unique metabolism. Investigating the genome of this strain revealed the potential of the strain to produce typical plant metabolites also produced by its host plant, *A. ferox*. Thus, indicating that the strain may have a unique metabolism and have the ability to produce new griseorhodins, macrolactams and other yet undiscovered bioactive compounds. As discussed earlier, MTB antibiotic-resistance is increasing and thus further

investigation into possible MTB drugs is encouraged. One such anti-MTB compound of interest is caprazamycin, that may be produced by *Streptomyces* strain S149. Additionally, the strain showed inhibition activity against *M. aurum*, a closely related species to MTB. The strain was furthermore isolated from the leaves of *S. frutescens* and may also possess a unique metabolism. Therefore, it may be producing a modified caprazamycin-like compound with activity against mycobacteria.

The first step purifying and characterising bioactive compounds is to scale up the production of the compound by optimising growth conditions. The more compound is produced in the original culture, the more compound can be extracted. The compound should then be extracted using the solvents from this study, or other solvents, if the solvents were not successful for extraction of the compounds. The extract can then be purified using chromatography techniques such as column adsorption chromatography. The purity and quantity of the compound can be evaluated using HPLC methods. The structure of the compound can be assessed by determining the solubility, melting point, UV-absorption spectrum, infrared spectra, mass spectrum (via GC-MS or LC-MS depending on compound), and nuclear magnetic resonance (NMR). The biological activities of each purified compound can be determined by measuring the minimum inhibitory concentration (MIC), the antioxidant activity via DPPH and FRAP assay, antitumor and anti-inflammatory activity (Rao *et al.,* 2017). Other biological activities can also be investigated depending on the potential activity unveiled through genome mining i.e. anti-HIV and neuroactivities. Much of the testing of the bioactivities of the compound(s) will need to take place at collaborators laboratories that have access to higher biosafety level labs and necessary equipment needed for certain assays.

Several methods have been developed to activate silent or poorly expressed biosynthetic gene clusters. Secondary metabolite production can be stimulated by changing the environment of the actinobacteria. This can be done by changing the culture conditions (OSMAC approach), growing the microbe under stress conditions (high salt, temperature or ethanol), addition of elicitors to growth media (e.g. quorum sensing, heavy metals etc.), or co-culturing with other microorganisms. Silent BGCs can also be activated using molecular techniques. This includes ribosome and polymerase engineering (e.g. creating *rpsL* or *rpoB* mutants), stimulating expression of the activator (e.g. LysR-type transcriptional regulator; LTTR), deleting suppressors via mutation, using artificial promoters via heterologous cloning, and refactoring of BGCs or promoters (Reen *et al.,* 2015; Zhang *et al.,* 2019). These approaches can be taken to stimulate the expression of the silent BGC's from strains A81 and S149. Heterologous expression of BGCs is a useful strategy to activate, improve or modify the natural product pathways present in actinobacterial isolates. Many of the BGCs found in

actinobacterial isolates are poorly expressed under laboratory conditions and thus making it difficult to isolate and characterise the bioactive compound it is producing. Heterologous expression of these BGCs can help to activate these silent pathways. However, cloning and efficient expression of these, often large pathways (ranging from 20 kb to over 100 kb), can be challenging and sometimes ineffective due to the current technologies available. Three main methods have been developed and used in recent years to try and express these large BGCs, and are the TAR, IR, and pSBAC vector systems (discussed in Chapter 1, Section 1.1.3.5). These heterologous expression systems can help to activate large cryptic pathways and improve the production of natural products. Nevertheless, genetic manipulation of these large BGCs are still difficult. Moreover, the high %G+C content of actinobacteria, and the long, highly similar intragenic and intergenic tandem repeats of the NRPS and PKS clusters make it difficult to genetically manipulate and express the BGCs.

Despite finding 4 novel species, 80% of the strains are likely to be species which are already known to scientists. These strains will need to be thoroughly identified using additional chemotaxonomic, phenotypical and molecular analysis as used in this study. Many potential novel compounds were found but problems with optimal production and purification was still evident. These problems will need to be investigated and rectified in future research. Investigation into the genome and potential compounds produced by the isolates may give an indication as to the culture conditions that should be used for optimal production of the bioactive compounds, as well as the methods to extract and purify the bioactive compounds of interest.

Nevertheless, this study was successful in discovering novel antibiotic-producing actinobacteria associated with two South African medicinal plants, *A. ferox* and *S. frutescens*. The actinobacterial isolates were screened for their ability to inhibit growth of human pathogenic strains, that are prone to develop antimicrobial resistance. Genetic screening of these antibiotic-producing isolates will further reveal their bioactive potential. In this study, the first reported medicinal plant endophytic actinobacteria, *Streptomyces* strain S149, was sequenced. The genomes of *Streptomyces* strain A81 and S149 were investigated to reveal the genetic potential of the isolates to produce antibacterial, antifungal, antiparasitic, antitumor, and other bioactive compounds. Combining some of the strategies mentioned, and leveraging new technologies, will help us to uncover the pharmaceutical potential of these actinobacterial isolates and help to develop new drug leads to combat not only antimicrobial resistance, but also other emerging diseases and illnesses.

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Appendix A: Media Compositions

All media used in this study was autoclaved at 121 °C, 15 psi for 15-20 minutes. The pH of the media was adjusted using HCl and NaOH, unless stated otherwise. Solid media was prepared using 15g/L of bacteriological agar or as stipulated. Agar was omitted from liquid media. Volumes of the media were adjusted using H_2O to 1 L, unless stated otherwise.

Table A1: Media and components used for isolation of actinobacteria from soil and plant samples

Media/Solutions	Components	Method
Humic acid-Vitamin (HV) medium	Humic acid (1 g/L), KCl (1.7 g/L), Na_2HPO_4 (0.5 g/L), $MgSO_4 \cdot 7H2O$ (0.5 g/L), $CaCO_3$ (0.02 g/L), $FeSO_4 \cdot 7H2O$ (0.01 g/L), Bacteriological Agar (10 g/L), Vitamin B solution (1 mL/L), cycloheximide (0.05 g/L), nalidixic acid (0.05 g/L), pH 7.2	Autoclave at 15 psi for 15-20 minutes. Add vitamin B solution, cycloheximide and nalidixic acid after the medium has cooled to 50 °C.
Complex Humic acid-Vitamin (CHV) medium	Humic acid (0.5 g/L), Soil extract (500 ml/L), Bacteriological Agar (10 g/L), Vitamin B solution (1 mL/L), cycloheximide (0.05 g/L), nalidixic acid (0.05 g/L), pH 7.2	Autoclave at 15 psi for 15-20 minutes. Add vitamin B solution, cycloheximide and nalidixic acid after the medium has cooled to 50 °C.
Vitamin B solution	Thiamine hydrochloride (0.5 g/L), Riboflacin (0.5 g/L), Nicotinic acid (0.5 g/L), Pyridoxine (0.5 g/L), Pantothenic acid (0.5 g/L), 4-Aminobenzaic acid (0.5 g/L), Biotin (0.25 g/L)	Dissolve vitamin B's in dH2O and filter with a 4uM filter. Alliquote 1 mL into eppendorf tubes and store at -20 °C.
Soil extract	100 g/L of mixed soil	Mix soil with dH2O. Autoclave at 15 psi for 15-20 minutes. Filter into a clean 1L bottle. Autoclave filtrate at the same parameters as above.
Starch-Casein-Nitrate (SCN) medium	Soluble Starch (10 g/L), Casein (0.3 g/L), KNO_3 (2 g/L), $MgSO_4 \cdot 7H_2O$ (0.05 g/L), $CaCO_3$ (0.3 g/L), $FeSO4 \cdot 7H2O$ (0.01 g/L), Bacteriological Agar (15 g/L), cycloheximide (0.05 g/L), pH 7.0	Autoclave at 15 psi for 15-20 minutes. Add cycloheximide after the medium has cooled to 50 °C.
Yeast Extract-Malt Extract (YEME) medium with Aloe ferox leaf juice	Yeast extract (4 g/L), Malt extract (10 g/L), Glucose (4 g/L), Bacteriological Agar (20 g/L), Aloe ferox leaf juice (25 g/L), cycloheximide (0.05 g/L), pH 7.3	Autoclave at 15 psi for 15-20 minutes. Add cycloheximide after the medium has cooled to 50 °C.
Actinomycete isolation medium with glycerol	Casein (2 g/L), L-Asparagine (0.1 g/L), $C_3H_5NaO_2$ (4 g/L), K_2HPO_4 (0.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.1 g/L), $FeSO_4 \cdot 7H_2O$ (0.001 g/L), glycerol (5 g/L), cycloheximide (0.05 g/L), pH 8.1	Autoclave at 15 psi for 15-20 minutes. Add cycloheximide after the medium has cooled to 50 °C.
Actinomycete isolation medium with glucose	Casein (2 g/L), L-Asparagine (0.1 g/L), $C_3H_5NaO_2$ (4 g/L), K_2HPO_4 (0.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.1 g/L), $FeSO_4 \cdot 7H_2O$ (0.001 g/L), glucose	Autoclave at 15 psi for 15-20 minutes. Add cycloheximide after the medium has cooled to 50 °C.

	(5 g/L), cycloheximide (0.05 g/L), pH 8.1	
Aloe ferox leaf juice medium	Aloe ferox leaf juice (25 g/L), Phytagel (20 g/L), cycloheximide (0.05 g/L), pH 7.0	Autoclave at 15 psi for 15-20 minutes. Add cycloheximide after the medium has cooled to 50 °C.
Aloe ferox leaf juice	Aloe ferox leaves	Peel leaves with a vegetable peeler. Grind up leaf pulp in a grinder until smooth. Autoclave leaf juice at 15 psi for 15-20 minutes.

Table A2: Composition of growth media and components used for phenotypic identification of actinobacteria and bioactive screening.

Media/Solutions	Components	Method
Cross-streak media (CSM; Sengupta *et al*., 2015)	Yeast extract (3 g/L), Peptone (3 g/L), Casein (3 g/L), Starch (8 g/L), K_2HPO_4 (0.5 g/L), $MgSO_4.6H_2O$ (0.5 g/L)	Adjust pH to 7.2. Autoclave at 15 psi for 15-20 minutes.
Bennett Medium (BM) with glucose	Glucose (10 g/L), Casitone (Difco; 2 g/L), Yeast extract (1 g/L), Beef extract (1 g/L), Bacteriological agar (20 g/L)	Adjust pH to 7.0. Autoclave at 15 psi for 15-20 minutes.
Bennett Medium (BM) with glycerol	Glycerol (10 g/L), Casitone (Difco; 2 g/L), Yeast extract (1 g/L), Beef extract (1 g/L), Bacteriological agar (20 g/L)	Adjust pH to 7.0. Autoclave at 15 psi for 15-20 minutes.
SCN	Soluble starch (10 g/L), Casein (0.3 g/L), KNO_3 (2 g/L), $MgSO_4.7H_2O$ (0.05 g/L), $CaCO_3$ (0.3 g/L), $FeSO_4.7H_2O$ (0.01 g/L)	Adjust pH to 7.0. Autoclave at 15 psi for 15-20 minutes.
ISP-1	Tryptone (5 g/L), Yeast extract (3 g/L), Bacteriological agar (20 g/L)	Adjust pH to 7.0. Autoclave at 15 psi for 15-20 minutes.
ISP-2 (YEME)	Yeast extract (4 g/L), Malt extract (10 g/L), Glucose (4 g/L), Bacteriological agar (20 g/L)	Adjust pH to 7.3. Autoclave at 15 psi for 15-20 minutes.
ISP-3 (Oats)	Oats (20 g/L), Trace salts solution (1 mL/L), Bacteriological agar (18 g/L)	Cook oats in 800 mL of H_2O. Strain oats water into a new container using a sieve. Add agar and adjust volume to 1 L using H_2O. Adjust pH to 7.2. Autoclave at 15 psi for 15-20 minutes.
ISP-4	Solution I: Soluble starch (10 g/500 mL) Solution II: K_2HPO_4 (1 g/ 500 mL), $MgSO_4.7H_2O$ (1 g/500 mL), NaCl (1 g/500 mL), $(NH4)_2SO_4$ (2 g/500 mL), $CaCO_3$ (2 g/500 mL), Trace salt solution (1 mL/500 mL), Bacteriological agar (20 g/L)	Solution I: Make a paste of the starch using a small amount of water and then adjust volume to 500 mL. Solution II: Adjust volume to 500 mL and adjust pH to between 7.0 and 7.4. Do not adjust pH if it is within this range. Mix solution I and II. Add agar and autoclave at 15 psi for 15-20 minutes.
ISP-5	L-asparagine monohydrate (1 g/L), Glycerol (10 g/L), K_2HPO_4 (1 g/L), Trace salts solution (1 mL/L),	Adjust pH to 7.0 with HCl. Autoclave at 15 psi for 15-20 minutes.

	Bacteriological agar (20 g/L)	
ISP-6	Peptone (15 g/L), Soybean proteose peptone (5 g/L), Ferric ammonium citrate (0.5 g/L), K_2HPO_4 (1 g/L), $Na_2S_2O_3$ (0.08 g/L), Yeast extract (1 g/L)	Adjust pH to 7.0 and autoclave at 15 psi for 15-20 minutes.
ISP-7	Glycerol (15 g/L), L-Tyrosine (0.5 g/L), L-Asparagine monohydrate (1 g/L), K_2HPO_4 (0.5 g/L), $MgSO_4.7H_2O$ (0.5 g/L), NaCl (0.5 g/L), $FeSO_4.7H_2O$ (0.01 g/L), Trace salts solution (1 mL/L), Bacteriological agar (20 g/L)	Adjust pH to 7.2 with HCl. Autoclave at 15 psi for 15-20 minutes.
ISP-9	$(NH_4)_2SO_4$ (2.64 g /L), KH_2PO_4.anhydrous (2.38 g/L), $K_2HPO_4.3H_2O$ (5.65 g/L), $MgSO_4.7H_2O$ (1 g/L), Trace salts solution (1 mL/L)	Adjust pH to 6.8 – 7.0. Autoclave at 15 psi for 15-20 minutes. Add appropriate filter sterilized carbon source* solution (10%) to cooled medium (< 60 °C) at a final concentration of 1%.
Trace salts solution	$FeSO_4.7H_2O$ (0.1 g/100mL), $MnCl_2.4H_2O$ (0.1 g/100mL), $ZnSO_4.7H_2O$ (0.1 g/100mL), H_2O up to 100 mL	Filter sterilize solution using a syringe and a 0.45 µM filter.

** The following carbon sources were added to ISP-9 medium to test carbon utilization of each source: D-Maltose, Sucrose, D-Raffinose, α-D-Lactose, D-Fructose, L-Rhamnose, myo-inositol, Citric acid and D-Malic acid. ISP-9 control plates contained no addition of a carbon source.*

Table A3: Composition of LB media for growth of human pathogenic bacteria.

Components	Method
Tryptone (10g/L), Yeast Extract (5g/L), NaCl (10g/L), Bacteriological Agar (15g/L)	Autoclave at 15 psi for 15-20 minutes. Omit the agar for LB broth.

Table A4: Solutions and method for CAS media preparation for siderophore testing. All glassware was rinsed with 3M HCl and dried before use.

Media/Solutions	Components	Method
Solution I	1mM $FeCl_3{\cdot}6H_2O$ in 10mM HCl, CAS (60.5mg in 50ml), CTAB (72.8mg in 40ml)	Add the $FeCl_3{\cdot}6H_2O$ solution to the CAS solution. Add the CAS solution slowly to the CTAB solution, with constant stirring. Autoclave and cool down to 50 °C.
Solution II	KH_2PO_4 (0.3g), NaCl (0.5g), NH_4Cl (1g), PIPES (30.24g), bacteriological agar (15g)	Prepare 700 ml salt solution containing KH_2PO_4, NaCl and NH_4Cl in 1L bottle. Add PIPES to the salt solution. Adjust the pH to 6.8 using KOH crystals. Add 15 g of bacteriological agar and adjust volume to 800 ml. Autoclave and cool down to 50 °C.
Solution III	Glucose (2g), Mannitol (2g), $MgSO_4{\cdot}7H_2O$ (493mg), $CaCl_2$ (11mg), $MnSO_4{\cdot}H_2O$ (1.17mg), M_3BO_3 (1.4mg), $CuSO_4{\cdot}5H_2O$ (0.04 mg), $ZnSO_4{\cdot}7H_2O$ (1.2mg), $Na_2MoO_4{\cdot}2H_2O$ (1mg)	Mix all components in 70ml water. Autoclave and cool down to 50 °C.

Solution IV	10% filter sterilized casamino acids	Dissolve 3g casamino acids in 30ml water. Filter sterilize solution using a 0.2 µm syringe filter.

Bring all solutions to 50 °C. Add Solutions I, III and IV to Solution II and shake briefly to mix. This should result in a blue coloured medium. Pour plates as per usual.

Appendix B: Geographical Maps

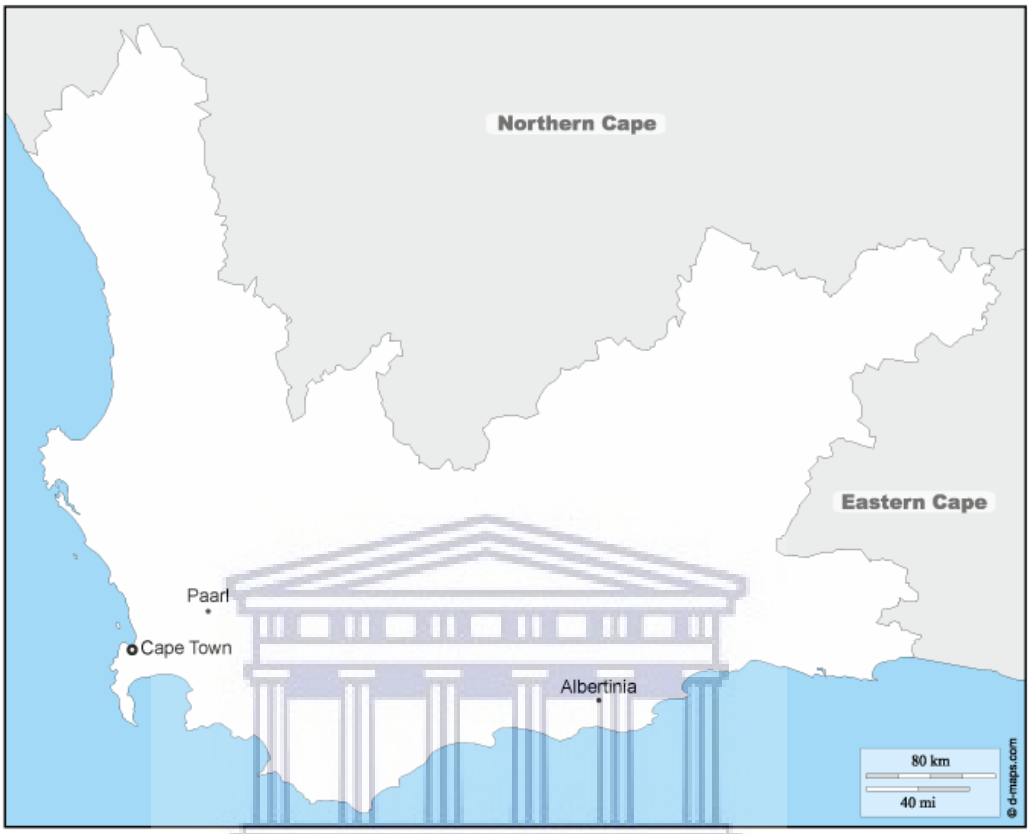


Figure B1: *Sampling sites for* Aloe ferox *and* Sutherlandia frutescens *soil and plant samples from Albertinia and Paarl, respectively.*

Appendix C: Cross-streak Results

Table C1: Cross-streak results of *Aloe ferox* isolates. - : growth not inhibited; +: growth inhibited up to 2 mm from isolate; ++: growth inhibited up to 5 mm from isolate; +++: growth inhibited up to 10 mm from isolate; ++++: complete growth inhibition

Isolate	*B. cereus*	*S. aureus*	*E. coli*	*E. faecalis*
A1	+	++	+++	+
A2	++	+	++	++
A3	++++	++++	++	++++
A4	+	+	-	-
A5	+	+	-	+
A6	+	-	+	-
A7	++	+	-	+
A8	+	-	+	+
A9	+++	+++	-	+
A12	++++	+++	-	-
A14	+	-	-	+
A15	-	-	-	-
A16	++++	+++	+++	+++
A17	+	-	+	-
A18	+	-	-	-
A19	+	++	-	-
A20	+	+	-	-
A21	+	-	+	-
A23	+	+	+	-
A24	+	+	-	+
A25	++++	+++	+++	+++
A26	++	++	+	+
A27	-	-	-	-
A28	+	-	-	-
A29	++++	++++	++	+++
A30	+++	+++	-	++
A31	-	-	++	++
A32	+	+	-	+
A33	+++	+	+++	++
A34	++++	++	++	++++
A35	+	-	-	-
A36	++	++	++	++
A37	+++	+++	-	+
A38	+++	++	-	+
A39	+	++	-	++
A40	++	++	-	++
A41	++	+++	+	+++
A42	+	+	-	-
A43	++	-	++	++
A44	++++	++++	+	+++
A45	++	+++	++++	+++
A46	++	++	-	++
A47	++	+	-	-
A48	-	-	-	-
A49	+++	+++	+	+++
A50	+	+	+	-
A51	+	+	-	+
A52	+++	+	+	+
A53	++	++	-	++

A54	++	++	+	+
A57	++	++	+	++
A58	-	-	-	-
A60	+++	-	+	+
A62	-	-	-	-
A63	++	-	+	+
A64	+++	+	+	+
A65	+	+	-	+
A66	++	+	+	++
A67	-	-	+	-
A68	-	-	-	-
A69	+	-	-	-
A70	+	+	+	+
A71	+	+	-	+
A72	-	-	-	-
A73	-	-	-	-
A74	-	-	-	-
A75	++	+++	+++	+++
A76	-	-	-	-
A77	++	++	++	++
A78	-	-	-	-
A79	-	-	-	-
A80	-	-	-	-
A81	++	++	-	+
A82	-	-	-	-
A83	++	-	-	-
A84	-	-	-	-
A85	-	-	-	-
A86	+	+	-	+
A87	-	-	-	-
A89	+	+	+	+
A90	+++	++	+	+
A91	++++	+++	-	+
A93	-	+	-	-
A94	-	+	+	+
A95	+++	++	-	+
A96	-	-	-	-
A97	+++	+++	++	+++
A98	-	-	-	-
A99	+	-	-	-
A100	+	++	++	+++
A101	+	+	-	+
A102	++	+	+	+
A103	+	-	-	-
A104	++	++	+	+
A105	++	++	-	++
A106	++++	++++	+	+
A107	++	+++	++	+++
A108	+	+	+	+
A109	+	+	-	+
A110	-	-	-	-
A111	++	++	-	+
A112	+	+	-	-
A113	+	+	-	-
A114	+++	++	+	++
A115	-	-	-	-
A116	++	+	++	+
A117	++	++	-	+

A118	+	-	-	-
A119	+++	+	-	+
A120	+++	+	+	+
A122	+++	+	+	+
A123	+	-	-	+
A124	+	-	-	-
A125	-	-	-	-
A126	-	-	-	-
A127	++	++	-	-
A128	++	++	-	+
A129	+	+	+	+
A130	++	+++	+++	+++
A131	++	+	-	-
A132	++	++	-	+
A133	-	-	++	-
A134	+	-	-	-
A135	++	-	-	-
A136	+++	+	++	++
A137	+	+	+	+
A138	-	-	-	-
A139	+	+	-	+
A140	++	++++	-	+
A141	-	-	-	-
A142	-	-	-	-
A143	+	+	+	+
A144	++	-	+	+
A145	+	+	+	+
A146	+	+	+	+
A147	-	-	-	-
A148	++	++	+	+
A149	+	-	-	-
A150	++	-	++	++
A151	+	+	-	+
A152	++	+	+	-
A153	++++	+++	+++	+++
A154	+++	+++	+++	+++
A155	+	-	-	-
A156	++	-	++	++
A157	++	++++	-	-
A158	++	+	++	++
A159	+++	+++	+++	+++
A160	++++	+++	+	+
A161	+	++	+	+
A162	+++	+	-	-
A163	++	++	+	+
A164	-	-	-	-
A165	-	-	-	-
A166	+	+	-	-
A167	-	-	-	-
A168	+	+	-	-
A169	-	-	-	-
A170	-	-	-	-
A171	+	+	+	+
A172	+++	+	++	++
A173	+	-	-	-
A174	+	+	-	-
A175	+	-	-	-
A176	+	+	-	-

A177	-	-	-	-
A178	-	-	-	-
A179	-	-	+	+
A180	++	++	-	-
A181	+++	+++	++	+++
A182	+	+	-	+
A183	++	+	+	+
A184	-	-	-	-
A185	-	-	-	-
A186	-	-	-	-
A187	+	+	-	-
A188	+	+	+	-
A189	++	+	+	+
A190	+	+	-	-
A191	++	+	++	++
A192	+	-	-	-
A193	++	-	+	+
A194	-	+	+	-
A195	+	+++	++	++
A197	+	-	-	-
A198	++	+	-	-
A199	+	-	-	-
A200	-	-	-	-
A201	-	-	+	+
A202	+	+	+	+
A203	+++	+++	+++	+++
A204	++++	++++	+	+
A205	++++	++++	+	+
A206	++	++	-	++
A207	-	-	-	-
A208	-	-	-	-
A209	-	-	-	-
A211	+++	+++	+++	+++
A212	++	++	+	+
A214	++	++	+	+
A215	++	++	+	+
A216	+	+	-	+
A217	-	-	-	-
A218	+++	+	+	+
A219	++	+	-	-
A220	++	++	+	+
A221	+	+	+	+
A222	-	-	-	-
A223	+	-	-	-
A224	-	-	-	-
A225	-	-	-	-
A226	+++	++++	+++	+++
A227	+	+	-	+
A228	-	-	-	-
A229	-	-	-	-

Table C2: Cross-streak results of *Sutherlandia frutescens* isolates. - : growth not inhibited; +: growth inhibited up to 2 mm from isolate; ++: growth inhibited up to 5 mm from isolate; +++: growth inhibited up to 10 mm from isolate; ++++: complete growth inhibition

Isolate	B. cereus	S. aureus	E. coli	E. faecalis
S1	-	+	-	-
S3	+	+++	++	+++
S5	+	++	+	+
S6	-	+	+	+
S7	+	+	+	+
S8	+	-	+	+
S9	-	-	-	+
S10	+	+	+	++
S12	+	-	-	-
S13	++	+	-	+
S14	++	++	++	++
S15	-	-	-	-
S17	+++	+++	-	-
S18	+	-	-	-
S19	+++	++	-	+++
S20	-	-	-	-
S21	-	-	-	
S22	+	-	-	-
S24	+	-	-	+
S25	++++	++++	++	+++
S26	+	-	-	-
S27	-	-	-	-
S34	-	-	-	-
S37	+++	+++	+	++
S39	+	-	-	-
S41	+++	++++	+	++
S43	++	++	-	+
S44	+	-	-	-
S46	-	-	-	-
S48	-	-	-	-
S50	++	++	++	++
S51	++	+	-	+
S53	+	+	-	-
S54	+	+	-	-
S55	+	+	-	-
S56	-	+	-	-
S57	+++	+++	-	++
S58	-	-	-	-
S60	++	+++	+	++
S61	+	++	+	+
S62	++	+	+	+
S63	-	-	-	-
S64	-	-	-	-
S65	+	+++	+	++
S67	+	-	-	-
S68	+	+++	+	+
S69	+	+++	+	+
S70	-	-	-	-
S72	+	+++	+	+
S73	-	-	-	-
S74	++	+	+	+
S75	-	-	-	-
S76	+	++	-	++

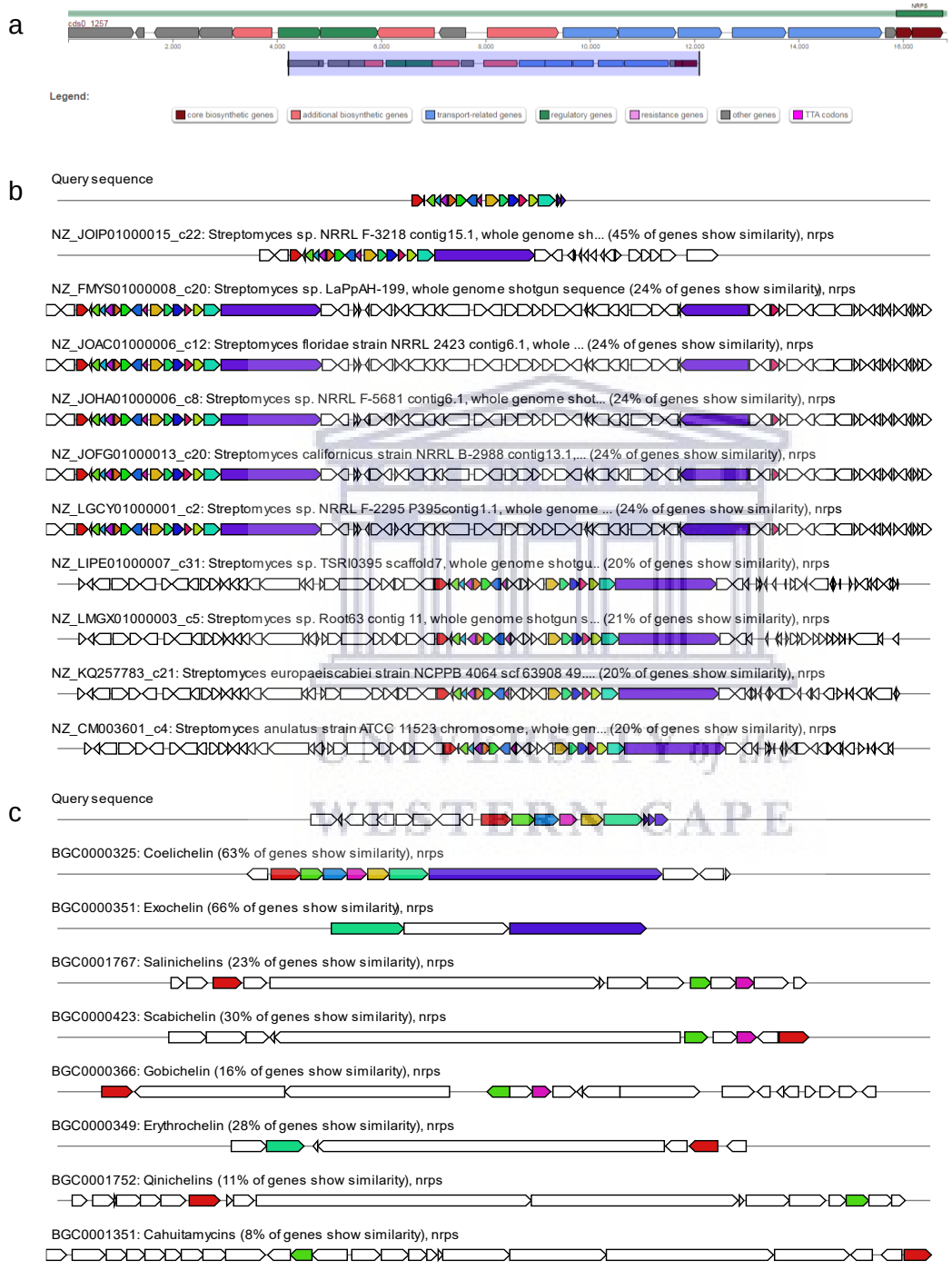
S77	+	-	-	-
S78	-	-	-	-
S79	+	-	-	-
S81	-	-	-	-
S82	-	-	-	-
S83	++	++	-	++
S84	+	-	+	-
S85	-	-	-	-
S86	-	-	-	-
S87	-	-	-	-
S89	-	-	-	-
S90	+	+	+	+
S91	+	-	-	-
S92	+	+	-	-
S93	+++	++++	-	+++
S94	+	+	+	+
S95	+	+	-	+
S96	-	-	-	-
S97	+	-	-	-
S99	+	+++	+	++
S100	+	+++	+	++
S101	+	-	-	-
S102	+	-	-	-
S104	-	-	-	-
S105	+	+	-	-
S106	+++	++	-	+
S107	+	-	-	-
S108	+++	+++	-	+
S109	-	-	-	-
S110	-	-	-	-
S111	-	-	-	-
S112	+	-	-	-
S113	-	-	-	-
S114	+	-	-	-
S115	-	+++	+	++
S116	+	+	-	-
S117	+	-	-	-
S118	-	-	-	-
S119	-	-	-	-
S120	+	-	-	-
S121	+	-	-	-
S122	+	-	-	-
S125	++++	+++	-	+
S127	-	-	-	-
S128	+	++++	+	++++
S130	+	+	-	-
S131	-	-	-	-
S132	+	-	-	-
S133	-	-	-	-
S134	-	-	-	-
S135	-	-	-	-
S136	-	-	-	-
S137	+	-	-	-
S138	+	-	-	-
S139	-	-	-	-
S148	-	-	-	-
S149	++	-	-	-
S155	-	-	-	-

S156	+	+	++	-
S158	-	-	-	-
S159	+++	++	+	++
S160	-	-	-	-
S165	-	-	-	-
S166	-	-	-	-
S170	++	++++	+++	+++
S171	++	+	-	-
S172	-	-	-	-
S173	++	+	-	-

Appendix D: Genome Annotation and BGCs

D1: Biosynthetic gene clusters from *Streptomyces* strain A81:

Figure D1.1: *a) Region 66.1 has gene clusters similar to NRPS gene clusters b) and had*



sequence similarities (45% and lower) to the NRPS gene clusters from Streptomyces *species. c) The query sequence had similarities to coelichelin (63%) and exochelin (66%). Results obtained from antiSMASH software.*

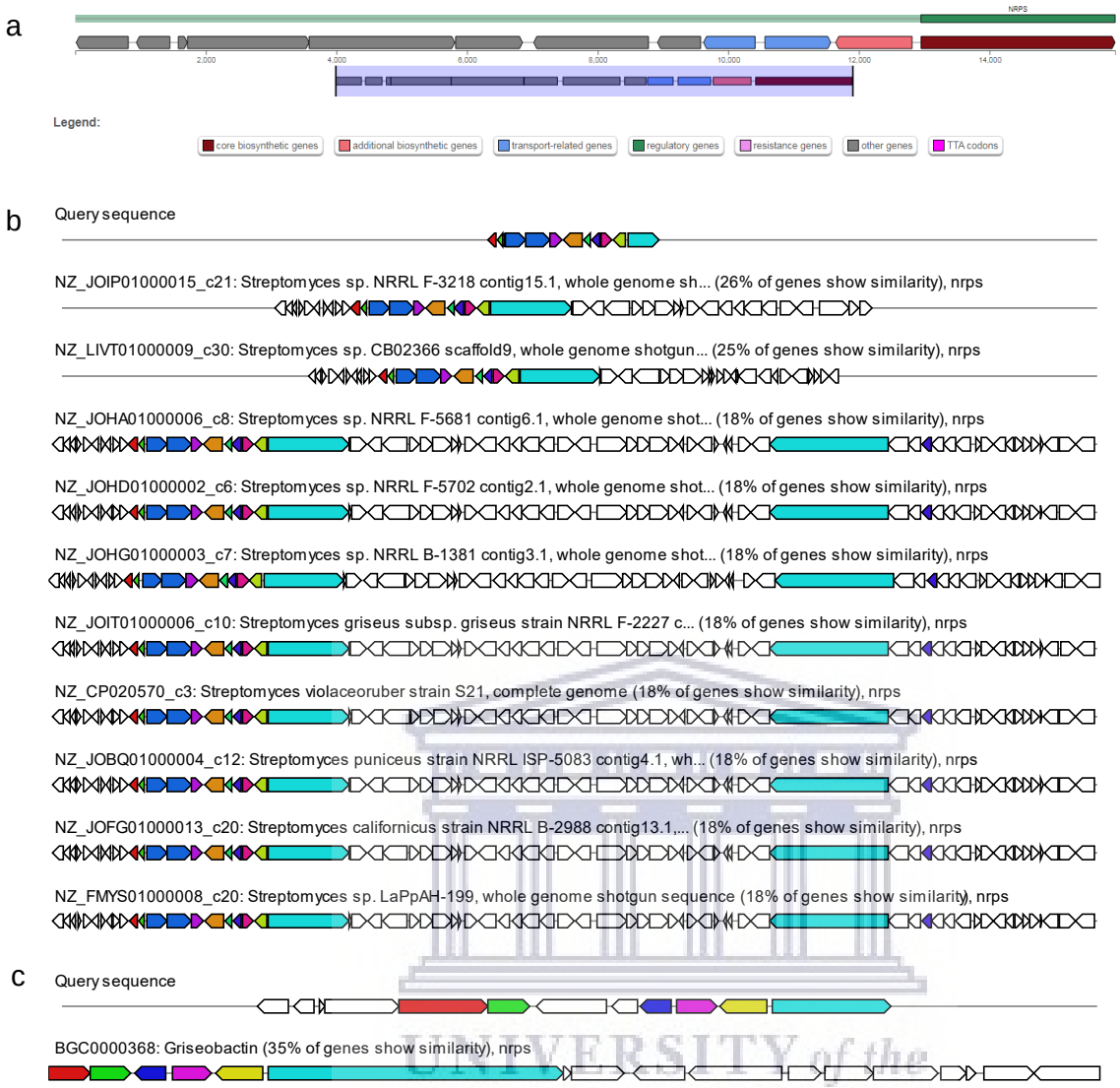


Figure D1.2: *a) Region 71.1 had similarities to NRPS gene clusters from b)* Streptomyces *species. c) The query sequence was 35% similar to genes involved in griseobactin synthesis. Results obtained from antiSMASH software.*

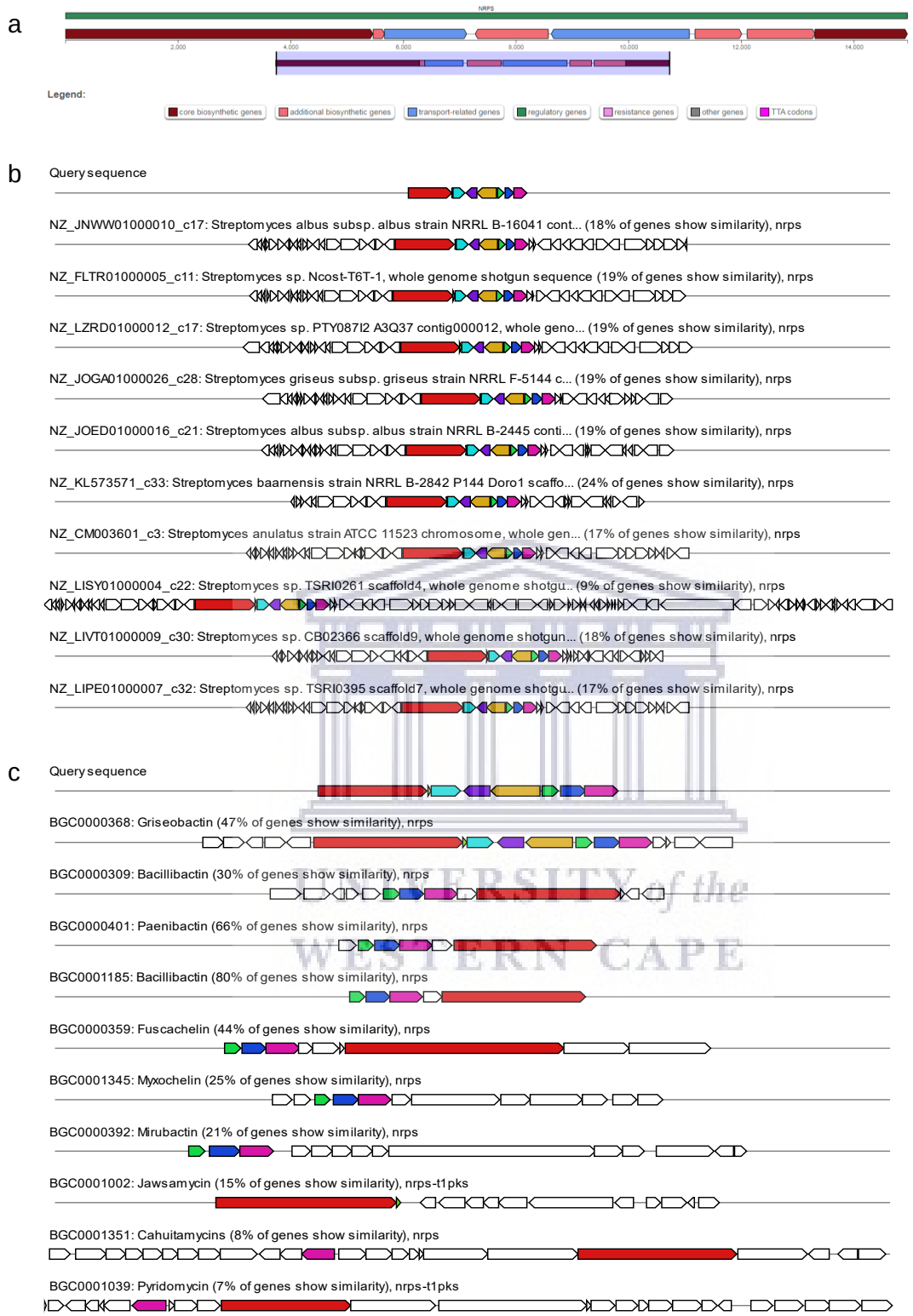
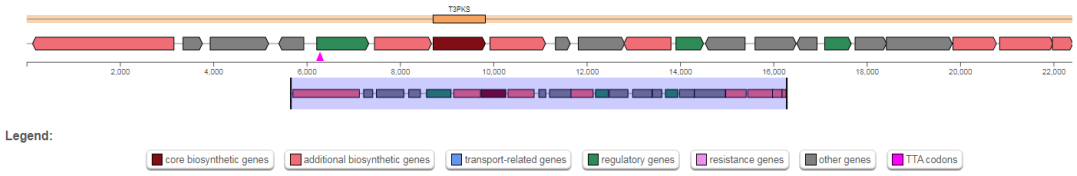
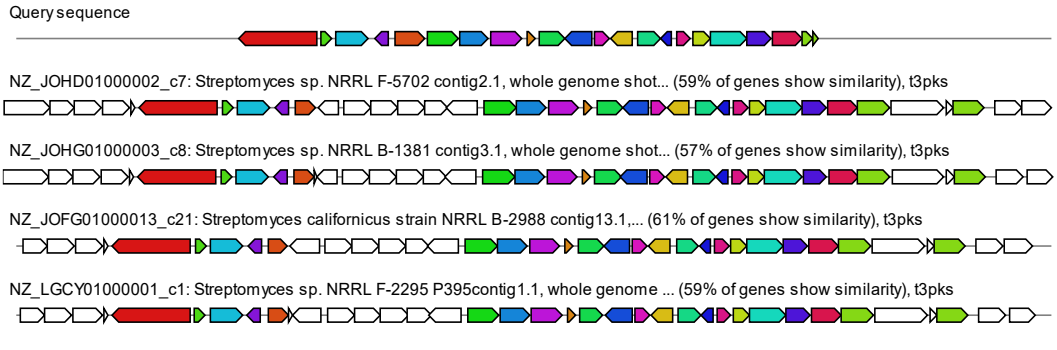


Figure D1.3: *a) Region 289.1 showed similarity to NRPS genes with low similarity to genes from* Streptomyces *strains. c) The biosynthetic genes had similarities to genes involved in griseobactin synthesis. Results obtained from antiSMASH software.*

a



b



c

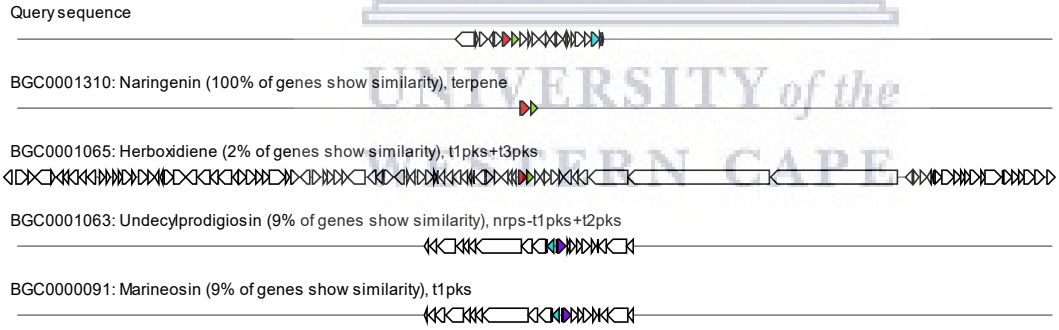


Figure D1.4: *a) Region 328.1 showed similarities to type-3 PKS biosynthetic genes and b) had 57% and higher similarities to genes from various* Streptomyces *species. c) The biosynthetic genes showed 100% similarity to naringenin biosynthetic genes. Results obtained from antiSMASH software.*

D2: Biosynthetic gene clusters from *Streptomyces* strain S149:

Figure D2.1: *a) Region 120.1 showed similarities to indole-type 3 PKS hybrid biosynthetic*

genes and b) had 100% similarity to genes from Streptomyces sp. NRRL S-475 *genes. c) The biosynthetic genes showed 60% similarity to 7-prenylisatin biosynthetic genes. Results obtained from antiSMASH software.*

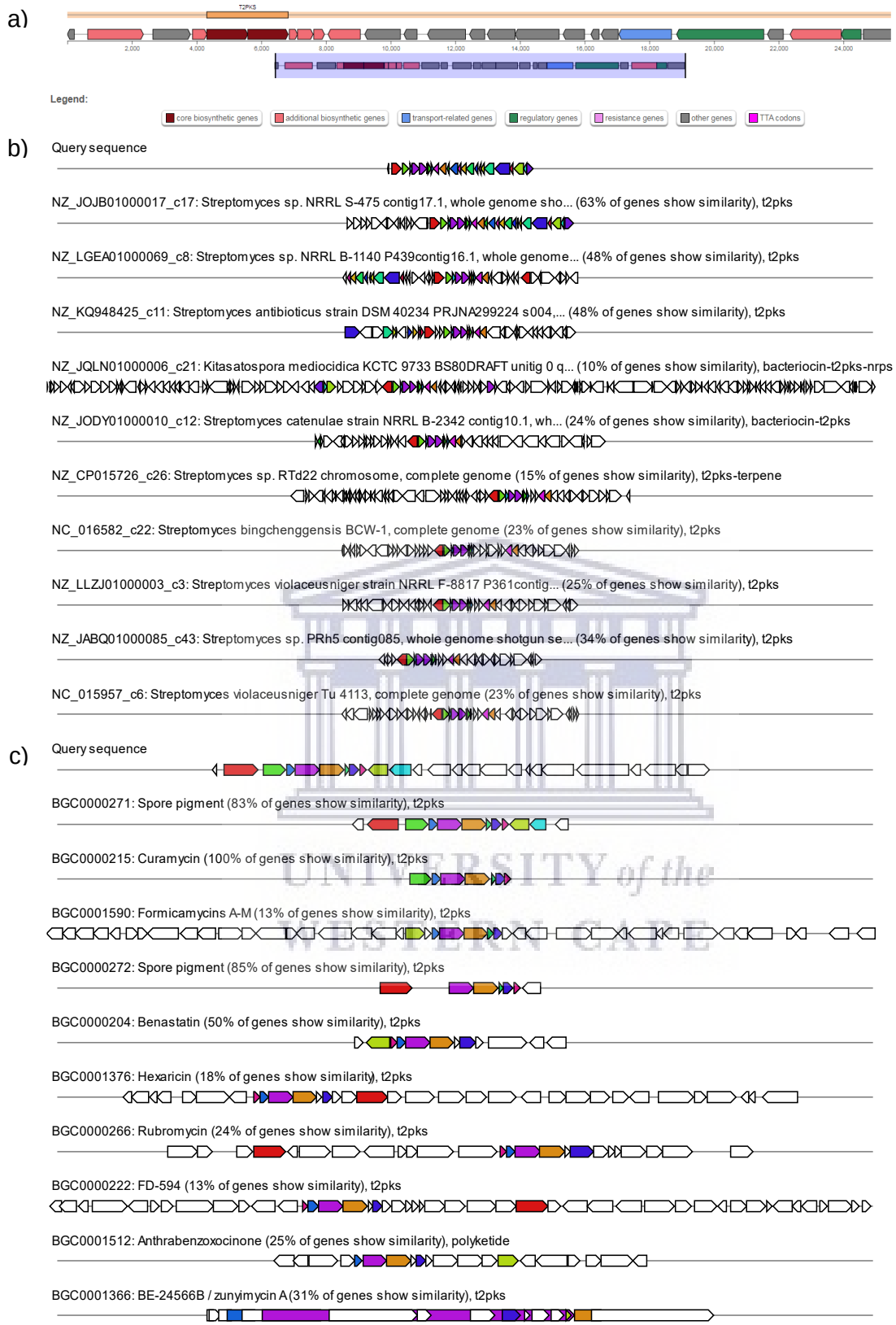


Figure D2.2: *a) Region 446.1 showed similarities to type-2 PKS biosynthetic genes and b) had similarities to genes from various* Streptomyces *species. c) The biosynthetic genes showed 100% similarity to the biosynthetic genes of curamycin. Results obtained from antiSMASH software.*

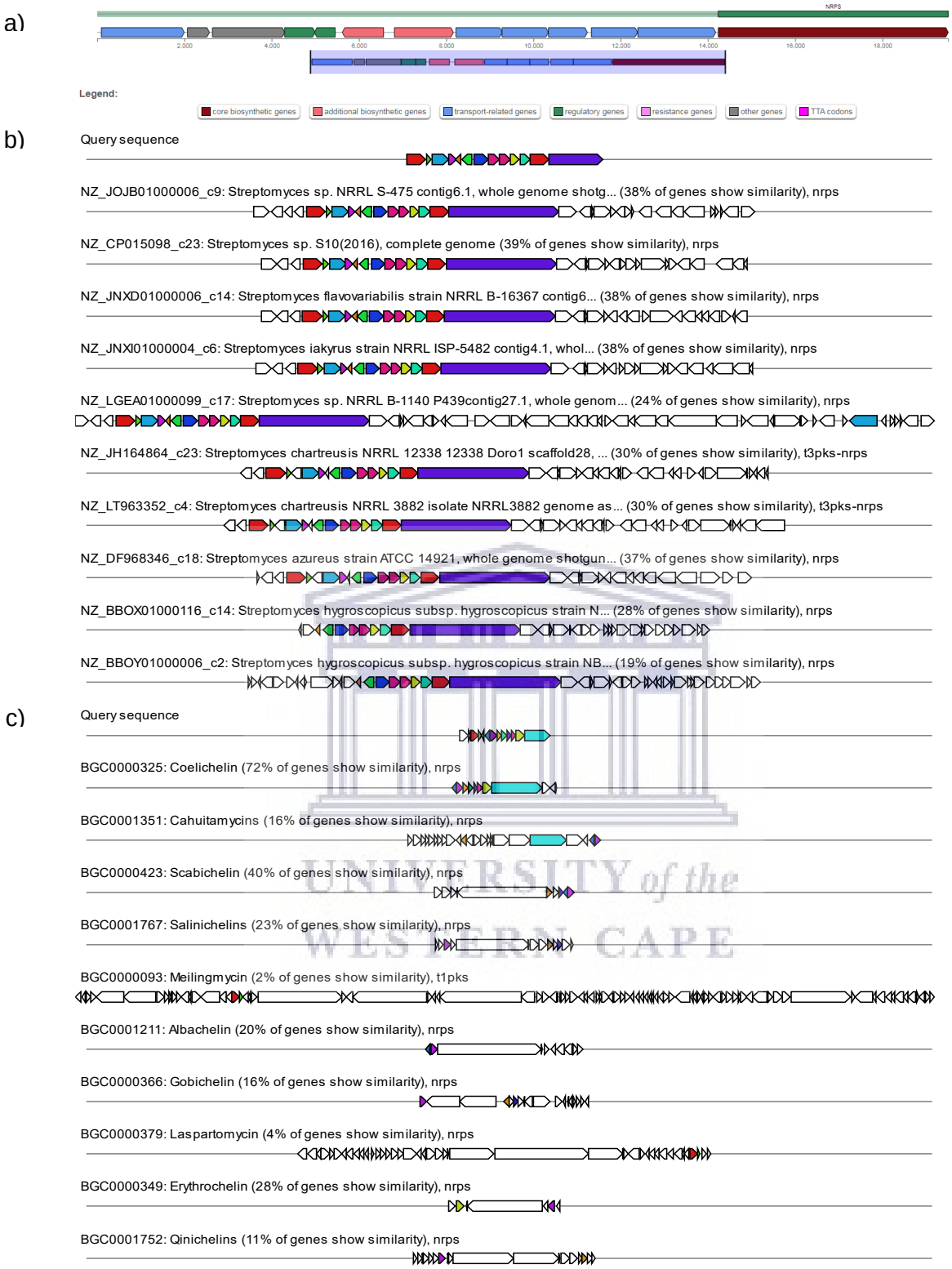


Figure D2.3: *a) Region 306.1 showed similarities to NRPS biosynthetic genes and b) had similarities to genes from various* Streptomyces *species. c) The biosynthetic genes showed 72% similarity to the biosynthetic genes of coelichelin. Results obtained from antiSMASH software.*

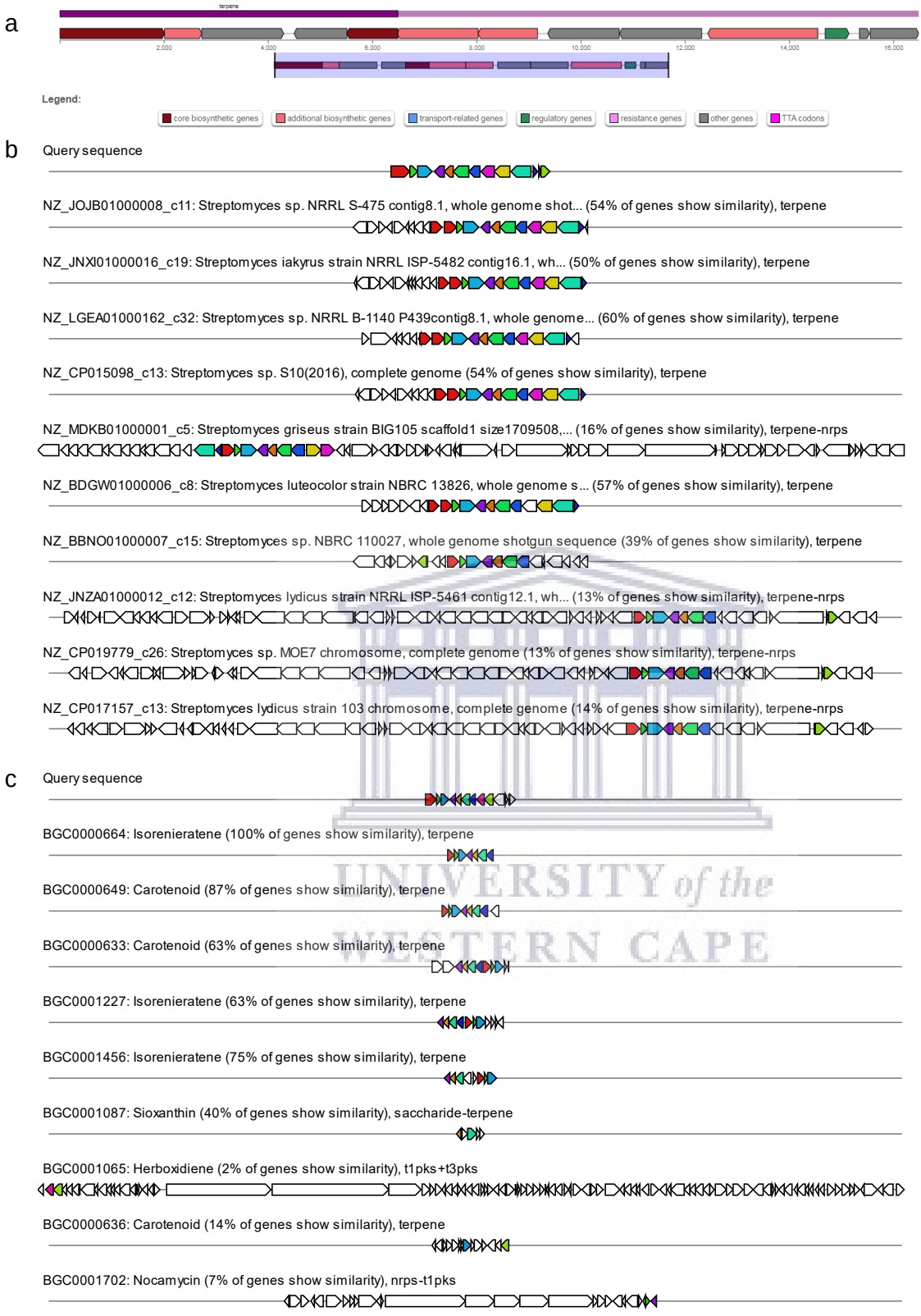


Figure D2.4: *a) Region 211.1 showed similarities to terpene biosynthetic genes and b) had similarities to genes from various* Streptomyces *species. c) The biosynthetic genes showed 100% similarity to the terpene biosynthetic genes of isorenieratene and 87% similarity to carotenoid biosynthetic genes. Results obtained from antiSMASH software.*

Figure D2.5: *a) Region 358.1 showed similarities to bacteriocin biosynthetic genes and b) had 100% similarities to genes from various* Streptomyces *species. c) The biosynthetic genes showed 42% similarity to the biosynthetic genes of informatipeptin. Results obtained from antiSMASH software.*

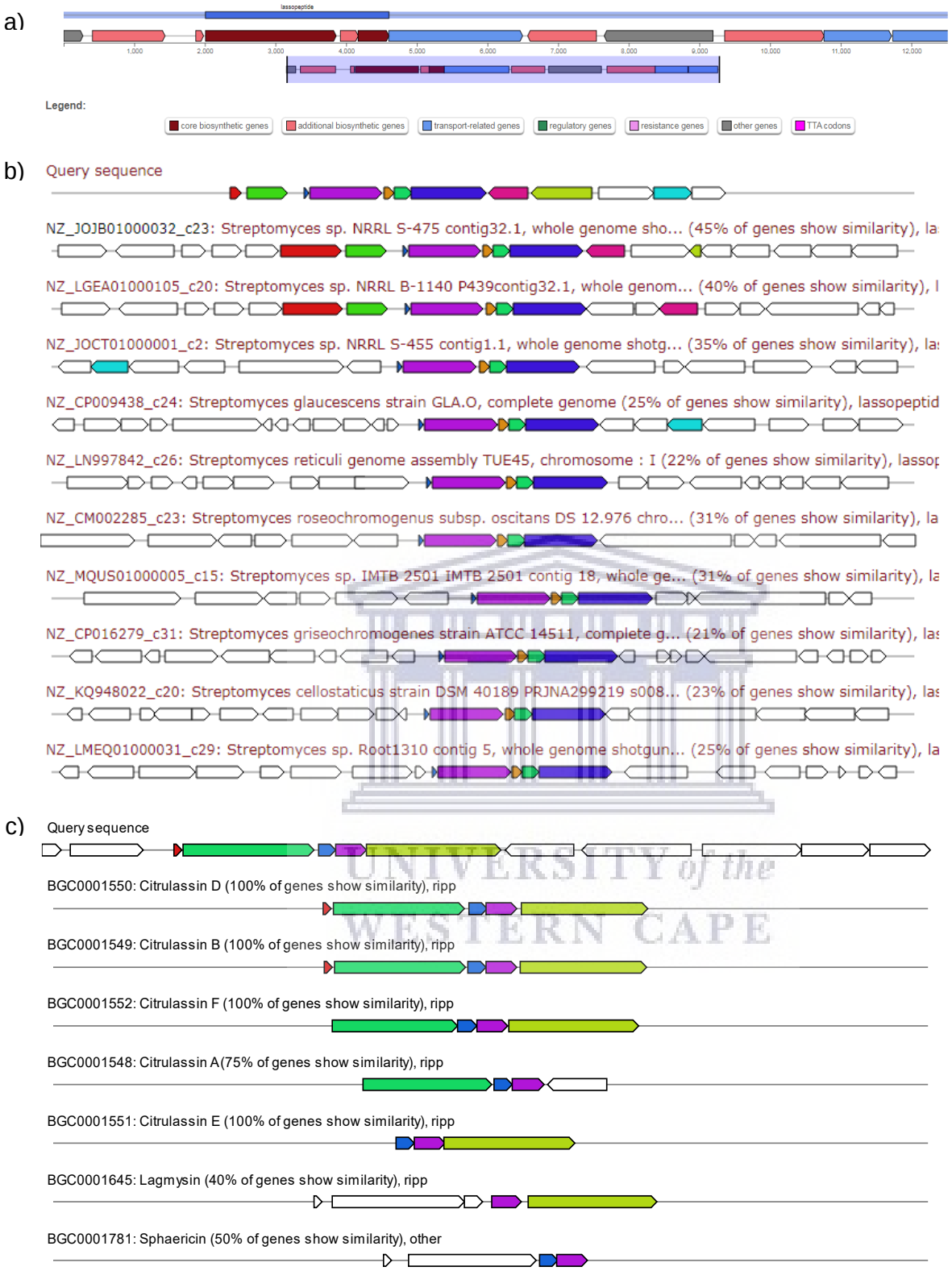


Figure D2.6: *a) Region 480.1 showed similarities to lasso peptide biosynthetic genes and b) had similarities to genes from various* Streptomyces *species. c) The biosynthetic genes showed 100% similarity to the biosynthetic genes of citrulassin D, B, F and E. Results obtained from antiSMASH software.*