A MOLECULAR-BASED ANALYSIS OF THE ANTIBIOTIC BIOSYNTHETIC POTENTIAL OF SELECTED MARINE *MICROMONOSPORACEAE* SPECIES

By

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DECLARATION

I, Brighton Mutepfe, do hereby declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, the views expressed in this thesis represent my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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ABSTRACT

Introduction

The discovery of the first antibiotic, penicillin, paved way for many other discoveries and developments much to the benefit of antibiotic treatment against bacterial infections. As years went by, the irresponsible use of antibiotic compounds in the medical, agricultural and veterinary fields, as well as lack of robust infection control protocols in clinical spaces, led to the emergence of antibiotic tolerant and resistant microorganisms. In an attempt to arrest the crisis, the discovery of new bioactive compounds that can be developed into potent novel antibiotics has been of importance. Amongst the many sources of bioactive compounds that have been researched for decades, microorganisms of the genus *Micromonospora* have been well documented as producers of potent bioactive metabolites, many of which have been successfully developed into novel commercial antibiotics. There is, however, a dearth of research information concerning the possibility of novel bioactive metabolites isolated from *Micromonospora* species from South Africa. Therefore, this study aimed to investigate the antibiotic biosynthetic potential of selected marine *Micromonospora* species isolated from the Algoa Bay region in Port Elizabeth, South Africa.

Method

A total of 30 *Micromonospora* strains isolated from Algoa Bay region, Port Elizabeth in South Africa were provided as frozen stock cultures at the Cape Peninsula University of Technology's Biocatalysis and Technical Biology (BTB) research unit. The strains were first cultured on SGG and 172 F solid and liquid media, with and without artificial sea water (ASW). The Gram stain was performed to ensure purity of strains and to evaluate microscopic morphology before extracting DNA. Multi-Locus Sequence Analysis (MLSA) of the *rpoB* and *gyrB* housekeeping genes was performed as well as 16S rRNA gene analysis. Phylogenetic analysis was performed using MEGA X and phylogenetic trees were constructed to this effect. Eight antibiotic biosynthetic gene clusters (BGCs) were screened for via PCR. Gaps in the current primer sets available for BGC screening were analysed. Genomic data for 44 *Micromonospora* strains was retrieved from EzBiocloud and antiSMASH and these assisted with primer designing after assessing primer-knowledge gaps. The designed primers were designed to target BGCs encoding for bacteriocins and lanthipeptides and were tested on five selected *Micromonosporaceae* strains. The antibacterial activity of the top five strains was also investigated using overlay studies on solid media cultures and bioautography studies in liquid media cultures. In addition, the efficacy of antibiotic extraction was tested through the use of five different antibiotic extraction techniques.

Results

Our results demonstrated that all the strains under study were viable *Micromonospora* species. Phylogenetic analysis of the five strains chosen for further analysis identified their closest related validly published type strain as *Micromonospora aurantiaca* ATCC 27029. Furthermore, one of the eight BGCs that were screened for, the Type II PKS BGC, was positive in 28 out of the 30 strains. Genomic information of the genus *Micromonospora* was retrieved from antiSMASH which assisted in assessing gaps in current primer knowledge. Ultimately this led to the design of new primers to target bacteriocin and lanthipeptide BGCs. Four of the five strains tested gave a positive PCR result, albeit with multiple bands. The multiple bands on the agarose gel signified non-specificity in the binding capacity of the designed bacteriocin BGC primers hence there was no exclusive and convincing evidence of existence of this gene cluster. Negative PCR results were observed for the Lanthipeptide primer set. Antibacterial activity analysis on solid and in liquid culture media proved that the five selected strains produced bioactive compounds that were active against Gram-positive (*Bacillus cereus* ATCC 10876) and Gram-negative (*Escherichia coli* ATCC 25922) pathogens as well as yeast (*Candida albicans* ATCC 24433).

Conclusion

The *Micromonosporaceae* species that were under investigation in this study show great potential as sources of bioactive metabolites with broad spectrum antibacterial activity as well as antifungal activity. These should be considered as suitable candidates for whole genome sequencing as well as comparative genome sequence analysis for greater insights into the *M. aurantiaca* group.

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DEDICATION

To my mother Lucy P. Mutepfe, my brother Vincent Mutepfe and my late father Thomas Mutepfe:

This piece of my academic thought process is a special dedication to you!! Your unconditional, unexplainable, deep love and unwavering support in all the spheres of my life is well appreciated. You are loved fam!

"We really are facing, if we don't take action now, a dreadful post-antibiotic apocalypse" **Dame Sally Davies.**

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GLOSSARY

LIST OF ABBREVIATIONS

16S rRNA	16S ribosomal Ribonucleic Acid
AGE	Agarose Gel electrophoresis
AIDS	Acquired Immune Deficiency Syndrome
ANI	Average Nucleotide Identity
AMR	Antimicrobial resistance
bp	Base pairs
BSA	Broad spectrum of activity
BTB	Biocatalysis and Technical Biology
CDC	Centres for Disease Control and Prevention
CDDEP	Centre for Disease Dynamics, Economics and Policy
cDNA	Complementary DNA
DNA	Deoxyribonucleic Acid
CRE	Carbapenem-resistant *Enterobacteriaceae*
GC content	Guanine-cytosine content
g/L	grams per litre
gDNA	Genomic DNA
GPL	Global Priority List
HIV	Human Immunodeficiency Virus
ICU	Intensive Care Unit
Kb	kilobases
meso-DAP	*meso*-diaminopimelic acid
MNPs	Marine natural products
MLSA	Multilocus sequence analysis
MRSA	Multidrug-resistant *Staphylococcus aureus*
MTT	[3-(4, 5-dimethyldiazol-2-yl)-2, 5 diphenyltetrazolium bromide]
NSA	Narrow spectrum of activity
OTC drugs	Over-the-counter drugs
PCR	Polymerase Chain Reaction
PKSs	Polyketide Synthases

PPL	Pathogens Priority List
R&D	Research and Development
rpm	revolutions per minute
sp.	Species
STI	Sexually Transmitted Infection
TB	Tuberculosis
TLC	Thin-layer liquid chromatography
USA	United States of America
UV	Ultraviolet
VISA	Vancomycin Intermediate *Staphylococcus aureus*
VRE	Vancomycin Resistant Enterococci
WHO	World Health Organization

CLARIFICATION OF TERMS

16S rRNA: A constituent of the prokaryotic ribosomal 30S subunit that is present in all bacterial species and important in investigating bacterial species-relatedness from an evolutionary perspective.

Actinobacteria: A Gram-positive phylum of bacteria with a very high guanine and cytosine content in their respective genomes.

Actinomycetes: A group of mycelial forming heterotrophic microorganisms under the order *Actinomycetales* and phylum Actinobacteria that produce a vast amount of biologically potent compounds.

Antimicrobials: Chemical compounds that are agents capable of acting against the growth of microorganisms or otherwise totally killing them.

Antibiotics: Type of antimicrobials that either halt or stop the growth of foreign or resident bacteria that cause infections.

Antibiotic resistance: The ability of bacteria to resist the effects of antibiotics that once slowed their growth or eliminated them.

Average Nucleotide Identity: A measure, between two genomes, of how similar they are with specific reference to their coding regions at nucleotide level.

Bacterial infections: The effect of the presence of bacteria in the body which are either foreign or normally resident but turning opportunistic to cause illness.

Bioactive secondary metabolites/compounds: Useful molecules of biological potency that are produced by microorganisms and are not necessarily a pre-requisite of the microorganisms' normal lifecycle.

Biosynthetic potential: The ability of a living organism to use its metabolism to formulate chemical compounds.

GC content: The ratio of the amounts of guanine to cytosine, usually expressed in a percentage, within the genome of a microorganism.

Genome: The total genetic make-up of an organism

Gram stain: A laboratory staining technique that is used to categorize bacteria mainly into two large categories, thus Gram positive and Gram negative microorganisms.

Housekeeping genes: These are genes in an organism's genome are typically expressed in both normal and abnormal conditions to maintain basic cellular activity and function thereof.

Integrons: Mobile genetic elements that possess gene-capturing characteristics enabled by site-specific recombination.

Marine environments: Habitats that are closely related to the seas and oceans with a great biodiversity relying on saltwater for sustenance.

***Micromonospora*:** A genus under the *Micromonosporaceae* family that is known to have filamentous, spore-forming and Gram-positive bacterial species.

Multilocus sequence analysis: A molecular biology technique that makes use of individual bacterial species' housekeeping genes.

Marine drugs: Pharmaceutical products that have their origin traceable from marine, and other closely associated, environments.

Microbes: Microorganisms that are usually bacterial (could also refer to fungi, viruses, and some protozoa).

Natural Products: Any compound or chemical in nature that is produced by a living organism.

Novel metabolites: Small undiscovered molecules that are by-products of the metabolic activities of microorganisms.

Over the counter drug: Medication that does not require presentation of a Doctor's prescription in order to buy it.

Plasmids: Small intracellular-based molecules of DNA which can replicate independently from the chromosomal DNA.

Polymorphisms: Differences in sequences, usually of closely related bacterial species.

Terrestrial environments: The earth's environments that are land areas together with their accompanying natural and human-made sub-surface features.

Transposons: Mobile genetic elements within a genome, capable of altering the genetic function of cells that carry them.

CHAPTER ONE

INTRODUCTION

1.1 Statement of research problem

The advent of antibiotics in the 1940s was a medically significant breakthrough that positively impacted on humankind's ability to treat bacterial infections (Davies & Davies, 2010; Hardy, 2016). Since then, the increased availability, use and abuse of the diverse array of antibiotics in the clinical, veterinary spaces and agricultural practices, has led to the emergence of antibiotic resistant pathogens that are harder to treat (Kumarasamy *et al.,* 2010; Meggersee & Abratt, 2014). Amongst the various avenues being explored by Scientists to counter the effects of antibiotic resistance and tolerance, development of new antibiotics from novel metabolites that are produced by microorganisms remains critical. The aim of this study is, therefore, to investigate the antibiotic biosynthetic potential of the genus *Micromonospora* isolated along South Africa's coastline.

1.2 Background to the research problem

There has been much progress, globally, around the isolation of bioactive metabolites from *Micromonosporaceae* (Boumehira *et al*., 2016; Hirsch & Valdes, 2010). Many of these metabolites have been developed into antibiotics such as neomacquarimicin, telomicin, micromonosporin A amongst many others (Boumehira *et al*., 2016). Despite notable output from *Micromonosporaceae*-centred drug discovery research, there have not been many studies focusing on *Micromonosporaceae* spp. isolated along South Africa's coastline.

The ongoing drug discovery and development research project at the Cape Peninsula University of Technology's Biocatalysis and Technical Biology research group has seen the isolation of more than 500 species of marine actinomycetes from sea sponges and sea squirts collected from the Algoa Bay region. The screening work previously performed on these isolates, however, sought to evaluate only their oxidative enzyme-producing capacity. This research will, therefore, seek to evaluate the antibiotic biosynthetic potential of 30 of the isolated *Micromonosporaceae* strains.

1.3 Research aim

- To use molecular techniques to determine the antibiotic biosynthetic potential of selected microbial strains of the family *Micromonosporaceae* that were isolated from sea sponges and sea squirts collected from Algoa Bay.

1.4 Research objectives

- To identify all *Micromonosporaceae* strains to the species level, using multi-locus sequence analysis.
- To perform a molecular screen for eight common biosynthetic gene clusters (BGCs) amongst the marine *Micromonosporaceae* strains present in the BTB culture collection.
- To analyse the genome sequences of *Micromonospora* strains to assist with the design of new primer sets.
- To identify gaps in the current knowledge around primer sets available for the molecular screening of antibiotic biosynthetic gene clusters and the design of new primer sets.
- To test the designed bacteriocin and lanthipeptide primer sets on five of the *Micromonospora* strains used in this study, for the presence of these gene clusters.

1.5 Research questions

- Is there laboratory-based evidence of the presence of viable *Micromonosporaceae* strains from samples in the BTB culture collection?
- Are there any identifiable *Micromonosporaceae* strains, to species level, by use of multi locus sequence analysis?
- Are there any identifiable knowledge gaps around primer sets available for the molecular screening of antibiotic BGCs and how would genome sequence analysis assist in the design of new primer sets?
- To what extent are the designed primers functional /beneficial in achieving the aim of the study?

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

"*When I woke up just after dawn on September 28, 1928, I certainly did not plan to revolutionize all medicine by discovering the world's first antibiotic or bacterial killer*." These were the humble words of the Scottish-born scientist, Sir Alexander Fleming, in retrospection of his accidental, yet significant discovery of the first antibiotic, penicillin (Hardy, 2016). This monumental discovery is undoubtedly one of the greatest discoveries during the history of humanity and it subsequently earned Sir Fleming a Nobel Prize in Physiology or Medicine in 1945 (Nobelprize.org, 2014). Alexander Fleming's discovery of penicillin paved way for greater discoveries, leading to the development of a diverse range of antibiotics. Humanity benefited immensely from these discoveries and people began living longer since clinicians finally managed to contain the crises of bacterial infections.

With progressing technology and research, scientists began to understand the pathophysiology of previously misunderstood pathologies, hence delivery of healthcare improved, and patients had better outcomes. Despite notable robust and accelerated developments in medical innovations through technology, bacterial infections that had been previously easy to manage suddenly became increasingly harder to treat due to the overwhelming emergence of antibiotic-resistant microorganisms. From that point of antibiotic discovery till now, a new crisis of antibiotic resistant infections has rendered the performance of modern medical procedures such as organ transplantation, surgery, chemotherapy and dialysis among others, challenging (White house, 2015).

Antibiotic resistance and tolerance are well attributable mainly to genetic and biochemical mechanisms within specific pathogenic bacterial species (Meggersee & Abratt, 2014). A global multi-dimensional approach involving clinicians, pharmaceutical retailers, scientists, patients and politicians is pertinent in efforts to control and reduce the effects of antibiotic resistance (Kumarasamy *et al.,* 2010). From a scientific perspective, continuous discovery of novel drug leads, in the form of novel metabolite-producing microorganisms, has become an important anchor in the fight against antibiotic resistance.

Habbu *et al.* (2016) noted the remarkable progress made in chemical synthesis and engineered biosynthesis of novel antibiotic compounds but went on to identify nature as the richest source of novel drug leads due to its vast diversity. Marine microorganisms have, over the past years, attracted unprecedented attention in biopharming prospects, especially marine actinobacteria. This is a phylum of microorganisms with a history of supreme biological relevancy for housing the *Streptomyces* genus, which accounts for up to 70% of

the world's antibiotics currently within the clinical space (Habbu *et al.,* 2016; Penesyan *et al.,* 2015; Zhang *et al.,* 2005). Under actinobacteria, the genus *Micromonospora* has attracted growing research interests ever since the isolation of several of this genus' strains as aminoglycoside-type antibiotic producers, most notably gentamicin from *Micromonospora echinospora* (Everest & Meyers, 2013; Kasai *et al.,* 2000). Since microorganisms of the *Micromonospora* genus are known to be abundantly found in aquatic habitats including beach sands and deep-sea sediments (Cross, 1981; Kawamoto, 1989), untapped marine environments such as the South African coastline are thus worth exploring as harbours of novel metabolite-producing *Micromonospora* species.

2.2 Antibiotics, their discovery and classification

2.2.1 What are antibiotics?

In 1941, Selman Waksman, a scientist famous for the discovery of the antibiotic streptomycin, formulated the noun “antibiotic” (Bhattacharjee, 2016; Clardy *et al.,* 2009). Waksman defined an antibiotic as a microorganism-derived chemical which, in solution, is characterized by selective potential of growth inhibition or complete elimination of some other microorganism (Kingston, 2004). As the science of antibiotic development broadened, scientists began making synthetic compounds with antibiotic properties, therefore also equally naming these compounds as antibiotics. This inclusion of synthetic antibiotics to fit into Waksman’s original definition of antibiotics faced resistance from Waksman and many other scientists. It was only with time that the noun antibiotic began being accepted as nomenclature to both natural and synthetic compounds (Bhattacharjee, 2016).

Bayarski (2011) loosely defined antibiotics as a group of drugs, which either retard or completely halt bacterial growth. Antibiotics however fall under a broader group of drugs called antimicrobial drugs, which include anti-parasitic, antiviral as well as antifungal agents. Bhattacharjee (2016) propagated that antibiotic administration in humans and animals is aimed at bringing positive therapeutic outcomes in the treatment of infections, therefore, antibiotics ought to be selective towards host tissues. This notion led to their widely accepted modern day definition, which defines antibiotics as molecules with an effect of slowing the growth of or totally killing pathogenic microorganisms while causing the minimum possible harm to host tissues (Aminov, 2010; Davies & Davies, 2010).

Some scientists have decided to take an all-inclusive route in defining antibiotics. For instance, Bhattacharjee (2016) defined antibiotics as chemicals targeting virulent agents on a selective inhibition basis, at the same time effecting as minimal harm to the host as possible. It is important to note that antiprotozoal, antiviral, antifungal as well as anticancer drugs all have mechanisms of action that mimic those of antibiotics hence an all-inclusive, non-restrictive definition to suit all these compounds. Such a definition has, however, faced criticism in some circles with scientists specifically pointing out, as an example, cancer as a pathology not initiated by a foreign pathogen such as other bacterial infections. Proponents of the all-inclusive definition of antibiotics have pointed out the Muehlenbachs *et al.* (2015) discovery of the cancer-causing tapeworm *Hymenolepis nana* in response to the aforementioned criticism. However, for the purposes of this study, the common restrictive definition of antibiotics will take effect since this study focuses on antimicrobial agents strictly against bacteria and not any other pathogens.

2.2.2 Classification of antibiotics

There are various approaches used in order to group antibiotics into various clusters. The individual characteristics that enable such classification include, but are not limited to, chemical and/or molecular structure, the antibiotic's mechanism of action as well its spectrum of activity (Calderon & Sabundayo, 2007).

2.2.2.1 Classification according to chemical structure

Adzitey (2015) identified the classes of antibiotics shown in Figure 2.1 as the major antibiotic classes from a chemical structure point of view. It should however be noted that more antibiotic classes not shown below, such as the streptogramins, do exist but do not house antibiotics which are as common as those of the classes shown below.

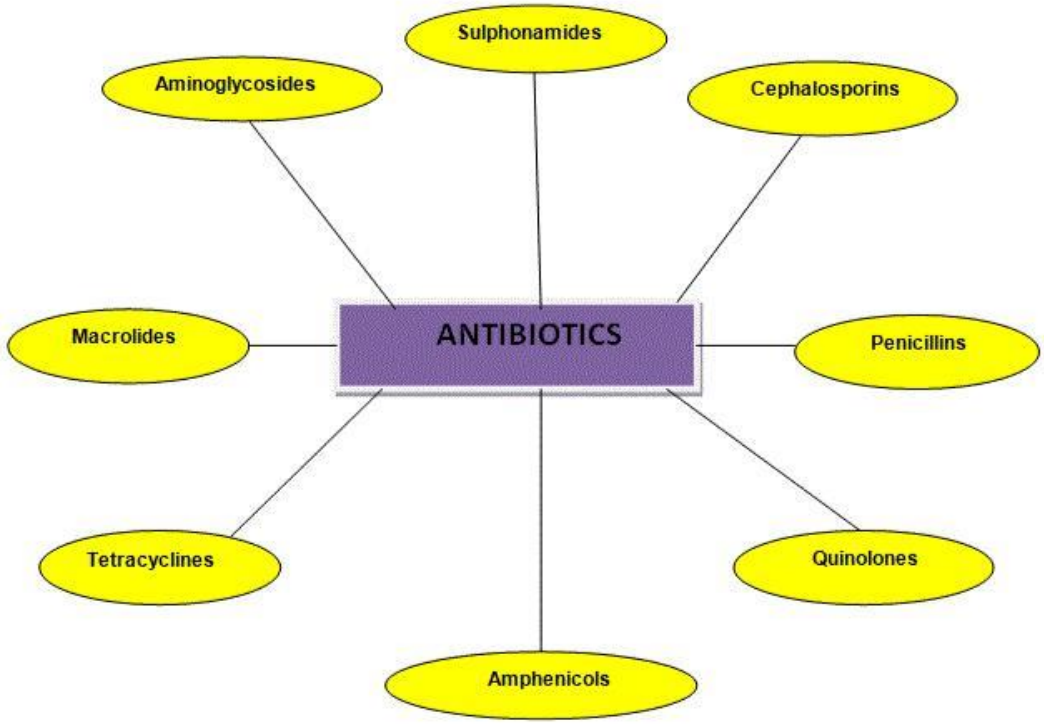


Figure 2.1: A representation of the 8 major, chemically-related, antibiotic classes in clinical practice. The individual antibiotic compounds within each of these classes have closely related chemical structures hence the above-depicted classification (Adzitey, 2015).

2.2.2.2 Bactericidal and bacteriostatic antibiotics

When it comes to their mechanisms of action, antibiotics can be broadly categorised as bacteriostatic or bactericidal (Calderon & Sabundayo, 2007). Antibiotics are said to have a bacteriostatic mode of action when they slow the growth rate of colonising bacteria as opposed to killing any colonising bacteria. The latter mode of action is regarded as

bactericidal (Adzitey, 2015). The following antibiotic classes are examples of bactericidal antibiotics and their specific targets within bacterial cells:

i. Penicillins and Cephalosporins: Function by targeting cell walls of a bacterial colony.
ii. Fluoroquinolones and Sulphonamides: Function by disrupting key bacterial enzyme pathways.
iii. Polymyxins: Function by destroying bacterial cell membrane integrity.

Bacteriostatic antibiotics take a slightly different, though universal, mode of action. Their specific target in foreign microorganisms is the protein-synthesis biochemical machinery. If protein synthesis is disrupted in colonising microorganisms, it subsequently means that all protein-dependent processes within bacteria will suffer thus leading to a decreased growth rate. Classes of antibiotics that operate as such include tetracyclines, aminoglycosides and macrolides (Calderon & Sabundayo, 2007).

2.2.2.3 Classification according to specific mechanisms of action

Classification of antibiotics according to their specific mechanisms of action, as shown in Table 2.1, is also common. The basis of such classification lies in the fact that bacterial infections require the viability of their individual bacterial cells within bacterial colonies in order for clinical manifestation to occur. This subsequently means antimicrobial agents need to target the pathogenic bacteria's cellular processes, in the treatment of bacterial infections. The way antimicrobials function becomes the basis of antibiotic classification according to mechanism of action (Korzybski *et al.,* 2013). Cellular processes which are key for disruption in order to halt growth or kill the pathogenic bacteria, include protein synthesis, nucleic acid synthesis, cell wall synthesis, folic acid synthesis as well as mycolic acid synthesis (ibid). With time, as they are exposed to the same antibiotics, bacterial cells develop counter mechanisms which in turn escalate the rate at which the aforementioned cellular processes occur, thus leading to antibiotic resistance. These mechanisms of resistance are explained in the sections to follow.

Table 2.1: Antibiotic classification according to mechanisms of action (Korzybski *et al*., 2013).

MECHANISM OF ACTION	ANTIBIOTIC CLASSES
Protein synthesis inhibitors	30-S ribosomal subunit inhibitors: Tetracyclines Aminoglycosides 50-S ribosomal subunit inhibitors: Chloramphenicol Clindamycin Macrolides Linezolid Streptogramins
DNA synthesis inhibitors	Fluoroquinolones Metronidazole
Inhibitors of cell wall synthesis	Vancomycin Cephalosporins Penicillins Carbapenems β-lactamase inhibitors Aztreonam Bacitracin Polymycin
Inhibitors of folic acid synthesis	Trimethoprim Sulphonamides
Inhibitors of mycolic acid synthesis	Isoniazid
RNA synthesis inhibitors	Rifampin

2.2.2.4 Classification according to spectrum of activity

Bacterial infections emanate from two kinds of broadly categorized microorganisms, *viz* Gram-positive and Gram-negative microorganisms (Baker-Austin *et al.*, 2006). Some infections are regarded as mixed infections, whereby the causative bacterial species are a mixture of Gram-positive and Gram-negative microorganisms (Sarpong & Miller, 2015). Many of the antibiotics in clinical use are considered to either have a broad spectrum of activity (BSA) or narrow spectrum of activity (NSA) (ibid). Adzitey (2015) further pointed out that those antibiotics that are specifically active against either Gram-positive or -negative bacteria are regarded as having NSA while those active against both Gram-positive and -negative bacteria are considered to have BSA. Some authors, however, argue that the term “narrow-spectrum antibiotics” can be used essentially to describe antibiotic compounds of limited activity, which target specific bacterial species not necessarily considering their Gram reaction. For instance, Guardabassi & Courvalin (2006), highlighted aminoglycosides and sulfonamides as narrow spectrum antibiotics as they only effectively target aerobic microbes. On the same basis, nitroimidazoles are also exemplified in literature as NSA antibiotics as they also only target anaerobic microbes (ibid).

It is, however, important to note that although the above explained are the most commonly used criteria of antibiotic classification; they do not represent the exhaustive list of antibiotic classification criteria. For instance, another criterion that can be used for antibiotic classification, according to Etebu & Arikekpar (2016), is the route of drug (antibiotic) administration. Classification according to the route of administration would focus on the three routes of antibiotic administration, which are oral, intravenous and topical (ibid). Such classification has a pitfall in the sense that choice of route of antibiotic administration can be determined by the clinical condition of a patient. For instance, an unconscious patient in need of antibiotic therapy can have an intravenous administration of an antibiotic, which is normally administered orally. It is, however, important to appreciate the overview in antibiotic classification provided by the above-described classification criteria.

2.2.3 Discovery and evolution of antibiotics

The discovery of antibiotics is noted in medical history as a significant breakthrough since many infectious diseases became curable and the fate of humankind took a remarkable turn (Aminov, 2010; Carlet *et al.*, 2012; CDDEP, 2015; Martinez, 2014; Ventola, 2015). Ever since their inception into the medical and agro-based practices, antibiotics have seen wide range of pertinent uses. Such uses include treatment of infections, prophylactic administration in surgically acquired hospital infections, oncology patients with minimal immune responses as well as agro-based uses for healthy livestock production (CDDEP, 2015). Nathan & Carl (2014) highlighted that modern medical practice has significantly improved due to availability of antibiotics in severe infections therapy as well as in medical and surgical procedures as prophylaxis.

This current study focuses on attempts to address the problem of antibiotic resistant and tolerant microorganisms with special focus on the discovery phase of antibiotic development. It, therefore, becomes a matter of paramount importance to look back from where it all began, thus the timeline of antibiotic discovery, evolution as well as the emergence of resistance. Such a review of these aspects of literature will allow for the appreciation of the gains and challenges of all era of the antibiotics story; including present day, as well as insights into the future.

2.2.3.1 Pre-antibiotic era

Since the ancient times, humankind had been seriously haunted by diseases, many of which were bacterial infections, which the medical fraternity found difficult to comprehend, let alone cure. The first bacterial infection treatments in history, as described by Keyes *et al.* (2003), saw ancient cultures resorting to substances such as mouldy bread, plants and soil among others for remedy. The period before the discovery of the first antibiotic was dominated by a pursuit of knowledge concerning the understanding of how diseases came about, their

progression and possible remedies. It is important to note the challenges that the scientist of the day had to encounter in the pre-antibiotic period. Not much literature existed since no breakthroughs had been made and technological research capacity was not as advanced as that of centuries that were to follow.

As pointed out by Otten (1986), the germ theory of disease proposed and expanded by Girolamo Fracastoro in 1546 and Marcus von Plenciz in 1762, respectively, became an important foundation in the comprehension of the relationship between microbes and diseases. The germ theory of disease postulated that many of the diseases, which humans and animals suffer from, are a direct result of the actions of specific microorganisms (Bastian, 1875). After the establishment of this theory and gradual consensus of the same, scientists shifted their focus from comprehending disease occurrence to finding effective ways of getting rid of these specific disease-causing “germs”.

The present day Gram-negative bacillus, *Pseudomonas aeruginosa*, is recorded in the antibiotic history as the firstly discovered producer of pyocyanase, a by-product with antimicrobial activity (Levy, 2002). This observation dates back to 1888 and the microorganism was known as *Bacillus pyocyaneus.* In the year that followed, the newly discovered pyocyanase failed at clinical trial level after showing signs of instability and considerable toxicity levels to host tissues. Although this immediately disqualified this compound as a breakthrough antibiotic, it reemphasized the fact that selectivity and stability were going to be paramount for any antibiotic candidate anticipated to successfully make it into the clinical space (Levy, 2002).

2.2.3.2 The golden age of antibiotics

Scientific research has proven that exposure of humanity to antibiotics dates back to a period way before the commonly known “antibiotic era” (Aminov, 2010). To be specific, this period of humanity’s first exposure to antibiotics has been identified as the period from the golden age of antibiotics onwards. Bassett *et al.* (2010) and Nelson *et al.* (2010) highlighted that the discovery of traces of tetracycline, as an example, dating as far back as 350-550 CE in human skeletal remains of Sudanese origin suggested diets which contained tetracycline enriched foods amongst these subjects.

However, the “modern antibiotic era” has Sir Alexander Fleming’s 1928 discovery of penicillin as its landmark (Ventola, 2015). This historic discovery came about when Sir Fleming realised that the growth of *Staphylococcus aureus* on a culture plate appeared to be halted by a certain unknown “mould juice” secreted by the fungus later identified as *Penicillium notatum* (Saga & Yamaguchi, 2009). Fleming’s discovery was, however, just the genesis of a long and rigorous set of laboratory experiments, which would include attempts to purify the

compound, decipher its chemistry as well as to produce it in large quantities before clinical trials could commence.

The discovery of penicillin was a development that would be received with mixed feelings amongst scientists. On the one hand, it created hype and excitement, but on the other hand, scepticism was inevitable. The previous failed experiences of pyocyanase and another compound, salvarsan, made scientists approach Fleming's penicillin very cautiously (Zaffiri *et al.*, 2012). On his own part, Fleming encountered difficulties cultivating the *P. notatum*, as well as purifying what he had perceived to be the active ingredient of the fungus (ibid). Fleming's experiences with *P. notatum* remain a key aspect in modern day research within the field of antibiotic development. It remains vital for any compound posing as a candidate for a novel antibiotic to be easily purified in large quantities.

The year 1935 was another landmark year within this golden era of antibiotics. Protonsil, a sulfonamide developed by Gerhard Domagk, became the first sulfonamide to be commercially available for clinical therapeutic use after successfully passing clinical trial (Otten, 1986). The therapeutic successes of Protonsil are well documented in history. Infections such as meningitis, pneumonia and childbed fever became easily curable and mortality rates from these drastically declined (ibid). It is important to note how the success or breakthroughs reported by other scientists, work as inspiration to colleagues within similar fields of research. Exemplifying this notion were Howard Florey and Ernst Boris Chain who, under the inspiration of Domagk's successful discovery of Protonsil, pursued the chemical elucidation of Fleming's penicillin, which enabled them to produce it in large purified quantities successfully (Zaffiri *et al.*, 2012).

In 1940, the discovery of Actinomycin became another landmark discovery, which marked the genesis of the "Golden age of antibiotics" which some authors refer to as the "Great antibiotic era" (Murphy *et al.,* 2012). This era resulted in the development of more than 100 antimicrobial drugs effective against infectious diseases as well as other ailments such as hypercholesterolemia and carcinomas. Most notably, 1942 saw the year when penicillin hit the commercial market and subsequently saving millions of lives. Salyers & Whitt (2005) pointed out sulpha drugs, streptomycin and penicillin as the first three antibiotics that found use in the clinical space after successful laboratory development.

Penesyan *et al.* (2015) and Ventola (2015) noted the immense therapeutic use of penicillin (Figure 2.2), amongst World War II soldiers in preventing and controlling the spread of infections. After the discovery of penicillin, many other antimicrobial agents were developed in years that followed. After penicillin, another notable antibiotic discovered was streptomycin, an anti-tuberculosis agent, which is a derived from *Streptomyces griseus* (Nanjwade *et al.,* 2010). This was subsequently followed by the development of other notable

antibiotics, from 1940s to 1960s, which included chloramphenicol, macrolides, glycopeptides among others.

Figure 2.2: A penicillin carton and vial on display at the science museum, in the United Kingdom, in present day (Retrieved from http://www.sciencemuseum.org.uk/broughttolife/objects/display?id=6091)

The era of antibiotic discovery was subsequently followed by a phase of improvement of these antimicrobial compounds (Saga & Yamaguchi, 2009). It became necessary to improve the various discovered antimicrobial classes since this increased their therapeutic efficacy (ibid). It is during this phase of drug discovery that scientists became too confident in antibiotic use to the extent of naively declaring that infections of bacterial origin had been defeated for good (Penesyan *et al.*, 2015).

2.2.3.3 Beyond the golden age of antibiotics

After the golden age of antibiotics, scientists began to realise that the efficacy of many antibiotics in the market was diminishing. Personnel within the medical and veterinary fields became reluctant and reckless, and the imprudent use of the newly developed antibiotics, such as penicillin continued unabated (Inglis *et al.,* 2005; Luangtongkum *et al.*, 2006).

Ironically, in 1945, Sir Alexander Fleming had warned of the possibility of the emergence of mutant forms of the microorganisms that were no longer susceptible to penicillin due to misuse of the drug (Ventola, 2015). Fleming's foresight became a reality, and by 1955 many countries resolved to upgrade the penicillin from an over the counter (OTC) drug to a prescription antibiotic (ibid). This move, however, did little to thwart the sporadic rise in resistance against penicillin, especially amongst staphylococci, since use of the drug had increased widely. The period between 1960 and 1964 then saw the pharmaceutical industry

and scientists alike becoming under pressure to save penicillin in the wake of growing resistance against this famous “miracle drug”. These efforts subsequently led to the development of methicillin, which is a semi-synthetic modification of penicillin (Inglis *et al.,* 2005).

Methicillin had been developed as a solution to the resistance against penicillin but, in no time, *S. aureus* became resistant against this drug thus the infamous multi-drug resistant or methicillin–resistant *Staphylococcus aureus* (MRSA); a menace that has burdened even modern day medicine (Chen *et al.*, 2017). It is important to note that in all antibiotic era; viz pre-golden age, golden age and post-golden age, the cycle of antibiotic discovery followed by emergence of resistance has been an ongoing one as shown in Figure 2.3. This observation is the basis of this current study; in the wake of continuous emergence of drug resistant microorganisms, the search for novel metabolites for the development of new antibiotics ought to be an equally continuous effort.

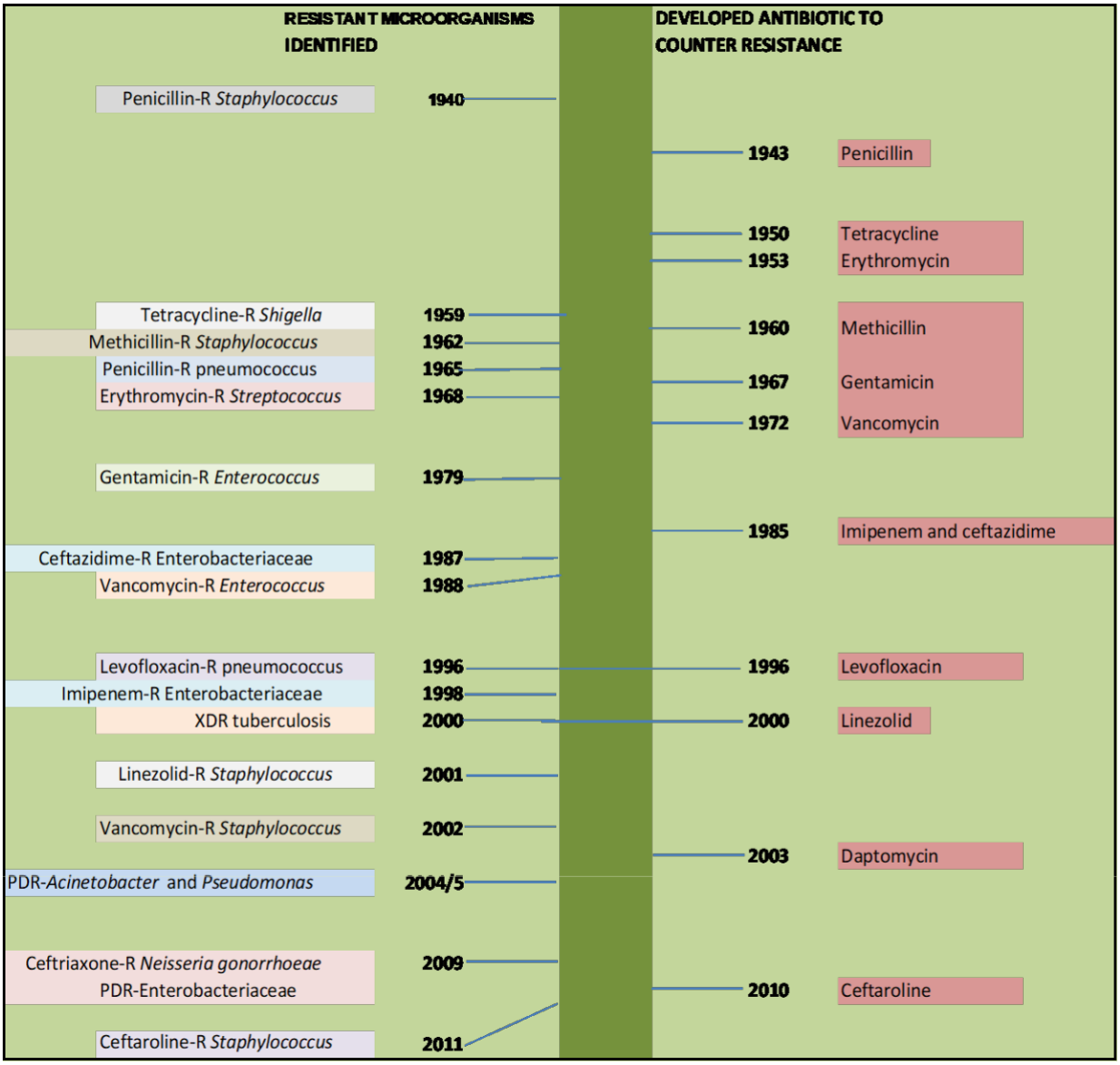


Figure 2.3: An account detailing the evolution of antibiotics through all three eras; pre, golden and post golden antibiotic discovery era. The cycle of major antibiotics discovered during specific years, pathogens which developed resistance against these, and subsequent antibiotics developed to counter such resistance is well outlined (Ventola, 2015).

2.3 Antibiotic resistance and its effects

2.3.1 Emergence and spread of resistance

The misconception that bacterial infections had been totally conquered by the discovery of antibiotics was discredited by the emergence of bacterial resistance to antibiotic therapy in the late 1970s. Scientists had become complacent and felt that the fight against antibiotic resistance was finally conquered. Ever since, the rapid emergence of antibiotic resistant organisms, and subsequently infections, has been noted as a serious public health threat, especially in clinical set-ups (Mulvey & Simor, 2009). Wright & Poiner (2012) pointed out the declining prioritisation and interest in novel antibiotic development research from a pharmaceutical perspective, as one other aspect that indirectly fuels the uncontrollable emergence of bacterial resistance to antibiotic therapy. Gould & Bal (2015) partly corroborated with Wright & Poiner (2012) as they summarised the push factors of antibiotic resistance as the following:

I. Wrong prescription and over-prescription of antibiotics to patients by clinicians.
II. A decreased developmental output of novel antibiotics by pharmaceutical entities due to:
    (a) Decreased incentives for scientists' research efforts.
    (b) Stringent legislative bottlenecks along the entire process of drug development.

The notion that there is a lack of adequate research incentives to support antibiotic discovery and development seems to be relatively common in literature (Grabowski *et al*., 1990; Simpkin *et al*., 2017; Towse *et al*., 2017; Williams & Bax, 2009). Until recently, there seemed to be limited political will, especially amongst developing countries, with respect to governments' funding towards antibiotic discovery and development projects (Simpkin *et al*., 2017). One of the many reasons that can be attributed to this development is the observation by Butler *et al*. (2013) and Chopra (2008) that many research and development (R&D) companies are inherently dumping antibiotic R&D in favour of other perceivably lucrative therapeutic ventures.

Antibiotic resistance is triggered at either biochemical or physiological levels, or even combinations of mechanisms falling under these two broad categories (ibid). The activity of resistance-conferring genes, called r genes, is what ultimately leads to antibiotic resistance. Liu and Pop (2009) noted the existence of a published genomic database, formulated from selected bacterial genomes, which shows more than 20 000 potential r genes. They further highlighted that these resistance genes are of up to 400 different categories, which shows

how broad and diversified antibiotic resistance potentially emanates, from a genotypic perspective. Such an observation indeed raises great concern of returning to the pre-antibiotic era amongst healthcare workers, scientists and medical pundits.

The Centre for Disease Dynamics, Economics and Policy (CDDEP) in its 2015 state of the world's antibiotics review clearly stated that the continuous emergence of highly antibiotic resistant microorganisms has caused serious global threats not sparing even first world countries (CDDEP, 2015). If any extrapolative conclusions can be made, to have first world countries who boast of world class healthcare systems suffering from threats of antibiotic resistance microorganisms, can only but point to a looming global crisis.

When bacteria in a specific colony are subjected to antibiotic therapy, given sufficient time, they ultimately win the contest of survival within their colonies hence the emergence of resistance. The rise in antibiotic resistance has been attributed to many factors, chief amongst them being their prolonged irresponsible medical and veterinary use, lack of adherence to infection control programs and antibiotic management programs (Giedraitiene *et al.,* 2011). Emergence of antibiotic resistant microorganisms translates into global threats when such microorganisms begin to spread. For instance, global human movement in the form of migration, emigration and tourism has been noted in literature as the primary vehicle of the spread of global antibiotic resistant microorganisms (CDDEP, 2015). The general increase in income globally also translates to increased access to antibiotics, thus an increase in both their appropriate and inappropriate use which contributes to a rise in antibiotic resistance (ibid).

In 2016, the Centres for Disease Control and Prevention, (CDC), published a shocking case of a patient in Nevada who succumbed to the carbapenem-resistant *Enterobacteriaceae* (CRE) which was found to be resistant to 26 antibiotics (Chen *et al.*, 2017) and progressed to cause septic shock. The specific CRE isolated and identified as *Klebsiella pneumoniae* was reported to be resistant to all aminoglycosides and polymyxins tested (ibid). The isolate was also reported to be resistant to tigecycline; a glycylcycline antibiotic developed to combat the exponential antibiotic resistant rate depicted by microorganisms such as *S. aureus, Escherichia coli* and *Acinetobacter baumannii* (Chen *et al.*, 2017; Rose & Rybak, 2006). This is a classic case of multi-drug resistant microbes becoming fatal. If efforts of developing new antibiotics do not match such levels of emergence of antibiotic resistance, the coming decades may lead to a total collapse of the healthcare systems globally.

Clinical setups have also been well documented to have a huge impact in the emergence and subsequent spread of antibiotic resistance. Brusselaers *et al.* (2011) propagated that clinical Intensive Care Units (ICUs) world over have become a dangerous source of antibiotic

resistant organisms due to below-par infection control, selective competence on resistant strains and emergence of new mutations amongst the already immune-vulnerable patients.

2.3.2 Mechanisms of resistance

The basis of the resistance of many bacteria to antibiotics lies within their genes (Mulvey & Simor, 2009). This genetic linked resistance can be either intrinsic resistance or extrinsic resistance (ibid). In simple terms, intrinsic resistance in bacteria is displayed when that specific bacteria resists the therapeutic effect of a group of antibiotics due to its natural genomic make up. Two good examples of such a mechanism of antibiotic resistance are depicted in the group streptococci which are predictably resistant to aminoglycosides as well as the resistance of Gram-negative bacilli to all vancomycins (Mulvey & Simor, 2009).

In contrast, acquired resistance is a result of alteration in a microorganism's genome. Such alterations can be in the form of mutations or acquisition of totally new genetic material in the form of or facilitated by plasmids, transposons and integrons (ibid). This mechanism of resistance is common in *Mycobacterium tuberculosis*, which is well known to resist the antibiotic isoniazid (Torrey, 2016). Ultimately, all these alterations in genomes of pathogens lead to biochemical modifications in bacterial cells as shown in Figure 2.4, thus effecting antibiotic resistance (Torrey, 2016).

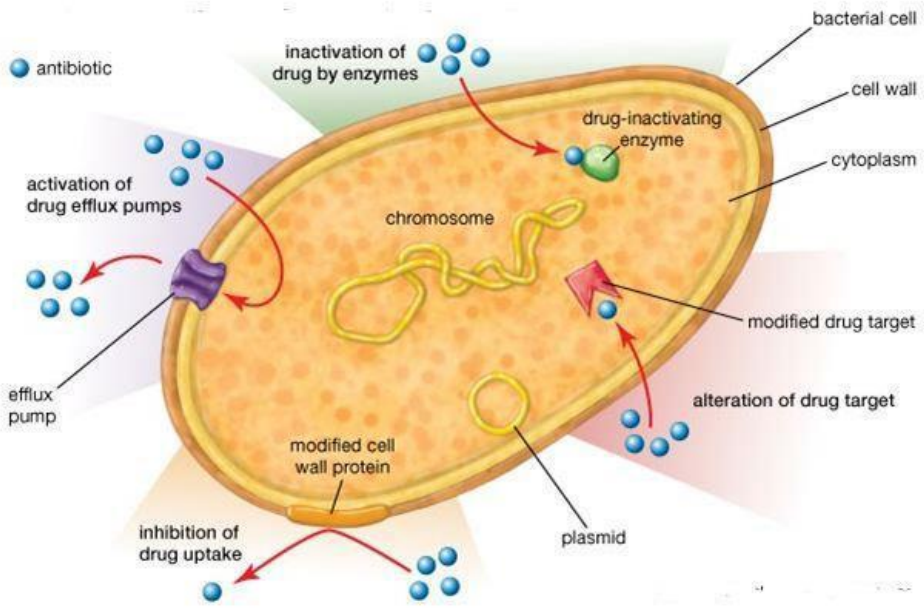


Figure 2.4: Examples of biochemical mechanisms of antibiotic resistance within bacterial cells. The cell wall of bacteria plays a crucial role in conferring resistance to colonising microbes. Mechanisms such as activation of drug efflux pumps eliminate antibiotics molecules before reaching their target organelles and total drug inhibition by cell wall protein modification are two key examples. Within the bacterial cell, several mechanisms which specifically serve to deal with antibiotic molecules which

would have successfully evaded cell wall inhibitory mechanisms become key in conferring resistance. Such examples, as shown above, include modification of the drug target and direct enzymatic inactivation of the antibiotic molecules (Retrieved from: Bbosa *et al*., 2014).

2.3.3 Socio-economic impact of antibiotic resistance

It is a matter of great concern to note that the discovery and development of new commercial antibiotics are now inversely proportional to the emergence and re-emergence of drug resistant infections (Martinez *et al.*, 2014). The rapid emergence of antibiotic resistance impacts public health care and subsequently derails efforts of governments to offer affordable, accessible and quality health care especially in third world developing countries.

As of March 2015, statistics from the United States of America revealed that approximately 2 million illnesses and 23 000 deaths were being annually reported, in the United States (US) only, due to antibiotic resistant infections (White house, 2015). The CDC further reported that such figures in the US served as a backdrop of loss in economic productivity to the tune of $US 35 billion. In the USA, the antibiotic resistant infections crises saw the Obama administration initiating a national action plan that sought to analyse and integrate interventions by key stakeholders to counteract antibiotic resistance (White House, 2015). The prioritisation of the fight against Antimicrobial Resistance (AMR) in the USA under President Obama brought about a promising chapter globally due to the USA's global influence and global public healthcare obligations. Such political will enhances research and development of novel antibiotics amongst scientists, a group of professionals who usually face unnecessary bureaucracy and bottlenecks in their attempts to influence policy to combat of AMR.

As of 2009, in Europe, 25 000 annual deaths were reportedly attributed to antibiotic resistant infections. This translated to a massive € 1.5 billion, in direct and indirect related annual losses (ECDC, 2009). Economists at the World Bank have put it on record that the impact of global antibiotic resistance, by the year 2050, is likely to be as devastating as the infamous 2008 global economic meltdown provided the current trends in AMR remain unchecked (Adeyi *et al.*, 2017). Unlike the 2008 global economic crisis which was buffered by a cyclical recovery phenomenon, a worst-case scenario of completely antibiotic resistant infections would hit hard developing nations as its costly impact will most likely persist, hence pushing an approximate 28 million people into abject poverty (Adeyi *et al.*, 2017).

2.3.4 Antibiotic resistance in the African context

African countries have not been spared from the reality of antibiotic resistance and its effects. For instance, it is known that the multi-resistant MRSA prevalence is considered relatively high in a number of African countries (Shittu & Lin, 2006). These include Morocco, Nigeria, Kenya and Cameroon (ibid). More notably, at the turn of the millennium, a South African

countrywide study revealed worrying statistics about antibiotic resistance in the context of tuberculosis (TB). The study noted that 1.8% cases of newly diagnosed TB patients, as well as 6.7% cases of known TB cases, were actually of a multi-drug resistant type (WHO, 2018).

As early as 2004, the World Health Organisation (WHO) had released alarming statistics concerning the South African incidence rate of TB. According to a WHO 2006 report, South Africa's TB incidence of 718 cases per 100 000 people in a population was the worst amongst the then known 22 high-burden countries. The WHO went on to further estimate that in 2012, up to 600 000 new TB cases would be recorded in South Africa (Brink, 2014). However, this turned out to be an overestimation since the number of new TB cases in South Africa in 2012 was later reported to be approximately 400 000 (Sotgiu *et al*., 2017).

The effects of antibiotic resistance in Africa are exacerbated by poverty and the prevalence of HIV/AIDS that are rife on the continent's developing countries. Cotton *et al.* (2008) pointed out the existence of increased antibiotic resistance infections in South Africa's poor communities amongst HIV/AIDS infected children. In a comprehensive study in South Africa, the top multi-drug resistant pathogens identified to increase child mortality in HIV positive children included *Streptococcus pneumonia, Moraxella catarrhalis* and the Gammaproteobacteria, *Haemophilus influenza* (Cotton *et al.,* 2008).

2.3.5 Negative clinical impact of antibiotic resistance

The reality of the negative impact of antibiotic resistance manifests itself in bacterial infections which increasingly become harder and harder to treat regardless of historically being successfully treatable. This can be seen as a gradual reversion to the pre-antibiotic era where patients died in their numbers due to lack of effective antimicrobials.

The CDC, in a 2015 report, outlined that *Enterococcus* species and *S. aureus* in their antibiotic resistant form were the most serious global scourge. In a rather seemingly dramatic, yet factual analogy, Michael *et al.* (2014) echoed the CDC's sentiments and highlighted that annual MRSA mortality rates in the USA are much more than HIV, emphysema, homicide and Parkinson's disease combined. Boucher *et al.* (2009) noted that the common antibiotics historically used in the treatment of MRSA, vancomycin and teicoplanin, had been long resisted by the MRSA. Evolution of resistance to conventional antibiotic therapy within MRSA birthed the Vancomycin intermediate *S. aureus* (VISA), another teicoplanin-resistant strain (Boucher *et al.*, 2009).

Vancomycin-Resistant Enterococci (VRE) are also known to be a therapeutic menace in modern day medical practice (Ventola, 2015). Before developing resistance to vancomycin, enterococci infections were historically treatable by this antibiotic. In its analysis of the antibiotic resistance threats facing the USA, the CDC (2013), highlighted that annual

mortality attributable to nosocomial VRE infections was up to 1300 deaths. Rossolini *et al.* (2014) however mentioned the existence of alternative drugs of choice for the treatment of VRE infections and these include quinupristin/dalfopristin and linezolid. Other drug resistant microorganisms causing specific infections as well as the drugs which were once effective against these, are given in Table 2.2.

Table 2.2: A summary of some of the common bacterial infections and the antibiotics that historically were a common therapy to such before alternative therapy had to be developed due to the antibiotic resistance crises in clinical practice.

Infectious causative agent and clinical infections	**Antibiotics previously effective**	**Alternative therapy in use**
Streptococcus pneumonia: • Meningitis and bacterial pneumonia. • Sinusitis, septicemia, ear infections.	• Penicillins (such as Amoxicillin). • Erythromycins (such as Azithromycin).	• Pneumococcal conjugate vaccine (PCV13).
Mycobacterium tuberculosis: • Often lung TB	• First-line drugs (isoniazid; rifampicin) • Second-line drugs (amikacin, capreomycin, kanamycin); • Fluoroquinolones	• TB resistant to first line drugs treated by second line drugs. • XDR-TB resistant to almost all drugs-therapy options limited.
Enterobacteriaceae (such as. *E. coli* and *K. pneumoniae*): • Difficult to treat *Enterobacteriaceae* infections.	• Carbapenems (drugs of last resort).	• Fosfomycin and Tigecycline (Alternatives though not significantly effective).

2.3.6 WHO global priority R&D list

Research and scientific development of antibiotics are regarded as a very important avenue of combating the ever-rising global scourge of antibiotic resistant infections. It is notable that developing novel antibiotics and antibacterial agents comes at a cost; thus, it makes sense to develop priority lists to counteract only the most threatening bacterial species at any given time. According to a news release on 27 February 2017, the WHO announced a set of 12 bacterial families regarded as the greatest threat to global public health (WHO, 2017). The WHO refers to such a list as a global priority list (GPL) (ibid).

Dr Marie-Paule Kieny, from the WHO's health systems and innovation arm, highlighted the fact that time is running out in the quest to get new effective antibiotics into the market hence the WHO's intervention in formulating and periodically updating its R&D priority lists through GPLs (WHO, 2017). Such interventions by the WHO are mainly necessitated by the fact that

if the discovery and development of novel antibiotics are determined by market forces only, new drugs will not emerge on the market timeously and these drugs may not be in line with urgent public health needs (ibid).

In a fact sheet published in October 2016, the WHO, however, stressed the fact that efforts to invest in the discovery and development of new antibiotics can be hindered if a global behavioural change is not realised in antibiotic prescription and use (WHO, 2016). A few of the changes that the WHO encourages to compliment an effective novel antibiotic development system include vaccination-based infection control, improved hygiene of food and water, robust promotion of safe sex practices as well as hand washing (ibid).

The latest global priority pathogens list (PPL) formulated by the WHO was as a result of the analysis of AMR, which was carried out using a multi-criteria decision analysis approach (WHO, 2017). Such an analytical approach is very effective since it brings into unison expert opinion and research-generated data in a transparent unbiased and productive manner. The WHO's recently adopted global PPL, for which an accelerated effort to develop new antibiotics against these pathogens is imminent, has three priority levels as shown in Figure 2.5; viz. critical, high and medium (WHO, 2017). The role of natural products in the discovery and subsequent development of these urgently needed antibiotics cannot be overemphasised.

2.4 Natural products in antibiotic discovery

Ever since ancient times, natural products have been the common foundation of disease therapy amongst humans, to the extent of nature being referred to as an ancient pharmacy by some authors (Montaser & Luesch, 2011). Lahlou (2013) noted that the broad term "natural products" can be broken down to microbes, minerals, animals and plants. Ganesan (2008) corroborated with this notion and further opined that the greater quantity of pharmaceutical armamentarium currently on the market is sourced initially from natural products in comparison to the ones that are not. In essence, many infections in the veterinary and clinical practice are treatable by a diverse range of pharmaceutical products that have origins traceable to natural products (Rojas *et.al.*, 1992; Tawiah *et al.,* 2012). The discovery of penicillin, according to Singh (2014), did not only revolutionise antibiotic discovery as a field but also provided a focal point of scientific research in natural microbial products.

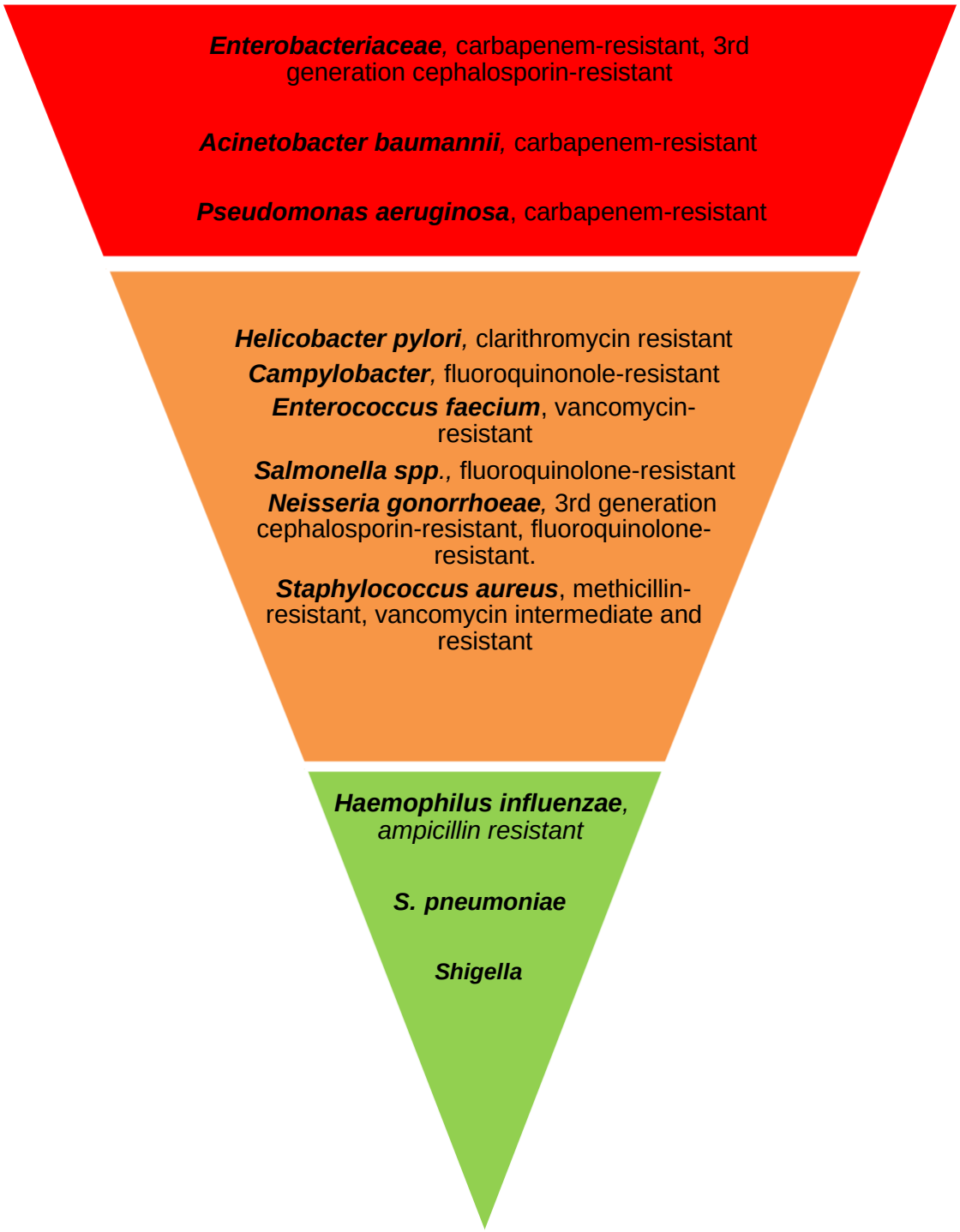


Figure 2.5: A representation of the WHO's latest research and development global priority list. Microorganisms that are regarded as critical targets against which new antibiotics are urgently needed are shown in the red zone, while the orange zone represents microorganisms of high priority. The pathogens in the green zone are medium priority targets for which new antibiotics are urgently needed.

Akhondzadeh *et al.* (2003) gave typical examples of herbs that have been exploited for their antimicrobial therapeutic uses successfully. Some of these important secondary metabolites include the chemotherapeutic vinca alkaloids, the cardiac failure therapeutic drug digitoxin sourced from the *digitalis* leaf, the antimalarial drug quinine sourced from the *cinchona* tree bark, as well as the qinghaosu tree bark antimalarial derivative, artemisinin. The natural products that are important in antibiotic discovery are broadly categorised into two classes, namely terrestrial derived and marine derived natural products.

Various ways exist for accessing natural products from their specific sources. Generally, water is used as the universal medium in which many of these extractions occur (Tawiah *et al*., 2012). Extractions of bioactive compounds from natural products usually begins with initial screening for bioactivity using extraction techniques that can be either in crude aqueous or alcohol phases (Cowan, 1999). These processes are typically followed by various extraction processes in the organic phase (ibid). Ethanol and methanol extraction methods are also commonly used to access bioactive compounds from natural products such as plant-derived aromatic bioactive compounds (Akhondzadeh *et al.*, 2003).

2.4.1 Terrestrial vs marine-based drug discovery

The period 1950 to 1960 saw an increase in research investment annually; to the tune of US$10 billion, specifically into drug discovery from microorganisms, by pharmaceutical entities (Murphy *et al.*, 2012). Such huge financial commitments led to massive discoveries of potent terrestrial microorganisms from a wide spectrum of environments ranging from arctic, cold temperate places, as well as tropical environments (Murphy *et al.*, 2012). The majority of these discoveries came from more temperate environments (ibid). It makes sense that terrestrial microorganisms became the undeniable focal point of drug discovery during these years. This hype amongst scientists to source bioactive metabolites from terrestrial microorganisms was quite huge despite 70% of the earth's surface being occupied by oceans thus providing vast marine spaces, which could be of equal research relevancy (Yu *et al*., 2015).

Murphy *et al.* (2012) pointed out that the oceans and their associated environments never received significant attention in microbial drug discovery due to a general notion that they were mere "storehouses" of most microorganisms that originated from terrestrial environments. Yu *et al.* (2015) corroborated with this observation and further pointed out that true marine microbes did exist albeit requiring cumbersome cultivation for scientific manipulation and analysis. With the emergence of antibiotic resistance, research into microbial drug discovery shifted focus to marine microorganisms. It is imperative to note how research-based evidence began to strongly endorse the marine ecosystem in general as a source of chemical compounds that are both novel and organic (Yu *et al.,* 2015).

2.4.2 The marine ecosystem in antibiotic development

2.4.2.1 Why focus on marine drugs?

Drugs which are referred to as “marine drugs” can be defined as those pharmaceutical compounds sourced from plants, animals and microorganisms which find marine spaces as their habitats (Doshi *et al.,* 2011). There is increased attention towards the marine ecosystem as a source of metabolites that are possibly novel drug leads for antibiotic development. The main reason for this attention from scientists is attributable to the vastness of the marine ecosystem that subsequently translates to more eco-diversity as compared with other ecosystems such as the terrestrial ecosystem. To put this fact into figures, Das *et al.* (2008) highlighted that the oceans on our planet have a coastline stretching to an approximate sum of 620 000km with a combined volume of $137km^3 \times 106km^3$ hence being the largest ecosystem.

Microorganisms that are habitual in marine environments occur in large numbers, generally. Murphy *et al.* (2012) highlighted that a millilitre of seawater could contain up to 10 million viruses, 1000 bacteria as well as 100 fungal species. These marine microbes are widely and diversely distributed within estuaries, seawater suspensions as well as sea sediments. The surfaces of macroorganisms also harbour marine microorganisms and so do their interiors (Murphy *et al.,* 2012). Stadler and Dersch (2016) highlighted that marine environments are habitats to a great deal of microbial populations and biodiversity. Ellis (2001) corroborated with this fact and further described the oceans as “*The Medicine Chest of the New Millennium*”. Since this current study seeks to investigate the biosynthetic potential of marine *Micromonospora* species, it becomes relevant to specifically review marine-based microorganisms generally, with a focus on their bioactivity.

2.4.2.2 Marine microorganisms as a specific source

Microorganisms, in general, are well documented as prolific sources of antibiotics in both terrestrial and marine environments. The discovery of penicillin from *Penicillium notatum* in 1928 paved way for further discovery of many other bioactive compounds from microorganisms. Notable examples of some antibiotics that have their foundations traceable to microorganisms, in general, include chloramphenicol, erythromycin, cephalosporin C, vancomycin, nalidixic acid and rifamycin among many others (Tawiah *et al.,* 2012). The cornerstone of drug development to counteract antibiotic resistance has seen, over the past two and half decades, the sourcing of bioactive metabolites from terrestrial microorganisms gradually declining in favour of marine microorganisms. Living microorganisms are now known to exist abundantly within oceans, contrary to assertions of the past, which labelled such environments as having a dearth of life and diversity (Fenical & Jensen, 2006). To put this notion into perspective, Penesyan *et al.* (2015) further opined that the cell count in a

millilitre of seawater could be as much as between 10^6 - 10^9 cells. This points out to an environment of research relevancy with vast microbial diversity and great potential in diverse metabolic activity. In general, marine microorganisms remain relevant in drug discovery due to their vast diversity as well as vastly evolved physiological and biochemical abilities, courtesy of selective pressure, that resulted in them exuding bioactive metabolites (Doshi *et al*., 2011). These bioactive metabolites are also useful in processes such as communication, reproduction and the microorganisms' self-protection (ibid). The South African coastline, therefore, is worthwhile exploring as a potential source of microorganisms capable of producing novel metabolites.

2.4.3 The South African coastline

South Africa has a biodiverse coastline that stretches over a distance of approximately 2 500km (Atkinson and Clark, 2005). The Namibian desert border with South Africa on the Western coast marks a point where the coastline starts (ibid). The coastline then runs southwards, around the African continent tip right to the South African-Mozambique border on the Indian ocean, as shown in Figure 2.6.

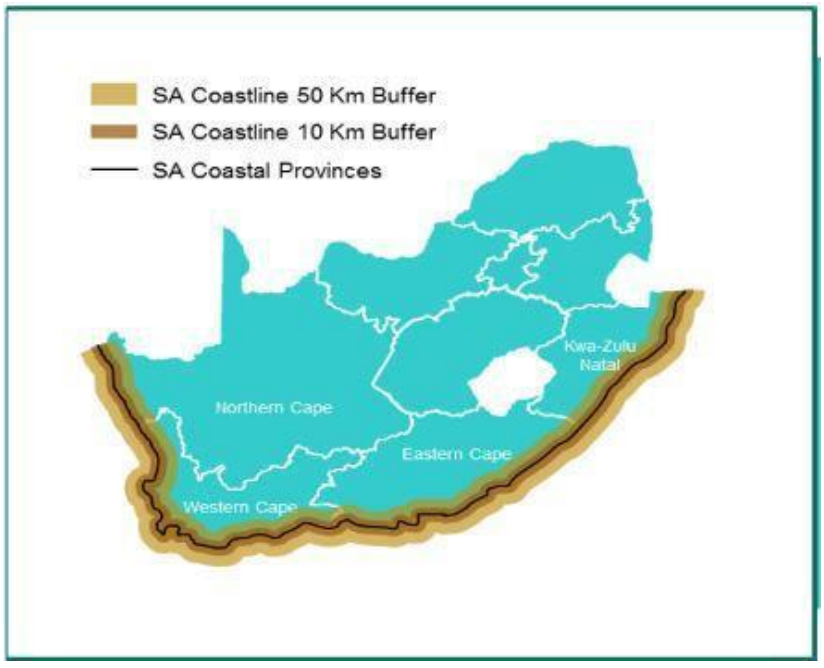


Figure 2.6: The South African 2500km long stretching coastline depicted by the brown trail. McCarthy and Rubidge (2005) described this coastline as a very smooth one containing very few, minimally modified natural harbours. Over the past 20 million years, South Africa has extensively undergone geographical upliftment resulting in this remarkably smooth coastline. McCarthy and Rubidge (2005) further pointed out that the South African coastline developed from the once intact underwater continental shelf, which was characteristic of limited gorges or deep ravines.

Doshi *et al.* (2011) highlighted the fact that the world oceans harbour a vast diversity of microorganisms of diverse pharmacological activity. Sharma & Ohja (2005) corroborated with this notion and further highlighted the fact that marine-derived microorganisms have historically provided over 25 000 bioactive compounds for further development into possible novel antibiotics. It, therefore, makes sense to regard the South African coastline as a very relevant research focus sampling area. This is because its vast area subsequently translates to vast biodiversity of marine species that it harbours (Atkinson & Clark, 2005).

2.4.4 Challenges in marine natural product drug discovery

Marine natural products (MNPs) are mainly sourced from microorganisms and invertebrates such as sea squirts, sponges, corals, algae, bryozoans and coelenterates amongst many others (Singh, 2014). Despite the success stories of drug discovery from marine ecosystems, challenges in this field of science are inevitable. For instance, marine microorganisms are generally not easily cultivatable on routine laboratory nutrient media. They are very slow growers hence requiring patience as well as strict culture media and growth conditions optimization in some instances (Singh, 2014).

Another challenge in the field of marine drug discovery and development is the high costs involved in the entire biotechnological process chain, from discovery of novel metabolites to clinical trials (Santhi *et al*., 2017). In some marine-based drug discovery projects, the costs involved in harvesting sources of targeted metabolites are huge inorder to suffice for clinical trial phases (ibid). A typical example is when the compound Bryostatin 1, a potential cancer chemotherapeutic agent sourced from *Bugula neritina*, had to go into the clinical trial phase after its discovery. As little as 18 g of this compound was produced from 13 tons of harvested *B. neritina*, a bryozoan, for the clinical trials (Mander & Hung-Wen, 2010). In such a situation, resources could be channelled to increase the harvest for the specific source microorganism but, Santhi *et al*. (2017) noted the devastating effect on the ecological set up such an intervention would have.

The rediscovery of already discovered compounds is also a challenge within the field of MNP drug discovery. The euphoria and optimism, which usually grips scientists, involved in MNP discovery projects, often turns into disappointment if the purportedly novel compounds turn out to be known compounds (Tulp & Bohlin, 2005). Singh (2014) reported a decline in funding towards natural product drug discovery in recent years within the pharmaceutical industry. Bologa *et al*. (2013) shared the same sentiments and further noted other key reasons for the de-emphasis of MNP drug discovery from a pharmaceutical perspective. These include a general perception of market saturation as well as the resentment at regulatory bottlenecks associated with the drug development from MNPs. Despite all these and many other challenges, successful antibiotic development from phyla such as

Actinobacteria have been documented. This study focused on the search for novel metabolites from *Micromonosporaceae,* hence it is important to assess the relevance of microorganisms of the phylum Actinobacteria as sources of MNPs.

2.5 Actinobacteria and their relevance to the study

2.5.1 Basic characteristics of actinomycetes

Actinomycetes are a group of Gram-positive bacteria that are classified under the order *Actinomycetales,* phylum Actinobacteria according to Bergey's manual of systematic bacteriology (Atlas, 1997; Chaudhary *et al.*, 2013; Qinyuan *et al*., 2016; Sharma *et al.*, 2014). The term 'actinomycetes' is also typically reserved for those genera that exhibit filamentous growth. Genera that do not exhibit filamentous growth are typically referred to as actinobacteria. Das *et al*. (2008) highlighted that the term 'actinomycetes' is a derivative of two individual Greek terms *viz* 'atkis and mykes' meaning ray and fungi, which translate to possessing bacteria-like and fungi-like characteristics but meeting enough criteria to fall under the kingdom bacteria. Under the Bacteria domain, the phylum Actinobacteria is amongst the largest known phyla hence making this phylum a generally biodiverse one of research relevancy (Barka *et al.,* 2016)

These microorganisms are known to be aerobic, forming spores when they grow as well as showing substrate and aerial mycelium growth (Chaudhary *et al.*, 2013). When it comes to cellular growth and development, actinomycetes take a rather different course as compared to other unicellular bacteria, which have cell enlargement and binary fission as their "*modus operandi".* In actinomycetes, the vegetative hyphae tend to develop into highly branched elongated filaments without necessarily undergoing cell division (Atlas, 1997). The end products of this unique developmental process are well-elongated bacterial cells which house genomic data in multiple copies (ibid). This process precedes another process that involves the formation of septa, thereby effecting cytoplasmic-chromosomal separation (Atlas, 1997). It is, therefore, through extensive cellular structural analysis, particularly hyphal cellular morphology and 16S rRNA gene analysis that taxonomists confidently concluded that actinomycetes differ from fungi and possess clear-cut prokaryotic characteristics (Qinyuan *et al.*, 2016).

One of the striking microscopic characteristics of actinomycetes is their being highly filamentous, a characteristic which befits their vast morphological differentiation (Atlas, 1997; Sharma, 2014; Ventura *et al.,* 2007). It is, however, important to note that in some instances, the filament growth patterns are not as pronounced, as typically expected, due to fragmentation and such a phenomenon is exemplified in some species of the genera *Nocardia*, *Rhodococcus* and *Gordonia*. Atlas (1997) also noted a common macroscopic identity confusion caused by the near resemblance of some actinomycetes to fungi during

certain stages of their lifecycle (Figure 2.7). Actinomycetes produce much narrower filaments compared to fungal hyphae, a pertinent characteristic that may be used to clearly distinguish between the two using light microscopy (ibid). Ventura *et al.* (2007) noted actinomycetes as a group of bacterial microorganisms with a rather complex life cycle that contributes to the diversity of both their phenotypic characteristics as well as the metabolites they produce.

Actinomycetes are also known to be microorganisms with relatively large genomes which resemble a very high guanine to cytosine (G: C) ratio and can either be linear or circular (Atlas 1997). Ventura *et al.* (2007) reiterated this outstanding genomic size in actinomycetes and further noted that these large genomes can double the size of *E. coli* genome, which is about five mega base pairs. Also of pertinence within actinomycetes genomes, are plasmids that are involved in genomic rearrangement and data transfer, fertility as well as antibiotic production (ibid). However, actinomycetes have attracted so much attention and research interest chiefly because of their proven ability to produce a wide range of bioactive secondary metabolites and their character as saprophytes (Yu *et al.,* 2015).

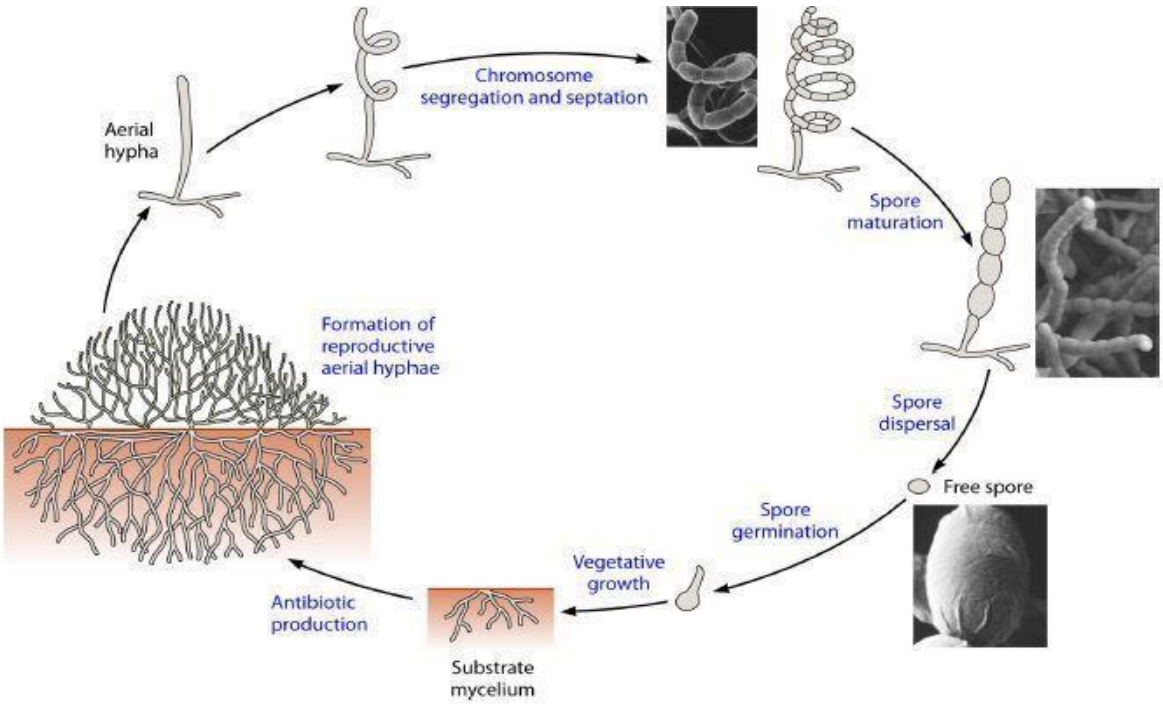


Figure 2.7: Life cycle of actinomycetes that produce spores. Historically, some scientists have considered actinomycetes to be an intermediate between fungi and bacteria. Mycelial production in actinomycetes indeed does resemble the same process in filamentous fungi but as shown, individual actinomycete cells have chromosomes, which are the foundations of sporulation in their spore-forming species. These individual bacterial cells are also susceptible to antibacterial agents hence confirming them as independently belonging to the bacteria kingdom (Barka *et al*., 2016).

2.5.2 Classification of actinomycetes

According to their scientific classification, actinobacteria represent a phylum under the bacteria domain, which subsequently houses the order *Actinomycetales.* It is each member of the order *Actinomycetales*, which is referred to as an actinomycete (Waksman *et al.*, 2010). Therefore, it is important to define these two terms to depict their differences contrary to their interchangeable use in some literature. Actinomycetes can be further classified based on a polyphasic approach that encompasses phylogenetic studies, phenotypic as well as genotypic characteristics. The taxonomy of actinomycetes is best achieved by comprehending bacterial phylogenetic relationships, which are subsequently founded in the 16S and 23S rRNA sequence data sets. This allows for the classification of bacterial species that are naturally very closely related, hence the reason for employing a polyphasic approach to actinomycete taxonomy (Hopwood, 2007).

2.5.2.1 Genotypic characteristics

Genomic methods are also an important component contributing to the polyphasic taxonomy of actinomycetes. As pointed out by Kirby and Le Roes-Hill (2009), many of the genomic-based methods used to decipher the genomes in the characterisation of actinomycetes are highly advantageous as they are applicable to both cultivable and uncultivable actinomycete strains. Genotypic taxonomy is approached effectively in a multi-dimensional approach, as shown below in Figure 2.8.

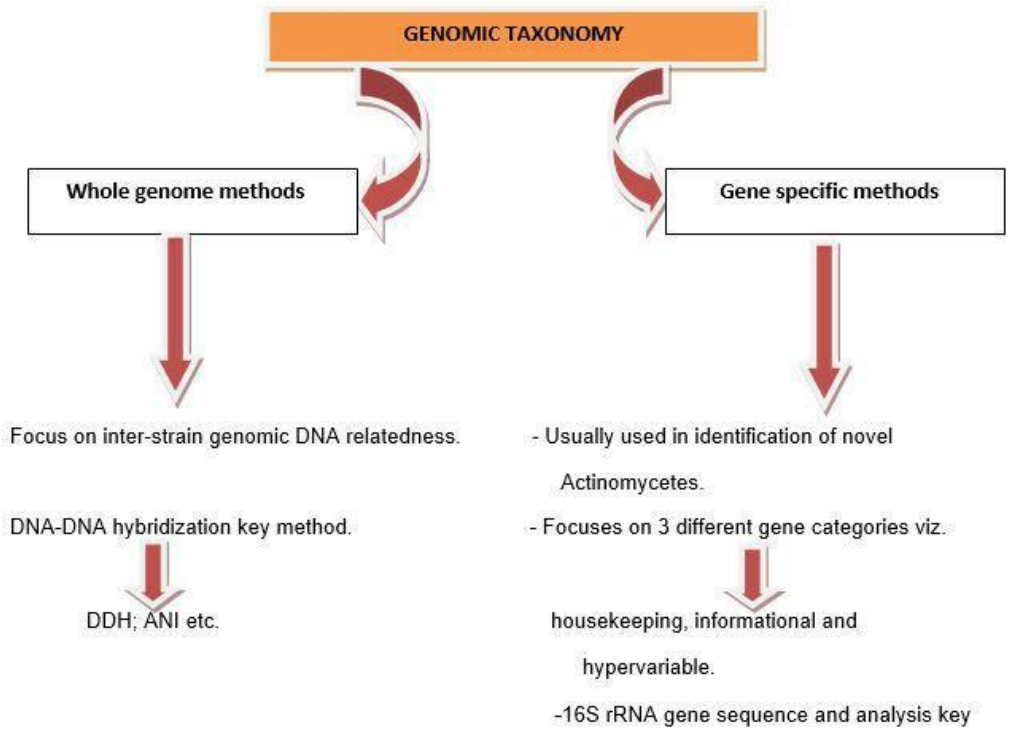


Figure 2.8: The two broad categories of genomic taxonomy in actinomycetes are shown. Gene specific methods give important information about bacterial cellular structure and function, cellular metabolism as well as virulence factors and surface proteins associated with specific related bacterial

species. On the other hand, DNA-DNA hybridisation becomes key in determining the DNA relatedness between two species. DNA relatedness can be quantified and a relatedness of below 70% between a species and its phylogenetic neighbour rules out significant relatedness; hence a conclusion of two unique species.

2.5.2.2 Multi-Locus Sequence Analysis (MLSA)

MLSA is an important tool that is useful in taxonomic differentiation (Glaeser & Kampfer, 2015). DNA sequence variations amongst specific housekeeping genes are analysed in an attempt to deduce a higher resolution of genus-centered inter-species relationships from a phylogenetic perspective (ibid). It should be noted that the widely used genetic marker for bacterial species relatedness, the 16S rRNA gene, has its shortcomings, which therefore make MLSA a complementary alternative (Papke *et al*., 2011). These include the fact that the 16S gene often falls short in fully discriminating multiple species within the actinobacteria genera since it appears to be too conserved. MLSA, therefore, aids in taxonomic differentiation in *Micromonospora* species. Despite its shortcomings, MLSA is also considered an effective substitute for DNA-DNA hybridization when effecting the delineation of multiple species within specific genera (Papke *et al*., 2011).n

2.5.2.3 Phylogenetics

MLSA is part of the broader field of phylogenetics. The biological field of phylogenetics is pivotal when it comes to the classification of actinomycetes. Edwards and Cavalli-Sforza (1964) defined phylogenetics as a field of life sciences that relies on DNA sequencing data to decipher the different evolutionary relatedness and closeness amongst groups of microorganisms. Phylogenetic analysis makes use of the 16S rRNA gene sequences obtained from a PCR using standard 16S rRNA primers. These sequences are then used to construct phylogenetic trees by use of various methods such as the commonly used neighbour-joining method (Papke *et al.*, 2011). Phylogenetic trees are central to phylogenetics as a field, and their ultimate goal in actinomycete classification would be to trace ancestral relationships amongst species within the actinobacteria phylum.

2.5.3 Distribution of Actinomycetes and their relevance

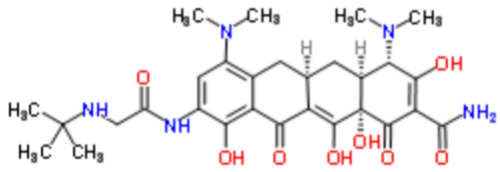
Actinobacteria are found both on terrestrial and in aquatic environments, with a predominant soil presence, where they have numerous ecological roles (Barka *et al.*, 2016; Jose & Jha, 2016; Bull *et al.*, 2005). Published information derived from genome sequences of Actinobacteria, to date, has shown that this phylum consists of microorganisms of a very complex and beneficially diverse character (Ventura *et al.,* 2007). Microbial and plant secondary metabolites have been reported as the best available sources of unique chemical diversity (Tiwari & Gupta, 2012). This, in turn, means actinobacteria can produce secondary metabolites that are chemically diverse, especially given their genomic complexity and subsequent diversity (Abdelmohsen *et al.*, 2015).

The development of the majority of antibiotics in the market in present day can be traced back to actinomycetes (Adegboye & Babalola, 2013; Murphy *et al.*, 2012; Udwary *et al.,* 2007). Janaki *et al.* (2012) corroborated this fact and further highlighted that approximately 70% of the known microbial-sourced antibiotics are actinomycete-derived products. Over the years, it has been further pointed out that the genus *Streptomyces* accounts for up to 60% of actinomycete-derived antibiotics (Bhattacharyya & Sen, 2004; Chaudhary *et al.,* 2013; Nanjwade *et al*., 2012; Udwary *et al.,* 2007). The genus *Streptomyces* has been thoroughly studied due to its economic significance. Other genera within actinobacteria responsible for the production of up to 10% of antibiotics include *Micromonospora, Streptoverticillium, Thermoactinomycetes* and *Actinomadura* (Nanjwade *et al.*, 2012).

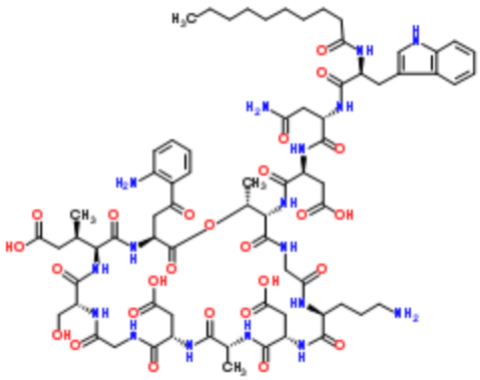
Ever since the discovery of streptomycin, researchers got encouraged to keep pursuing research projects focusing on beneficial natural products sourced from actinomycetes (Jose & Jha, 2016). However, it is important to note that interest in actinomycetes research declined at some stage and most projects on the aforementioned subject now only went as far as isolation and bioactivity screening of these actinomycetes (ibid). The major reasons attributed to this decline in interest were the dearth of adequate information with respect to genomics as well as the specific actinomycetes' biosynthetic abilities (Abdelmohsen *et al*., 2015). Jose and Jah (2016) however noted that interest in actinomycetes research had improved amongst scientists due to the advancement of technologies such as next-generation sequencing and genome editing.

Actinomycete-derived secondary metabolites have diverse antimicrobial properties, and this has maintained these microorganisms a case of research relevancy over the years. These properties include their display of antibacterial, antifungal, antiprotozoal, anticancer and antiviral characteristics against corresponding groups of pathogenic organisms (Janaki *et al.,* 2012). The chemical structures of tigecycline, daptomycin and streptomycin, some of the well know antibacterial derivatives of actinomycetes, are shown in Figure 2.9.

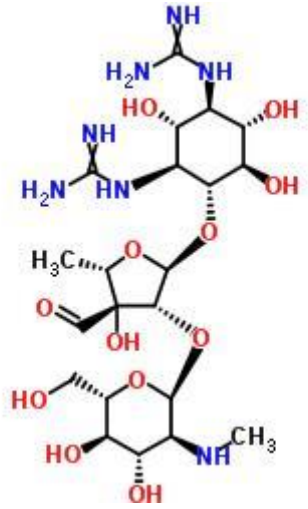
Amongst the many actinomycetes with a history of producing bioactive metabolites of pharmaceutical interest, the genus *Micromonospora* remains an auspicious one. This genus stands out as a bio-factory for novel metabolite compound mining, and some of its well-known antimicrobial products include gentamycins, rifamycins, and erythromycin B among many more others (Boumehira *et al.*, 2016).



A) Tigecycline



B) Daptomycin



C) Streptomycin

Figure 2.9: Image A shows the antibiotic tigecycline, which is used in the therapy of a number of infections such as *S. aureus, E. coli* and *A. baumannii* infections. Daptomycin, a lipopeptide antibiotic effective against mainly *S. aureus* bacteraemia, is shown in image B as a chemical structure. It is marketed under the name cubicin and is also used in resolving *S. pyogenes* and *S. agalactiae* infections. Streptomycin (Image C), is clinically used in treating TB, endocarditis and *Mycobacterium avian* complex infections amongst many others.

2.6 The Genus *Micromonospora*

2.6.1 Taxonomic classification and general characteristics

In 1923, Ørskov proposed the genus called *Micromonospora,* which is the subsequent type genus of the *Micromonosporaceae* family (Boumehira *et al.*, 2016; Kirby & Meyers, 2010; Maldonado & Quintana, 2015; Shen *et al.,* 2014). As far back as 1905, *Micromonospora chalcea* was the first species of the genus *Micromonospora* to be isolated by Foulerton and colleagues and subsequently named *Streptothrix chalcea* (Foulerton, 1905). Stackebrandt *et*

al. (1997) put forward a proposition for the class *Actinobacteria*. This classification was based on chemical and molecular data and it highlighted the existence of five different subclasses within *Actinobacteria*. Hirsch and Valdes (2009) further highlighted that the order *Actinomycetales* houses the suborder *Micromonosporineae* which further houses the family *Micromonosporaceae* as shown in Figure 2.10 (Koch *et al.,* 1996; Stackebrandt *et al.,* 1997).

Apart from the genus *Micromonospora,* the family *Micromonosporaceae* constitutes other multiple genera including *Actinoplanes, Catenuloplanes, Dactylosporangium, Couchioplanes* and *Pilimelia* (Boumehira *et al.*, 2016; Hirsch & Valdes, 2009). The genus *Micromonospora* consists of more than 60 species of valid nomenclature as highlighted by Boumehira *et al.* (2016).

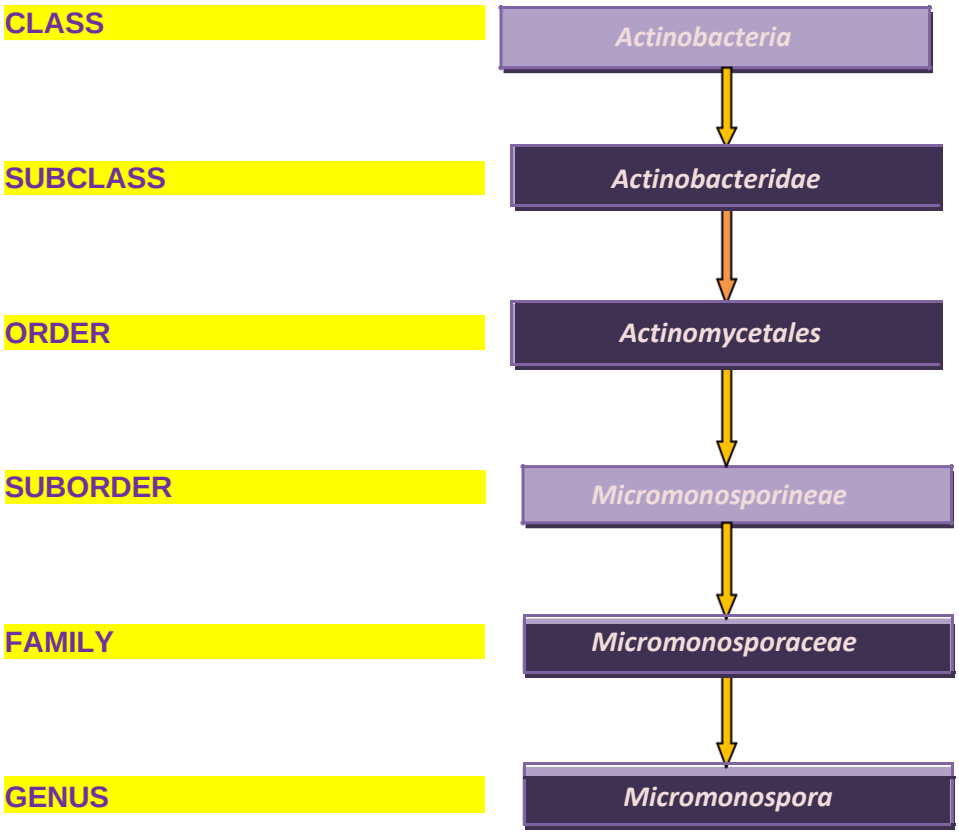


Figure 2.10: Taxonomic hierarchy showing the important taxonomic rank, which depicts how the genus *Micromonospora* fits in the class *Actinobacteria*.

Species belonging to the genus *Micromonospora* are filamentous, aerobic to microaerophilic, Gram-positive, chemo-organotrophs that usually lack aerial mycelium (Boumehira *et al*., 2016; Kirby & Meyers, 2010; Vobis, 1992). Suarez and Hardisson (1985) happen to be two of the few researchers who dealt with the presence and involvement of aerial mycelia in *Micromonospora* species. The occurrence of this phenomenon is regarded as extremely

rare, from a taxonomic perspective, due to the fact the individual spores in *Micromonospora* species are always attached to either the vegetative mycelia or the substrate mycelia (Maldonado & Quintana, 2015). An important genotypic trait that *Micromonospora* species. adopt by virtue of being actinomycetes, is that of a genome resembling a high Guanine: Cytosine (G: C) ratio (over 55%) (Trujilo *et al*., 2014).

2.6.2 Differential characteristics of *Micromonospora*

Microorganisms of the genus *Micromonospora* possess a considerable number of characteristics that are generally consistent with actinomycetes. However, there exists certain morphological, biochemical and physiological properties that help distinguish microorganisms of the genus *Micromonospora* from other actinomycetes. Kawamoto (1989) highlighted that some of these differential characteristics include how they grow on various media, their ability to use specific carbon sources as well as the mycelia-related pigmentation. However, Kirby and Le Roes-Hill (2009) argued that including mycelia-related pigments as a diagnostic feature of *Micromonospora* would be insufficient since these pigments, many at times, rather indicate various pH environments.

Another important differential characteristic of *Micromonospora,* as noted by Holt *et al.* (2000), is the unique structure of the peptidoglycan in their cell wall. The presence of *meso*-diaminopimelic acid (DAP) within the peptidoglycan polymer in the cell walls of *Micromonospora* makes these microorganisms unique. Hirsh and Valdes (2010) concurred with this assertion and further pointed out that *meso*-DAP in *Micromonospora* cell wall is sometimes found together with glycine as well as a *meso*-DAP 3-hydroxy derivative. It is important to note that mycolic acids are not part of the cell walls in *Micromonospora,* thus another important differential characteristic (Kawamoto, 1989).

There are specific main phospholipids, which are also found in the cell walls of *Micromonospora.* These include phosphatidylinositol, phosphatidylinositol mannosides and phosphatidylethanolamine (Kirby & Le Roes-Hill, 2009). Further to the unique cell wall in *Micromonospora,* the spores associated with this genus are of diagnostic relevance. The vegetative mycelium grows develops alongside non-motile spores that are formed in a singular fashion and is often observed as a black gelatinous mass on a colony that ranges from yellow, red or orange (Kirby & Le Roes-Hill, 2009).

2.6.3 Selective isolation and laboratory culturing of *Micromonospora*

2.6.3.1 Selective isolation of *Micromonospora* colonies

As characteristic of many actinomycetes, members of the genus *Micromonospora* are also widely distributed in disparate environments, most notably marine environments, albeit a not clearly defined ecological role within this environment (Maldonado & Quintana, 2015).

Humus-rich soil is also a common habitat for these microorganisms, whereby they serve as decomposers of organic matter (Qiu *et al.*, 2008). It is important to note that it requires efficient and effective laboratory techniques for selective isolation of the desired *Micromonospora* species from within a multitude of other microorganisms that are part of *Micromonosporaceae* ecological niches.

To enable effective isolation of microorganisms of the genus *Micromonospora* from soil samples for laboratory analysis, there are various techniques employed. These include the tunicamycin method developed by Wakisaka *et al.* (1982), pre-treatment heat methods, (Hayakawa *et al.*, 1991), a wet-heat treatment technique as described by Terahara *et al.* (2013) amongst many others.

Various isolation techniques of *Micromonospora* species from soil samples exist and the method of choice depends on many factors including convenience, affordability and intended use of isolated colonies. After making the desired choice of appropriate selective media, culturing is done and despite selectivity of media used, undesired microorganisms tend to grow alongside the *Micromonospora* species or the actinomycetes isolated, depending on the selectivity of the medium used. If one desires isolation of actinomycetes in general, from a processed and cultured soil sample, then colony morphology becomes key. The aerial mycelium consistent with actinomycetes results in fluffy colonies that have colours that range from red, pink, orange, blue, yellow or red (Kirby & Le Roes-Hill, 2009). If the medium used is specifically selective towards *Micromonospora* species, then bright orange colonies are representative.

2.6.3.2 Laboratory culturing post isolation

After *Micromonospora* species are successfully and selectively isolated from their natural habitats, it is imperative to identify appropriate nutrient culture to maintain the integrity of *Micromonospora* strains for further analysis and/or manipulation. Two such examples of nutrient media that are favourable for the growth and subsequent maintenance of *Micromonospora* strains are 172F and SGG media in both their solid and liquid forms (Kirby & Le Roes-Hill, 2009). *Micromonospora* strains in both these media typically grow as distinct orange colonies, which can sometimes be brown or deep brown, depending on the presence as well as the stage of sporulation as exemplified in diagrams A and B in Figure 2.11. A Gram stain is routinely performed to avoid a scenario whereby a mixed culture of microorganisms is mistakenly concluded to be pure *Micromonospora* colonies, and the microscopic result shows Gram-positive, highly filamentous colonies as shown in Figure 2.12.

A **B**

Figure 2.11: Typical growth characteristics of *Micromonospora* in both liquid and on solid media. Image A shows distinct orange colonies on 172F solid agar streaked out as per the technique outlined by Le Roes-Hill and Kirby (2010). Image B also shows bright orange distinct orange *Micromonospora* colonies after inoculation into 172 F liquid medium and incubation at 25^{o}C for nine days.

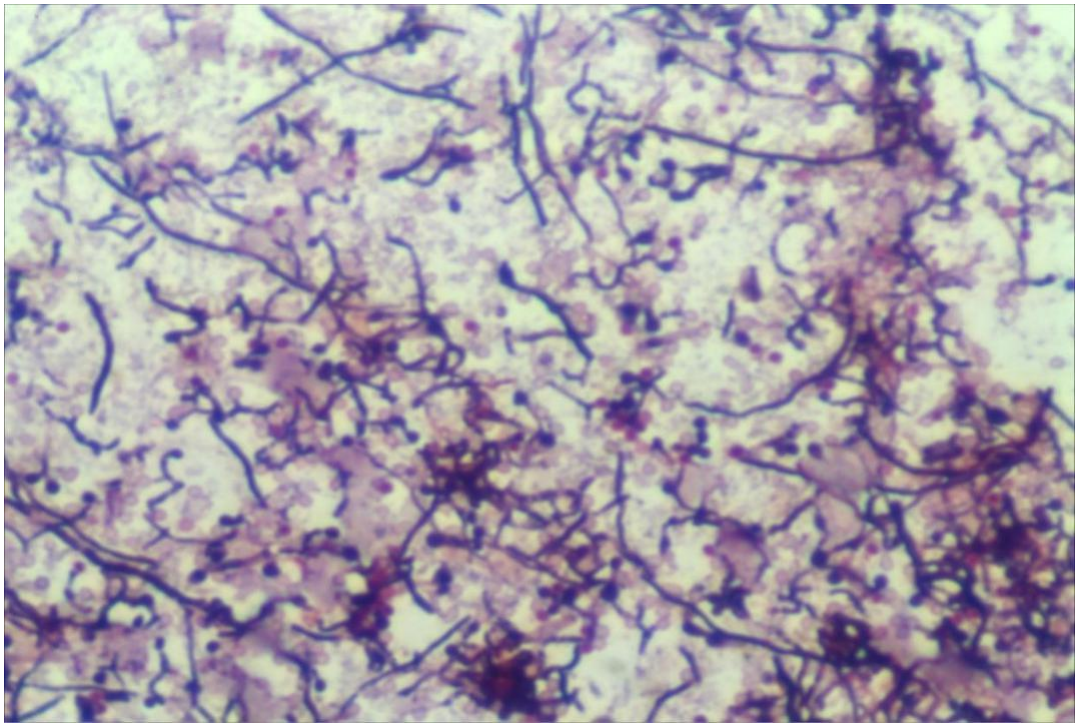


Figure 2.12: An example of a microscopic picture of the Gram stain reaction of one of the *Micromonospora* strains studied in this project. Gram-positive filaments and numerous spores can be seen at 100× magnification, under oil immersion.

2.6.4 Bioactive products and antibiotics sourced from *Micromonospora*

The discovery of secondary metabolites and their subsequent derived pharmaceutical products with antibacterial activity, amongst members of the genus *Micromonospora,* dates

back to the year 1942 (Kirby & Meyers, 2010). Amongst the pioneering discoveries from this genus is the antibiotic Micromonosporin, a chromoprotein isolated from soil *Micromonospora* in 1947 by Waksman *et al.* (1947). However, the reputation of this genus as a promising candidate for the screening of more bioactive compounds, amongst scientists, skyrocketed on the backdrop of gentamicin's isolation from *Micromonospora purpurea* (Wagman & Weinsten, 1980).

2.6.4.1 Gentamicin

The research interest for antibiotics sourced from *Micromonospora* species grew tremendously after the successful discovery of the antibiotic gentamicin in 1963 (Weinsten *et al*., 1963), and its subsequent introduction into the market in 1971 (Chen *et al.*, 2014). Gentamicin is a broad spectrum, aminoglycoside-complex (Figure 2.12) antibiotic used for the treatment of several infections such as urinary tract infections, endocarditis, pelvic inflammatory disease, bone infections and sepsis among many others (Chen *et al*., 2014). The two specific *Micromonospora* species from which gentamicin was developed from are *Micromonospora echinospora* NRRL 2985 and *Micromonospora echinospora* NRRL 2953 (the species *echinospora* formerly called *purpurea*) (Piepersberg, 2007; Wagman & Weinstein, 1980; Weinstein *et al.*, 1963)

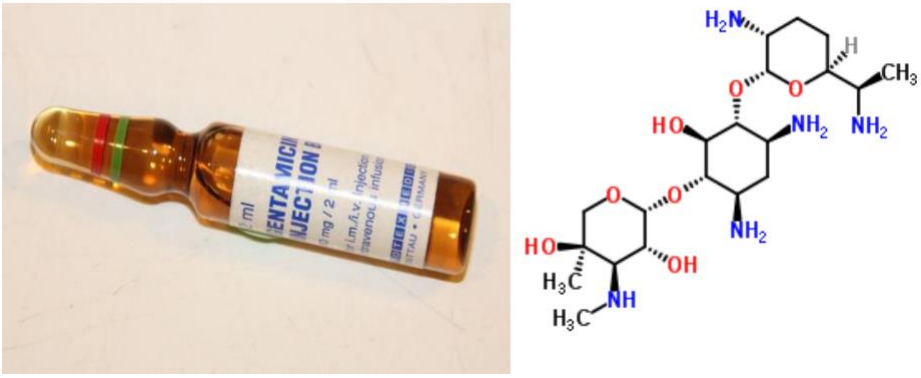


Figure 2.13: Image A shows an intravenous gentamicin solution which is clinically widely used. The chemical structure of the gentamicin complex is also shown in image B. This complex is anchored upon the central diaminogenouscyclitol (2-deoxystreptamine (2DOS) 4, 6- disubstituted, coupled with the auxiliary sugars garosamine and purpurosamine.

2.6.4.2 Other antibiotics from the genus *Micromonospora*

After the genus *Streptomyces,* it has been posited that the genus *Micromonospora* harbours the most bioactive metabolites within the actinomycete genera (Boumehira *et al*., 2016; Parekh *et al*., 2005; Hirsch & Valdes, 2010). Some antibiotic classes that are sourced from *Micromonospora* as listed by Hirsh and Valdes (2010) include the enediyne, oligosaccharide

and aminoglycoside antibiotics. Parekh *et al.* (2005) also further noted ribofuranosyllumichrome, retymicin, saquayamycin and galtamycin B as some individual known *Micromonospora*-sourced antibiotics. Wagman *et al.* (1976) also noted the *Micromonospora floridensis*-derived actinomycin complex as another success story when it comes to antibiotics sourced from *Micromonospora* species. Many more examples which give testimony to the genus *Micromonospora* as a true antibiotic "goldmine" are listed in Table 2.3.

Table 2.3: Examples of some antibiotic molecules discovered from microorganisms of the genus *Micromonospora* over the past four decades (Boumehira *et al.*, 2016).

Antibiotic	Class	Strain	Source	Year of discovery
Rosamycin	Macrolides	*M. rosaria* NRRL 3718	Soil, Texas, USA	1972
Rifamycins	Ansamysins	*M. lacustris* ATCC 21975	Mud, Connecticut, USA	1975
Erythromycin B	Macrolides	*Micromonospora* sp. 1225	-	1976
Tetrocarcins	Aminoglycosides	*M. chalcea* KY11091	Soil, Miyagi, Japan	1980
Clostomicins	Macrolide	*M. echinospora* subsp. *armeniaca* KMR-593	Soil, Niigata, Japan	1986
Crisamicin A	Naphthoquinones	*M. purpureochromogenes* subsp. *halotolerans* RV-79-9-101.	Mud sample, Philippines	1986
Deoxydynemicin A	Anthraquinone	*M. globosa* FERM P-10651	Soil, Japan	1990
Pyrrolosporin A	Macrolides	*Micromonospora* sp. ATCC 53791	Soil, Puerto Viejo, Peru	1996
Micromonosporin A	Macrolide	*Micromonospora* sp.	Thailand	2004
Telomycin	Macrocyclic peptide lactone	*M. schwarzwaldensis*	Soil, Black Forest, Germany	2013
Neomacquarimicin	Carboxylic polyketide	*Micromonospora* sp. NPS2077	Marine sponge, Japan	2014

2.7 Applications of Genomics in drug discovery

Important information that is central to the phenotypic, hereditary and biochemical behaviour of humans and animals is contained within nucleic acid sequences. Heather and Chain (2016) defined nucleic acid sequences as long polynucleotide chains that are built up by smaller successions of base pairs in specific orders. The ability for scientists to utilize

effective sequencing methods (from a cost, time and accuracy perspective) is imperative in all forms of research including drug discovery from *Micromonospora* species. It is thus important to look at the genesis of methods used in DNA sequencing, the evolution thereof, before reviewing their role in drug discovery.

2.7.1 Evolution of DNA sequencing

The breakthrough in the quest to understand DNA as a nucleic acid came about when Watson and Crick successfully deciphered its three-dimensional structure in 1953 (Watson & Crick, 1953). As pointed out by Heather and Chain (2016), it is important to note that at this stage scientists had already successfully established methods for protein chain sequencing. These methods were, however, limited when it came to nucleic acids sequencing. This led up to the development of first-generation sequencing techniques, with a focus on pure RNA sequencing sourced from ribosomal or transfer RNA of microbial origin. The techniques used by researchers at this stage were analytical chemistry-based and they had limitations in establishing nucleotide order, despite being reliable in determining composition of sequences (Holley *et al.*, 1961). First-generation DNA sequencing was able to decipher nucleotide composition but not nucleotide order in long stretches of DNA. In 1977, Sanger's chain-termination technique was developed. This famous technique also referred to as the dideoxy technique, saw the use of chemically derived analogues of dNTPs for improved sequencing (Sanger *et al.*, 1977).

Second-generation sequencing largely differed from first-generation sequencing in that radio- or fluorescently-labelled dNTPs were not used during nucleotide identification (Nyren & Lundin, 1985 #47). A luminescent-based pyrophosphate determining technique was the foundation of the inference of nucleotide sequences in second-generation sequencing (ibid). The introduction of third-generation sequencing followed and it became the pioneering technology in the sequencing of non-amplified DNA, thereby reducing biases which had characterised previous technologies (Schadt *et al.*, 2010). It is, however, the introduction of Next Generation Sequencing (NGS) technologies, which became the game-changer in bioinformatics.

2.7.1.1 Next-generation sequencing (NGS)

The introduction of NGS technologies in 2005 positively influenced the work of scientists pursuing genomic-centred research globally (Morozova, 2008). NGS technologies were introduced onto the market to bridge the gaps encountered by using conventional Sanger sequencing. It is important to note that NGS technologies brought about a revolution in bioinformatics, especially in processes such as whole-genome sequencing and resequencing (Morozova, 2008). Some of the commercially available NGS technologies, as described by Metzker (2005), as well as their principles of functionality, are discussed below.

2.7.1.2 The 454/Roche FLX platform

This technology was developed to address the *in vivo* amplification step of target genomic fragments characteristic of conventional Sanger sequencing. This *in vivo* amplification step was previously achieved by effecting cloning within bacteria as hosts and its major pitfalls were being labour intensive and the emergence of biases originating from the host bacteria (Sanger & Coulson, 1975). The 454 NGS technology addressed this gap by using emulsion PCR, which is more efficient when it comes to *in vitro* DNA amplification. This technology was ceased in 2017 and its read length was between 200-300 base pairs (bp) with a throughput of approximately 80-120 mega-bases (Mb) per run.

2.7.1.3 The Illumina/Solexa platform

The Illumina/Solexa NGS technology is one of the most widely used of the NGS technologies within many research laboratories globally (Kchouk *et al.*, 2017). This technology consists of a flow cell, which is the site of activity for all enzymatic processes and imaging steps (Buermans & Dunnen, 2014). The different sub-categories, into which the Illumina NGS technology can be divided, are based on the separate lanes used for every specific Illumina platform. For instance, the MiSeq platform makes use of a single lane; the HiSeq2500 operates with two separate lanes while the HiSeq2000 and HiSeq2500 platforms make use of eight separate lanes (Buermans & Dunnen, 2014). It is important to note how the Illumina MiSeq platform has, over the years, improved accessibility of small genome sequencing services to many researchers since it strikes a good balance between cost-effectiveness and labour intensiveness and efficiency.

The principle behind the Illumina NGS technology revolves around the flow cell; which is a solid surface upon which single stranded DNA (ssDNA) fragments are affixed in a DNA amplification step. This attachment of ssDNA fragments precedes a subsequent step that involves the bridge amplification of the established solid phase (Morozova, 2008). Progressive growth of the Illumina NGS technology has continued to be seen on the market as more R&D is carried out. For instance, in 2014 Illumina introduced the NextSeq500 and

the HiSeq X Ten platforms. The NextSeq500 platform came with increased outputs of between 40 Gg to 120 Gg in a period of 30 hours (Buermans & Dunnen, 2014).

Although base substitution errors are frequently common due to the inevitable application of modified polymerases and reversible terminators in Illumina sequencing, this technology remains efficient currently with a read length of between 75-300 bp and a massive throughput of up to 600 Gigabyte (Gg) per run within approximately 3-10 days (Hodzic *et al.*, 2017). The continuous improvement in efficiency and increased capacity of NGS technologies such as the Illumina platform allows for effective sequencing of multiple genomes from various microbes, such as *Micromonospora* species, in order to find novel drug leads. Effective location and identification of biosynthetic gene clusters (BGCs) which possibly encode for novel metabolites within those genome sequences, is very key in drug discovery (Medema *et al*., 2011). With the advancement of bioinformatics, tools such as the antibiotics & Secondary Metabolite Analysis Shell (antiSMASH) have become very useful in identifying BGCs encoding a wide range of known classes of secondary metabolite compounds of research relevancy (ibid). antiSMASH is a useful online bioinformatics tool (available at http://antismash.secondarymetabolites.org) that compares unknown BGCs to a wide range of known BGCs in a bid to explore possible relatedness (Walsh & Fischbach, 2010).

Another useful BGC mining tool, which has found prominence in bioinformatics, is BAGEL. This web-based bioinformatics database is useful in locating ribosomally synthesised and post-translationally modified peptides (RIPPs) (De Jong *et al*., 2006). After successfully obtaining genomic data from NGS, it is tools such as BAGEL that are put to use in the identification of BGCs encoding RIPPs such as lanthipeptides. In some cases, in bioinformatics, the annotation of Open Reading Frames (ORFs) within genome sequences becomes a challenge specifically when it comes to distinguishing genes of similar functionality albeit with limited or zero sequence homology (Besemer & Borodovsky, 2005). BAGEL also comes in handy in solving such dilemmas. Other bioinformatics tools which are useful in BGC identification and manipulation in drug discovery include ClusterFinder, EvoMining and SMURF amongst many others (Weber & Kim, 2015).

2.7.2 Comparative genomics

Darling *et al.* (2008) and Touchman (2010) defined comparative genomics as an arm of genomics which focuses on gathering information with regards to specific genomic parameters amongst organisms before making comparisons of such parameters. These genomic parameters include genes and their order, whole genome sequences, regulatory sequences, as well as other key genomic parameters (Xia, 2013). Fields *et al.* (2016) further pointed out that the benefits of comparative genomics to fields of biological research,

including drug discovery, lie in the ability of scientists to interrogate genomic data amongst closely related organisms.

The field of comparative genomics is principled upon the observation that features which are shared between different organisms, be it morphological or biochemical, are usually coded for by housekeeping genes which resist evolutionary biology (Hardison, 2003). It, therefore, makes sense that the alignment of different genome sequences to figure out the possibility of shared ancestry as well as extent of conservation amongst these sequences is an important foundation in comparative genomics.

Various tools are used in the field of comparative genomics. Such tools include DNA-DNA hybridization (DDH), which revolutionised prokaryotic classification through the verification of different organisms' genomic clusters (Ciufo *et al*., 2018). DDH allows taxonomists to roughly ascertain genomic similarity between strains despite the method being tedious and prone to errors. As an enhancement to the process, genomic data can be digitally derived; thus, Digital DDH. Another good example of a comparative genomics tool is ANItools web, which is the web version of the ANItools, developed by Han *et al.* (2016). Average Nucleotide Identity (ANI) is a calculation-based method used to ascertain genomic boundaries within different species. The average amino acid identity (AAI) is yet another tool that can be used to investigate relatedness between two sets of genomes (Konstantinidis & Tiedge, 2005).

2.7.2.1 Relevance of comparative genomics

A key characteristic of any antibiotic compound of beneficial therapeutic efficacy is its ability to target those genomic sequence sections which are highly conserved and commonly expressed, amongst various species of pathogenic bacteria (Fields *et al.*, 2016). Robust identification of these specific genomic sequence portions becomes achievable by the application of comparative genomics. Such information can be, therefore, used to extrapolate any sequence homology between target bacterial genomic sequences and bacterial proteins of known function. Huang *et al.* (2014) highlighted that such an approach ultimately serves to identify genes of interest within pathogenic bacteria, their extent of expression during different stages of causing infection and how essential they are. This information becomes the cornerstone of drug development, a process which takes into account the important aspect of high-value targets within pathogenic bacteria (Fields *et al.,* 2016).

Another useful application of comparative genomics is in the elucidation of BGCs in drug discovery. Doroghazi and Metcalf (2013) reported on work done which showed how important comparative genomics could be in understanding the ecology and diversity of actinomycete-derived natural products. Actinomycetes are characteristic of highly similar genomic sequence sections which code for non-ribosomal peptide synthetases (NRPSs) and

polyketide synthases (PKSs). In their study of *Streptomyces coelicolor* as a model, Doroghazi and Metcalf (2013) used comparative genomics to decipher up to 102 closed genomes within various actinomycetes genera. This enabled them to figure out occurrence of the common BGCs. They concluded that the use of comparative genomics on a wide scale is key to the formulation of hypothesis necessary in the field of drug discovery and development (ibid).

2.8 Conclusion

A thorough review of literature undoubtedly shows that antibiotic resistant infections have been in our midst for long, causing serious clinical ramifications and disrupting the global socio-economic order. Amongst the various interventions to correct the problem, continuous novel drug discovery from microorganisms of the genus *Micromonospora*, from previously untapped sources such as the South African coastline, remains key. Knowledge of bioinformatics to the scientist of the day can never be underestimated in the quest to fulfil the aim of this study. Literature describes the behaviour of *Micromonospora* species in both their natural habitats and within laboratory environments. This knowledge provides the basis of formulation of effective laboratory methodology to answer the objectives of this research study, as outlined in the following chapter.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Microbial strains and reagents used in this study

The microbial strains used in this study consisted of thirty actinobacterial strains obtained from the Biocatalysis and Technical Biology (BTB) research group's culture collection. These bacterial strains were isolated from sea sponges and sea squirts previously collected from the Algoa Bay region, located in the Eastern Cape province of South Africa. Rhodes University provided marine samples and actinobacterial strains were isolated by Dr Marilize Le Roes-Hill. Thirty strains that exhibited 'typical' *Micromonospora*-like morphology (orange colony with black spore mass) and which exhibited antimicrobial activity (as determined during a preliminary screen), were selected for this study.

The identities designated to the microbial strains are as follow:

02-118#4,#5, #8; 02-128*#1, #3; 02-138#3, #6; 02-139#9, #14, #18; 02-158#10; 02-203#1; 02-209#2, #4; 02-221#16, #26; 02-231#25;02-251#1, #10, #135, #136, #16, #253, #274, #322; 02-251*#36; 03-013#19; 04-015#16, #8; and 04-044 RT1.

Key: 02-118, 02-128, 02-138, 02-139, 02-158, 02-203, 02-209, 02-221, 02-231, 02-251, 03-013, 04-015 and 04-044 are the codes assigned to different marine samples collected by Rhodes University; the * indicates strains isolated after a pre-treatment step (material heated at 60 C prior to standard isolation); the numbers following the # indicate the isolate number.

All reagents used in this study were of laboratory grade unless otherwise stated. Chemicals and solvents used in this study were obtained from Merck-Millipore and Sigma-Aldrich, unless otherwise stated.

3.2 Solid and liquid media culturing of bacterial strains

The solid and liquid media used in this research project include SGG media, SGG media supplemented with 50% (v/v) artificial seawater (ASW), 172 F media as well as 172 F media with ASW. The culture media were prepared as summarised below according to methods outlined by Goodfellow and Fiedler (2010):

a) SGG agar (g/L distilled water): 10 glucose, 10 glycerol, 2.5 corn steep solids, 5 peptone, 10 potato starch, 2 yeast extract, 3 calcium carbonate, 1 NaCl, 20 bacteriological agar (pH 7.0).
b) 172 F agar (g/L distilled water): 10 glucose, 5 yeast, 10 starch, 2.5 tryptone, 2.5 tryptone, 20 bacteriological agar (pH 7.0).

c) ASW (g/L distilled water): 24.6 NaCl, 0.67 KCl, 1.36 $CaCl_2.2H_2O$, 6.29 $MgSO_4 7H_2O$, $MgCl_2$, $NaHCO_3$ (pH 8.0).

For liquid media cultures: Initially, all strains that had been isolated from the marine samples were stored as stock cultures at -80^{o}C and had to be cultured on fresh media to determine viability before further analysis. These frozen cultures were in 20% (v/v) glycerol stocks that had been made by mixing 600 µl of each strain's cell culture with 40 µl of a 50% (v/v) sterile glycerol. Volumes of 100 µl of all strains were inoculated into the four different liquid media as described above and these were then incubated at 25^{o}C while shaking on a rotary shaker at 160 rpm for a period of 5-10 days. After successful growth of the strains in liquid culture, stock cultures were prepared by inoculating 240 µl of culture into 360 µl of the 50% (v/v) glycerol. These were then frozen at -80 ^{o}C and -20^{o}C for further use.

3.2.1 Streaking out of bacterial strains onto solid agar media

Each actinobacterial strain was streaked out onto the four different agar media (100 µl each culture) described in section 3.2. This streaking was systematically performed from a heavy vertical inoculum into horizontal lines, followed by a W streak across these established horizontal lines (Figure 3.1) as described by Kirby and Le Roes-Hill (2009). Strains were maintained on these agar plates for use during the study.

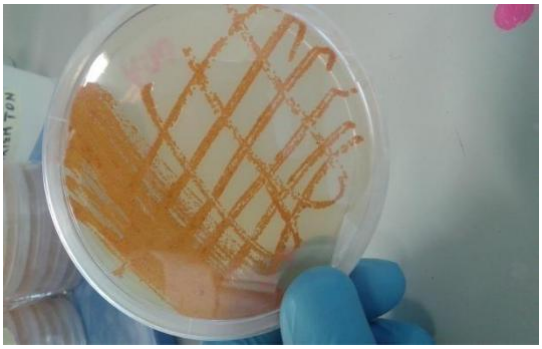


Figure 3.1: Pattern of streaking out of actinobacterial strains shown after successful growth. This method is used as an effective method of streaking out actinobacteria on solid agar media, *Micromonospora* species included, in order to get pure colonies after incubating under optimal growth conditions (9-11 days at 25^{o}C).

3.3 Gram stain of liquid cultures

A standard Gram stain was performed on all liquid cultures, as described by Bartholomew & Mittwer (1952) to ensure that the cultures used in this study were not contaminated.

3.4 16S rRNA gene sequence analysis

3.4.1 Isolation of genomic DNA

The DNA extraction method that was used is as described by Mandel and Marmur (1968). All the reagents used in the isolation of genomic DNA were prepared in-house before isolation commenced.

3.4.1.1 DNA extraction reagents

The reagents used for the isolation of genomic DNA include a cell suspension buffer, lysing solution as well as other solutions. The protocols that were followed in making up of all these solutions are as follow:

a) Cell suspension buffer**:** For 100 ml – 1 ml of 1M Tris-HCl (pH 8.0); 0.2 ml of 0.5 M EDTA; 12 g sucrose; made up with distilled water to 100 ml.
b) Lysing solution: (2×) For 100 ml – 10 ml of 1 M Tris-HCl (pH8.0); 4 ml of 0.5 M EDTA; 6 ml of 5 M NaCl; 8 ml of 25% w/v SDS; [2 ml β- Mercaptoethanol and 500 µl of 20 mg/ml proteinase K - added after autoclaving].
c) Other solutions used: 5 M $NaClO_4$ (700g/l); isopropanol; 75% ethanol; 3 M sodium acetate; TE buffer; choloform-isoamyl alcohol (24:1 v/v); phenol: chloroform (2:1 v/v); 10 µl RNase A (10 mg/ml) in 90µl TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA at pH 8.0).

3.4.1.2 DNA Extraction Methodology

Prior to DNA isolation, the bacterial strains were cultured in liquid media as previously described. After successful culturing (cell mass production clearly visible), bacterial cells were collected in 2 ml Eppendorf tubes. Dry lysozyme (8 mg/ml) was added to the bacterial cells as well as 200 µl of 5 M $NaClO_4$. The mixture formed a precipitate which was heated to 50^oC to get the precipitate back into solution. This was mixed uniformly to allow the cells to lyse. Cells were lysed overnight (12-14 hrs) at 60^oC.

The following day, 300 µl phenol: chloroform was added, and an emulsion was formed by shaking by hand before further shaking for 20 minutes on a vortex mixer. The samples were then centrifuged in a micro centrifuge at 12000 rpm for 10 minutes at room temperature. The aqueous layer was transferred to a clean, sterile 1.5 ml tube and the extraction was repeated twice more. Afterwards, the aqueous layer was transferred to a clean, sterile 1.5 ml tube; 0.6 volumes of isopropanol were added to the aqueous phase of the last extractions and mixed gently by inversion. DNA was spooled with sterile toothpicks and transferred to a clean microfuge tube to which 500 µl of 76% (v/v) ethanol was added. Samples were allowed to stand for 10 minutes. After the 10 minute incubation, the wash step was repeated one more

time. The precipitate was then dried at 37^{o}C for 15 minutes. After the drying step, the precipitate was dissolved in 400 µl of 1 × TE buffer (overnight at 4^{o}C).

After the overnight incubation, 5 µl of RNase A was added and incubation at 37^{o}C carried out for 1 hour. Extraction was performed with 100 µl chloroform-isoamyl alcohol before centrifuging the mixture and subsequently saving the aqueous layer; 0.1 volumes of 3 M sodium acetate were added, mixed with sample (by inversion) and overlaid with two volumes 95% (v/v) ethanol and DNA was then collected by spooling using sterile toothpicks. The isolated DNA was suspended in 500 µl of 1× TE buffer, pH 8, and stored at 4^{o}C until use.

3.4.2 Verification of isolated genomic DNA

To determine whether DNA extraction was successful for all samples, a 1% (w/v) agarose gel was prepared and electrophoresed as per the manufacturer's instructions (Bioline). In order to prepare the agarose gel, 1× TAE buffer needed to be first prepared from a 50× TAE buffer working stock [242 g Tris, 57.1 ml acetic acid, 100 ml of 500 mM EDTA (pH8) in a final volume of 1 L distilled water]. The 1× TAE working solution was then prepared from the 50× stock solution by diluting 20 ml of the 50× TAE stock with 980 ml of distilled water. The agarose gel was then prepared in the 1x TAE buffer (1 g agarose powder dissolved in 100 ml 1×TAE buffer) and supplemented with 10 µl of 1 µg/ml ethidium bromide to allow for the visualisation of the DNA under UV. The gel was viewed under UV light (254 nm) in a Uvitec UV gel documentation system.

3.4.2.1 Quantification of Isolated genomic DNA

The quantification of the isolated genomic DNA was performed on the Genova Life Science Spectrophotometer at an absorbance of 260 nm. The spectrophotometer was initially blanked with 1 ×TE buffer (pH 8.0), which was the respective buffer used for re-suspension of the final extracted DNA. One microliter of each of the isolated DNA samples was pipetted onto the spectrophotometer's nanodrop sample-detection area before reading the value that signified the DNA quantity in µg/ml.

3.4.3 Amplification of the 16S rRNA gene

The 16S rRNA gene was amplified for sequence analysis using a polymerase chain reaction (PCR) with a pair of standard 16S rRNA gene amplification primers as described by Cook and Meyers (2003). The primers used were the forward (F1) primer and the reverse (R5) primer, which have sequences as given below:

F1 (Forward Primer, 20-mer); 5'–AGAGTTTGATCITGGCTCAG-3'

R5 (Reverse Primer, 21-mer); 5'–ACGGITACCTTGTTACGACTT-3'

Note: I = inosine

The PCR was set up with the different components in specific proportions as shown in Table 3.1. The components needed to be defrosted on ice and the PCR reaction was subsequently set up on ice. The PCR reaction was performed in a Standard Techne Touchgene Gradient PCR machine under the phase-specific conditions, as summarised in Table 3.1.

Table 3.1: Setup and reaction cycle conditions of the individual 16S rRNA gene amplification PCRs.

PCR SET UP		PCR CYCLE CONDITIONS			
PCR ingredients	**Volume (µl)**	**Step**	**Temp/^{O}C**	**Duration**	**Cycles**
Forward (F) primer (10 µM)	2.5	Initial denaturation	96	2 sec	30
Reverse (R) primer (10 µM)	2.5	Denaturation	96	30 sec	30
Taq Ready Mix	25	Annealing	57	30 sec	30
Sterile nuclease-free water	18	Extension	72	2 min	30
Template DNA (Undiluted)	2	Final Extension	72	5 min	1
Total volume	50	Hold	10	∞	1

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To determine whether amplification of the target 16S rRNA gene was successful, a 1% (w/v) agarose gel was run as per the manufacturer's instructions (Bioline). A 25 µg/ml Fast DNA ladder (Biolabs) was also included upon loading the gel. The gel was viewed as previously described in section 3.4.2 and sample lanes evaluated for the presence of expected band size (1423 bp) (Kirby & Le Roes-Hill, 2009).

3.4.4 16S rRNA gene sequencing and analysis

3.4.4.1 16S rRNA gene sequencing

Prior to sequencing of the amplified 16S rRNA gene, the individual amplicons were purified using the MSB® Spin PCRapace kit (Stratec Molecular) as per the manufacturer's instructions. Gene sequencing services for the 16S rRNA gene were outsourced to inqaba biotec^{TM}, a genomics company based in Pretoria, South Africa. All necessary procedures and protocols from the quality control of submitted PCR amplicons to the generation of raw gene sequence data were performed according to the standard operating procedures (SOPs) of inqaba biotec^{TM}.

3.4.4.2 Analysis and processing of raw sequence data

The sequences, post 16S rRNA gene sequencing, were provided by inqaba biotec^{TM} in the form of .abi data files. The Chromas Lite (Version 2.6) programme (Technelysium) used to

open these files, was downloaded from the Technelysium website: http://www.technelysium.com.au/chromas.html. The chromatograms of the sequences were edited and during this process, incorrect base-calls were changed by typing in a new letter or deleted accordingly to correct any base coding anomalies. The edited chromatograms were then exported as .seq files into DNAMAN Version 4.13 (Lynnon BioSoft).

The reverse sequences were converted, saved and re-loaded to enable the final assembling of the full sequence. After uploading the sequences, the sequences were assembled and used for further analysis. The consensus sequences were used to determine the novelty of the individual strains by performing a BLAST analysis at http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome. In addition, sequences were also submitted to EzBiocloud (https://www.ezbiocloud.net) to determine relatedness to type strains (Yoon *et al*., 2017).

3.5 Multi-Locus Sequence Analysis (MLSA)

Multi-locus sequence analysis was performed by targeting four genes within the five best *Micromonospora* strains' individual genomes (strains selected based on antibacterial activity studies; see section 3.8). The genes targeted included *recA, rpoB, atpD* and *gyrB.* The PCR protocols used were a variation of the protocols outlined by Carro *et al.* (2011).

The *gyrB* gene was amplified by targeting two overlapping regions that would enable obtaining a final composite sequence measuring up to 1110 nucleotides (Garcia *et al.*, 2010). This meant that targeting this gene for amplification required two sets of primers, as shown in Table 3.2. All PCR reactions effected in the MLSA study followed the protocol outlined in Table 3.3 with the specific annealing temperatures for the specific genes highlighted in Table 3.2.

The amplicons were analysed using 1% (w/v) agarose gel electrophoresis as previously described in section 3.4.2. Prior to sequencing of the amplified genes, the individual amplicons were purified using the MSB® Spin PCRapace kit (Stratec Molecular) as per the manufacturer's instructions. As for the MLSA genes, sequencing services were outsourced to inqaba biotech™ and sequence data was provided in the form of .abi data files and a similar process of data manipulation and analysis was followed as described previously.

Table 3.2 Primer information for the primers used in the MLSA study

PRIMER	SEQUENCE	GENE TARGETED	ANNEALING T/^{O}C	EXPECTED AMPLICON SIZE
ATPDF1[g]	5'-AAGGGSMASGTMTTCAA-3'	*atp*D	57^{O}C	850 bp
ATPDF2[g]	5'-TGGTCSATYCACCGCAAG-3'	*atp*D	57^{O}C	850 bp
RAU[j]	5'-GGYAARGGYKCBGYNATGCG-3'	*rec*A	57^{O}C	510 bp
RAD[j]	5'-CTTVRMSCGGGTGCGGTT-3'	*rec*A	57^{O}C	510 bp
MYCOF[I]	5'-GGYAAGGTCACSCCCSAAGGG-3'	*rpo*B	56^{O}C	575 bp
MYCOR[i]	5'-ARCGGCTGCTGGGTRATC-3'	*rpo*B	56^{O}C	575 bp
GYF3[gh]	5'-ACSGTCGACTTCGACTTCCA-3'	*gyr*B	54^{O}C	900 bp
GYR3B[gh]	5'-CAGCACSAYCTTGTGGTA-3'	*gyr*B	54^{O}C	900 bp
GYF1[gh]	5'-TCCGGYGGYCTGCACGGCGT-3'	*gyr*B	62^{O}C	500 bp
GYR1B[gh]	5'-CGGAAGCCCTCYTCGTGSGT-3'	*gyr*B	62^{O}C	500 bp

Table 3.3 PCR protocol used in the MLSA study for the amplification of all target genes

PCR SET UP		PCR CYCLE CONDITIONS			
PCR ingredients	**Volume (µl)**	**Step**	**Temp/^{O}C**	**Duration**	**Cycles**
Forward (F) primer (10 µM)	2.0	Initial denaturation	94	2 sec	1
Reverse (R) primer (10 µM)	2.0	Denaturation	94	30 sec	35
Taq Ready Mix	25	Annealing	Refer to Table 3.2	1 min	30
Sterile distilled water	19	Extension	72	2 min	30
Template DNA	2	Final Extension	72	7 min	1
Total volume	50	Hold	4	∞	1

3.6 Phylogenetic analysis

For the phylogenetic analysis of the amplified DNA sequences, the top twenty sequences from BLAST or EzBioCloud analysis were used along with the sequences of the strains of interest. MEGA version X (Kumar *et al*., 2018) was used to align amplicons with sequences downloaded from NCBI and/or EzBioCloud. The aligned sequences were used in generating a neighbour-joining (NJ), minimum evolution and maximum likelihood tree with a bootstrap value of 1000. All three trees were analysed to determine whether the grouping on the NJ tree are conserved. For the MLSA, a concatenated sequence of the 16S-gyrB-rpoB was

generated for the five strains and the top 20 hits. The alignment and tree generation was performed as previously described. In both cases, the NJ tree was used for presentation.

3.7 PCR screening for the presence of Biosynthetic Gene Clusters (BGCs)

From the DNA isolated in section 3.4.1, eight known BGCs were screened for in separate PCRs using primer sets as given in Table 3.4. Information pertaining to the specific genes that were being targeted as well as their respective expected band sizes are summarised in Table 3.4.

Table 3.4: Primer sets for the amplification of targeted sequences in antibiotic BGCs

Biosynthetic gene targeted	Primer sets	Expected band size	Reference
Cytochrome P450 hydroxylase, polyene antibiotics	F PEH-1 5'-TGGATCGGCGACGACCG(G/C)(A/G/C)(T/C)CGT-3' R PEH-2 5'-CCG(T/A)A(G/C)AG(G/C)A(T/C)(G/C)CCGTCGTACTT-3'	350 bp	Hwang *et al*., 2007
Epoxidase, polyether ionophore	EPO-F: 5'-GGSTGGCARYAYCGYTTYCC-3' EPO-R: 5'-SCCRTGSCCGTRSAYSGGRTTG-3'	700 bp	Wang *et al*., 2011
P450 monooxygenase, glycopeptide	Foxy: 5'-CTGGTCGGCAACCTGATGGAC-3' Roxy: 5'-CAGGTACCGGATCAGCTCGTC-3'	591 bp	Wood *et al*., 2007
Ketosynthase alpha and ketosynthase beta, Type II polyketides	ARO-PKS-F: 5'-GGCAGCGGITTCGGCGGITTCCAG-3' ARO-PKS-R: 5'-CGITGTTIACIGCGTAGAACCAGGCG-3'	492-630 bp	Wood *et al*., 2007
3-amino-5-hydroxybenzoic acid synthase, ansamycins	ANSA-F: 5'-CC(C/G)GC(G/C)TTCAC(C/G)TTCATCTC-3' ANSA-R: 5'-AI(G/C)(C/T)GGAICATIGCCATGTAG-3'	641 bp	Wood *et al*., 2007
Isopenicillin N synthase, β-lactams	Pcb03-F: 5'- CGAGTCCTGGTGCTACCTGAACC-3' Pcb03-R: 5'- TCATCGACACGTCCAGGTGGTC-3'	355 bp	Bervanakis, 2008
dTDP-glucose synthase, aminoglycosides	StrD01-F: 5'- CTTCGCCATGTATCTCGGCGACAA-3' StrD01-R: 5'- TGCCGGTGTCCTTCCAGTAG-3'	370 bp	Bervanakis, 2008
Ketosynthase, Type I polyketides	KSM-F: 5'- GCSTCCCGSGACCTGGGCTTCGACTC-3' ATM-R: 5'- AGSGASGASGAGCAGGCGGTSTCSAC-3	750 bp	Bervanakis, 2008

3.7.1 PCR reaction components and conditions

The reaction setups for the BGC PCRs and the individual cycle conditions are given in Table 3.5.

The amplicons were analysed by gel electrophoresis using the same setup as for previous PCR analyses. Sequencing was not performed, as this section was merely a screening experiment.

3.7.2 Primer alignments with target genes

Target genes from the *Micromonospora* genomes were accessed via a searchable sheet for all coding sequences on EzBiocloud. After accessing these target genes, multiple sequence alignments between these target genes and the BGC primer pairs (Table 3.4) were set up in DNAMAN version 4.13. This exercise sought to investigate whether or not there was any alignment signifying binding of primers to intended targets and if not, whether there would be alternative BGCs that could be explored.

3.8 Primer design and testing of designed primers

3.8.1 Retrieval of genomic data: EZBioCloud

In order for the gaps in primer knowledge to be established for the purposes of primer designing, the genomic data of published species of the *Micromonospora* genus was retrieved and assessed. This was performed by searching EZBioCloud (Yoon *et al*., 2017), an online database available at www.ezbiocloud.net, using "*Micromonospora*" as the search term. The "Genome" option was then selected as a filter in order to retrieve data of only those species that had published genomic data. Each species was individually selected to explore its genomic information. Strain and genome information was retrieved and tabulated into an Excel spreadsheet. This information included, for each species, Taxon name, Strain name (Strain information) and status, Number of coding regions (CDSs), mean length of CDS and mean length of the intergenic region. Sequences of contigs provided as FASTA files were also downloaded.

3.8.2 Predicting of BGCs: antiSMASH

antiSMASH bacterial version, available online at https://antismash.secondarymetabolites.org, was used to predict the known and unknown gene clusters within the downloaded genome FASTA files (Medema *et al*., 2011). Data was input from extracted FASTA files and analysis options were made to include known clusters blast, cluster blasts, subcluster blasts, smCoG analysis, active site finder, detect TTA codons and whole-genome PRAM analysis. For each species, the particular BGCs and their types, that did not have any known predictable "most similar clusters" were identified and tabulated. These would form the basis for primer design.

Table 3.5 PCR protocols and cycle conditions for BGC analysis

Gene targeted	25 µl PCR SETUPS						PCR CYCLES CONDITIONS																	
	20 µM Forward Primer (µl)	20 µM Reverse Primer (µl)	2× Kappa Taq Readymix (µl)	1mg/ml BSA or DSMO or Glycerol (µl)	Sterile dH_2O (µl)	DNA Template (µl)	Initial Denaturation			Denaturation			Annealing			Extension			Final Extension			Hold		
							T /^{O}C	D sec	C	T /^{O}C	D sec	C	T /^{O}C	D sec	C	T /^{O}C	D sec	C	T /^{O}C	D sec	C	T /^{O}C	D sec	C
Cytochrome P450 hydroxylase	0.5	0.5	12.5	2.5 of 1mg/ml BSA	8	1	96	300	1	96	60	45	60	30	45	72	45	45	72	300	1	4	∞	1
Epoxidase	0.5	0.5	12.5	1.5 DSMO	9	1	95	480	1	95	45	32	59	45	32	72	60	32	72	600	1	4	∞	1
P450 monooxygenase	0.5	0.5	12.5	-	10.5	1	96	120	1	96	45	30	60	30	30	72	120	30	72	300	1	4	∞	1
Type II PKS	0.5	0.5	12.5	-	10.5	1	96	120	1	96	45	30	64	30	30	72	120	30	72	300	1	4	∞	1
Ansamycins	0.5	0.5	12.5	-	10.5	1	96	120	1	96	45	30	56	30	30	72	120	30	72	300	1	4	∞	1
Isopenicillin N-synthase	0.5	0.5	12.5	-	10.5	1	94	480	1	94	60	30	65	60	30	72	120	30	72	600	1	4	∞	1
dTDP-glucose synthase	0.5	0.5	12.5	-	10.5	1	94	480	1	94	60	30	65	60	30	72	120	30	72	600	1	4	∞	1
Ketosynthase	0.5	0.5	12.5	1.25 DSMO, 5 Glycerol	4.25	1	94	300	1	94	45	30	60	60	30	72	120	30	72	420	1	4	∞	1

3.8.3 Retrieval of gene sequences and primer design

Twenty selected *Micromonospora* strains were used to identify unique BGCs for which there are no known published primer sets specific for this genus. Only those genomic regions coding for lanthipeptides and bacteriocins were considered as the basis of primer design. The respective regions were individually selected, and their core BGCs were further selected to reveal comprehensive gene details. The required nucleotide sequences were then retrieved for each coding region. The specific genes targeted for primer design were the Lant_dehydr_C gene for lanthipeptides and the DUF692 gene for bacteriocin.

Primer design was then achieved by performing separate multiple sequence alignment of the lanthipeptides and bacteriocins using DNAMAN version 4.13 (Sievers *et al.*, 2011). The consensus sequences were then obtained for each of the target genes and these would provide the basis for the design of the forward and reverse primers for the lanthipeptides and bacteriocins. Following the rules for effective primer design, specific regions on the consensus sequences were located the designing of the forward and reverse primers. The primers were designed to be a maximum of 28 bp and to be able to amplify a product of at least 200bp (Yu *et al*., 2013). In order to analyse the designed primers to ensure their proper functionality, the IDT oligoAnalyzer tool available at https://www.idtdna.com/pages/tools/oligoanalyzer was used. After the design and analysis of primer pairs, a request for oligo-synthesis was submitted to Inqaba Biotec in Pretoria, South Africa.

3.8.4 Testing of designed primers

The chosen five *Micromonospora* strains used in this study were going to be the focal point for the testing of the designed primers. The primers were first tested on genomic DNA samples of the chosen five *Micromonospora* strains using the PCR programme given in Tables 3.6 and 3.7. Optimisation of PCR conditions, specifically the addition of BSA and employing of the gradient PCR method, were performed depending on initial amplification.

After running the PCRs for the target genes, 1% (w/v) agarose gels were prepared in both cases and electrophoresis was subsequently performed for 1 hour at 100 V. Viewing of these gels was performed under UV light by the Molecular Imager Gel Doc XR+ Imaging system (BIO-RAD). The amplicons were then purified using the MSB® Spin PCRapace kit (Stratec Molecular) as per the manufacturer's instructions. Sequencing of these amplicons was outsourced to inqaba biotec^{TM}. The sequence data was then processed as outlined in section 3.4.4.2 and inferences were drawn from the findings.

Table 3.6 Setup and reaction cycle conditions of the Bacteriocin gene amplification PCR

PCR SET UP		PCR CYCLE CONDITIONS			
PCR ingredients	**Volume (µl)**	**Step**	**Temp/^{O}C**	**Duration**	**Cycles**
Forward (F) primer (10 µM)	2.5	Initial denaturation	96	2 sec	30
Reverse (R) primer (10 µM)	2.5	Denaturation	96	30 sec	30
Taq Ready Mix	25	Annealing	51	30 sec	30
Sterile nuclease-free water	18	Extension	72	2 min	30
Template DNA	2	Final Extension	72	5 min	1
Total volume	50	Hold	10	∞	1

Table 3.7 Setup and reaction cycle conditions of the Lanthipeptide gene amplification PCR

PCR SET UP		PCR CYCLE CONDITIONS			
PCR ingredients	**Volume (µl)**	**Step**	**Temp/^{O}C**	**Duration**	**Cycles**
Forward (F) primer (10 µM)	2.5	Initial denaturation	96	2 sec	30
Reverse (R) primer (10 µM)	2.5	Denaturation	96	30 sec	30
Taq Ready Mix	25	Annealing	55	30 sec	30
Sterile nuclease-free water	18	Extension	72	2 min	30
Template DNA	2	Final Extension	72	5 min	1
Total volume	50	Hold	10	∞	1

3.9 Antimicrobial studies

3.9.1 Antibacterial activity in solid media

3.9.1.1 Preparation for testing antibacterial activity

Each of the bacterial strains under study (total of 30) was stab-inoculated into the following agar plates (using sterile toothpicks): SGG, SGG with ASW, 172 F and 172 F with ASW (that were prepared as described in section 3.2). The plates were incubated for 11 days at 30^{O}C to allow the actinobacteria to grow and produce their antibiotics.

3.9.1.2 Inoculation of test bacteria

Three microbial test strains were used in the antibacterial activity analysis of actinobacteria on solid agar media. These were *Escherichia coli* ATCC 25922 (Gram-negative), *Bacillus cereus* ATCC 10876 (Gram-positive) and *Candida albicans* ATCC 24433 (eukaryote). The test strains used were first incubated at 30^{o}C (*B. cereus* and *C. albicans*) or 37^{o}C (*E. coli*) for 24 hours, from liquid cultures onto nutrient agar plates to ensure their growth and viability by the time of analysis.

A day prior to the overlays, a large loopful of each of the test strains was inoculated from their respective agar plate cultures into 5 ml nutrient broth. These mixtures were vortexed vigorously to disperse the bacteria until a turbid suspension was produced. These nutrient broth cultures were incubated overnight at their respective temperatures with shaking on a rotary shaker, 160 rpm (14-18 hours). Test strain cultures consistent with high cellular density were diluted with sterile nutrient broth before taking a reading on the spectrophotometer at 600 nm. The reference guideline-ratios for the dilution of test bacteria cultures was as outlined by Kirby and Le Roes-Hill (2009). These reference guideline dilution ratios used were derived from *Mycobacterium aurum, E. coli* and *Enterococcus faecalis*. The test strains used had their cell densities theoretically estimated to these given standards (Kirby & Le Roes-Hill, 2009), before making dilutions accordingly.

a. *M. aurum* dilute 1-in-6
b. *E. coli dilute* 1-in-10
c. *E. faecalis* (and most other enteric bacteria) dilute 1-in-4

The *B. cereus* and *C. albicans* strains were diluted as per the *M. aurum* and *E. faecalis* dilutions respectively.

3.9.1.3 Antimicrobial activity analysis (overlay technique)

Nutrient agar was prepared according to the manufacturer's instructions, but with a 0.7% (w/v) agar content, and 6 ml of the agar was aliquoted into 10 ml McCartney bottles to form sloppy agar. The sloppy agar was then autoclaved and thereafter cooled to 60^{o}C in a water bath. The optical density of the test strain cultures (OD_{600}), was taken into account to ensure homogeneity in the concentration of cells in the sloppy agar from one overlay experiment to the next. The volume of test culture used per 6 ml sloppy-agar overlay was such that when multiplied by the volume of bacterial culture used per sloppy-agar tube by the OD_{600} of the culture, the following approximate standard values were obtained (these values have been determined empirically and vary for the different test bacteria).

a) *M. aurum* 160 OD_{600}.µl
b) *E. coli* 4 OD_{600}.µl
c) *E. faecalis* 160 OD_{600}.µl

An appropriate amount of test culture, as determined by prior calculations, was pipetted into a sloppy-agar tube and gently vortexed. This mixture was then poured onto a stab-inoculated actinobacterial culture plate. The plate was then gently swirled in order to distribute the sloppy agar evenly over the entire surface of the plate (from the edge of the plate right up to the edge of each actinobacterial colony on the plate). The sloppy-agar was then allowed to solidify and all plates were then turned with the agar-side up. The plates were incubated overnight at the test strains' respective temperatures.

For all strains that were tested, the medium on which the strain was grown and the duration of the incubation before the overlay test was performed were recorded. For those strains exhibiting antibacterial activity, the diameter values of the actinobacterial colony and that of the zone of growth inhibition were measured, to enable the determination of the area of the inhibition zone (mm^2). Based on the activity of the strains under study in solid media cultures (overlay technique), the five best strains were chosen for analysis of antibiotic production in liquid cultures as well as multi-locus sequence analysis (as described in section 3.5).

3.10 Antibiotic production in liquid cultures

3.10.1 Preparation of secondary metabolite extracts for use in bioautography

For the five strains selected for antibiotic production in liquid cultures, 10 ml of 172F liquid media cultures were prepared in duplicate following the 172F liquid media preparation protocol outlined in section 3.2. These were inoculated with the respective strains and then kept shaking at 160 rpm, on a rotary shaker for 5-7 days. Incubation was performed at a temperature of 25^oC-30^oC to enable optimal cell mass production. These cultures were used as pre-cultures to inoculate the flasks for testing antimicrobial production.

Aliquots (100 µl) of each of the pre-cultures were inoculated into 10 ml liquid cultures (in duplicate; 172F liquid media; 10 flasks per strain). These were then incubated at 25^oC-30^oC with continuous shaking at 160 rpm. After 3, 5, 7, 10 and 12 days of growth, two flasks of cultures per each strain were removed from the incubator and an equal volume (10 ml) of ethyl acetate added to each flask. These cultures were then incubated overnight at 30^oC while shaking at 160 rpm. The cultures were then removed from the shakers and allowed to settle. The upper ethyl acetate layer was removed and placed in a clean glass McCartney bottle. The ethyl acetate was allowed to evaporate in a fume hood and the dried extract recovered for use in bioautography. A set of two flasks with media only was included in the extraction process that would serve as a media control.

3.10.2 Bioautography

The dried extracts prepared above were re-suspended in 200 µl ethyl acetate for use in bioautography. The bioautography method used in this study is a variation of the one

outlined by Betina (1973). This protocol suits non-polar compounds that are not readily soluble in 40% (v/v) DMSO. Five microliter volumes of the concentrated extracts were spotted onto clearly labelled silica gel 60 F_{254} TLC plates (Merck Millipore). These TLC plates had grids drawn onto them, with small square areas measuring 1 cm × 1 cm. The spots were left to dry to completeness before the TLC plates could be used in bioautography.

The three test strains used in bioautography included *B. cereus* ATCC 10876*, E. coli* ATCC 25922 and *C. albicans* ATCC 24433. *B. cereus* and *C. albicans* were cultured in nutrient broth that was made according to the manufacturer's instructions. *E. coli* was cultured in Tryptic Soy Broth (Sigma-Aldrich) that was also prepared according to manufacturer's instructions. *C. albicans* and *B. cereus* were both incubated at 30^{o}C with shaking at 160 rpm overnight while *E. coli* was incubated at 37^{o}C, also with shaking at 160 rpm. After confirmation of the purity of the cultures through standard Gram staining, the optical densities of the test bacterial cultures were determined by use of a Rayleigh UV-9200 spectrophotometer at a wavelength of 600 nm, before adjusting the OD_{600} to 0.5 using the respective sterile liquid media used during culturing.

The test bacteria were then applied to the surfaces of the TLC plates with sterile absorbent cotton wool, placed in plastic sealable containers lined with moist paper towel and incubated at 37^{o}C overnight (*E. coli*) and 30^{o}C for the *Bacillus* and *Candida* strains. Thiazolyl blue (MTT, Sigma) dissolved in phosphate-buffered saline (4.26 g $Na_2HPO_4.7H_2O$, 2.27 g KH_2PO_4, 8.00 g NaCl per litre, pH to 7.0) at a final concentration of 0.25% (w/v) was then sprayed onto the TLC plates and incubated at the respective temperatures for 2 to 3 hours. Colour changes were monitored and recorded.

3.10.3 Accessing secondary metabolites using other extraction techniques

Based on the activity profiles of each of the five strains observed in section 3.9.2, an optimal incubation time and growth medium were selected and applied to each of the five strains for further antimicrobial studies. These studies were premised upon the application of four different extraction techniques to see whether different extraction techniques would result in accessing different bio-activities (eight flasks were prepared per strain).

3.10.4 Extraction techniques

3.10.4.1 Whole culture extraction

An equal volume of ethyl acetate was added to each culture after the strain's specific optimum incubation period and further incubated at 30^{o}C overnight while shaking at 160 rpm. The ethyl acetate layer was removed and subsequently dried down under a fume hood. The dried extract was stored at 4^{o}C prior to use in bioautography.

3.10.4.2 DIAION treatment and extraction

For this extraction, 0.5 g DIAION (Supelco) [a non-polar copolymer styrene-divynilbenzene adsorbent resin] was added to the culture after each strain's specific established optimum incubation period and further incubated at 30^{o}C overnight whilst shaking at 160 rpm. The following day, the cultures were filtered, and the filtrate discarded. The cells and DIAION were then washed twice with distilled water. The washed cells and resin were transferred to 50ml flasks and 10ml methanol was added. This mixture was then incubated at 30^{o}C with overnight shaking at 160 rpm. The extracts were then filtered and subsequently dried to completeness in the fume hood. The dried extracts were stored at 4^{o}C.

3.10.4.3 Ethyl acetate extraction on cells and filtrate

The culture was filtered after each strain's specific established optimum incubation period to enable extraction on the cells and filtrate separately. For the filtrate, an equal volume of ethyl acetate was added, and incubation repeated as in section 3.9.4.1. For the cells, 5 ml of ethyl acetate was added, and incubation was similarly performed as for the filtrate. The ethyl acetate layers were removed, dried down and stored at 4^{o}C.

3.10.4.4 Ammonium sulphate precipitation and dialysis

For each of the strains, the culture was first filtered after the specific established optimum incubation period and the filtrate retained. Ammonium sulphate (5.59 g) was added to 10 ml of filtrate and the resulting mixture was stirred at 20^{o}C for 1 hour. Centrifugation at 10000 x *g* was performed using the Sigma 1-14 bench top centrifuge (Lasec) for 10 minutes and the pellet was retained. The pellet was re-suspended in 10 ml 50 mM potassium phosphate buffer (pH 7.5) and dialysis was performed against 50 mM potassium phosphate buffer at 4^{o}C overnight using the Thermo Scientific SnakeSkin Dialysis tubing. The dialysate was removed, and extraction was performed with an equal amount of ethyl acetate as for other extractions. The ethyl acetate was then removed, dried down and stored at 4^{o}C for further use in bioautography.

3.10.5 Bioautography

After all the extracts had been obtained using the different extraction techniques outlined in sections 3.9.4.1 to 3.9.4.4, re-suspension of these extracts in 200 l ethyl acetate was performed. The extracts were then spotted onto a TLC grid and bioautography was performed using the same test strains and following the same protocol as outlined in section 3.9.2.

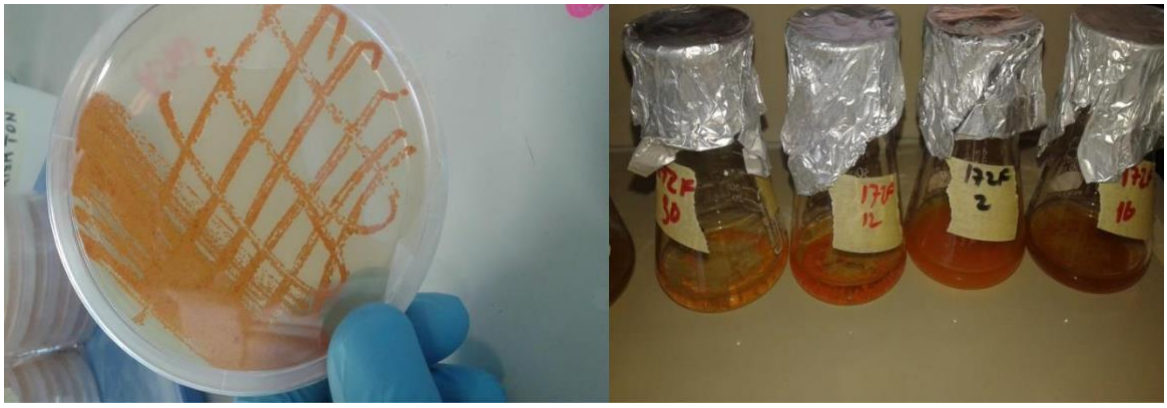
In the following chapter, the results that were obtained from the methods outlined in this chapter are presented. These are then fully discussed in Chapter 5 of the study.

CHAPTER 4

RESULTS AND FINDINGS OF RESEARCH STUDY

4.1 Growth of strains in solid and liquid cultures

The growth and morphological characteristics of the *Micromonospora* strains were macroscopically examined after their respective incubation periods. These observations were made in both liquid and on solid media with examples shown in Figures 4.1 A and 4.1 B. On 172F and SGG solid media, distinct orange colonies were observed (Figure 4.1 A) on the agar plates. It was also observed that the bacterial colonies initially assumed a light-orange shade on solid media before progressively changing to deep-orange, red and in some cases even brown.

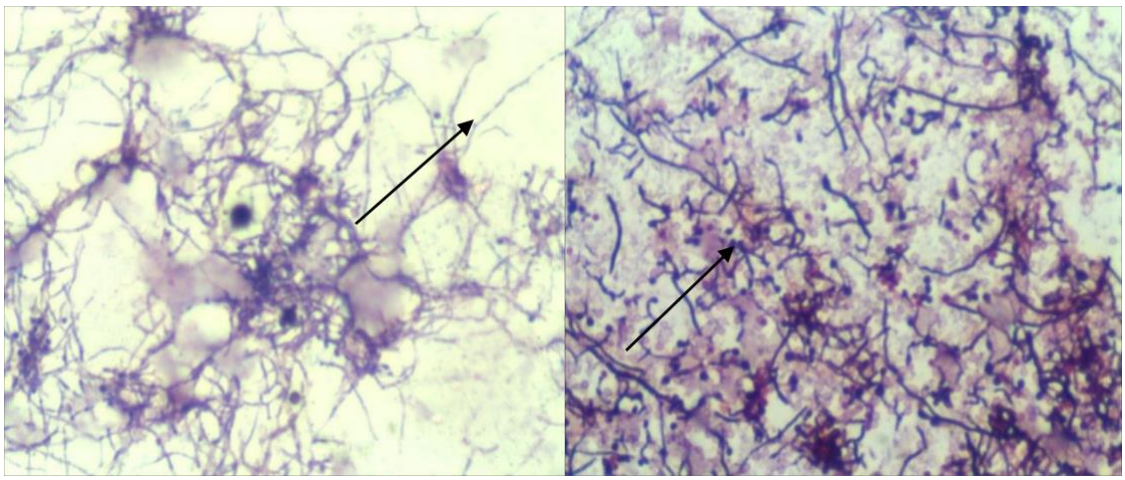


A **B**

Figure 4.1: A) shows growth of *Micromonospora* colonies (strain 02-139#18) on 172 F solid agar after an 11-day incubation period at 25^{O}C. B) shows four different *Micromonospora* strains in 172 F liquid medium after incubation at 25^{O}C with continuous shaking at 160 rpm. In image B, the difference in colour shades of the orange *Micromonospora* strains is visible.

4.2 Gram stain of liquid cultures

Upon viewing the Gram-stained slides at 100× magnification under oil immersion, all the strains were found to be Gram-positive, filamentous strains. Two examples, as seen under oil immersion, are given in Figure 4.2.



A **B**

Figure 4.2: As shown in A), strain 02-203#1 is Gram-positive and highly filamentous under oil immersion (100× magnification). B) shows strain 02-251#1's Gram-positive result, also under oil immersion. The two strains are clearly in different stages of sporulation as depicted by the numerous dot-like spores in B compared to the isolated spores in A (spores shown by arrows in each images).

4.3 Verification of genomic DNA isolation

After DNA isolation, agarose gel electrophoresis was performed on all strains and Figures 4.3, 4.4 and 4.5 were generated upon viewing the agarose gels under UV light (254 nm) in a Uvitec UV gel documentation system. The keys below each gel image reference the numbers annotated on the specific gel to the original identity of the individual samples as given in section 3.1. Figure 4.5 shows an agarose gel picture generated from the re-isolation of DNA from samples 10, 11, 15, 26 and 27.

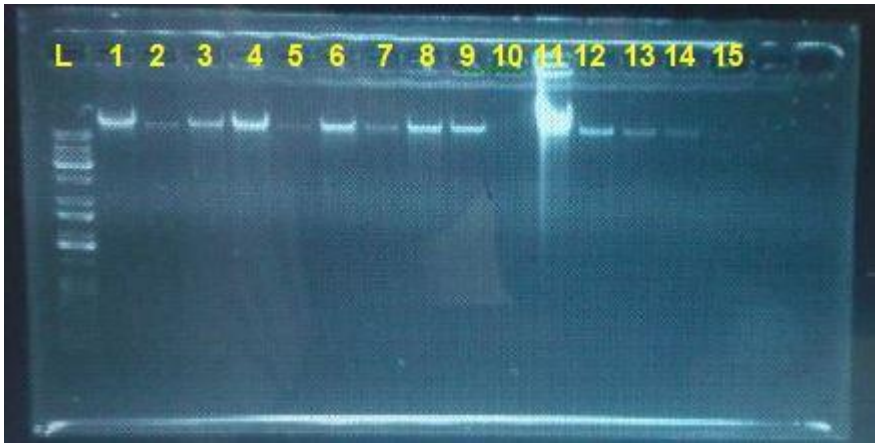


Figure 4.3: Verification of DNA for isolates 1 to 15. The bands on the gel show the respective DNA isolated from the respective strains as given in the key.

KEY: L:Fast DNA Ladder; **1**: 02-158#10; **2**: 02-138#6; **3**: 02-128*#3; **4**: 02-139#9; **5**: 02-209#4; **6**: 02-138#3; **7**: 02-139#18; **8**: 02-118#4; **9**: 02-251#1; 1**0**: 02-221#16; **11**: 02-251#253; 1**2**: 03-013#19; **13**: 02-128*#1; **14**: 02-118#5; **15**: 04-044RT1

Figure 4.4: Verification of DNA for isolates 16 to 30. The bands on the gel show the respective DNA isolated from the respective strains as given in the key.

KEY: L: Fast DNA Ladder; **16**:02-251#136; **17**: 04-015#8; **18**: 02-118#8; **19**: 02-139#14; **20**: 02-203#1; **21**: 02-209#2; **22**: 02-221#26; **23**: 02-231#25; **24**: 02-251#10; **25**: 02-251#135; **26**: 02-251#16; **27**: 02-251#274; **28**: 02-251#322; **29**: 02-251*#36; **30**: 04-015#16

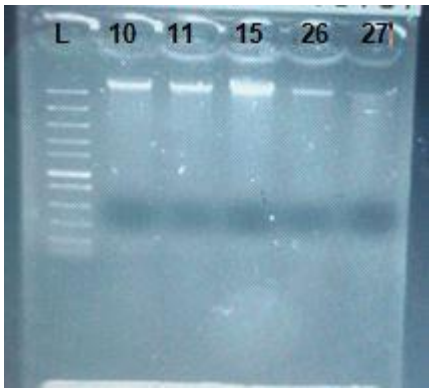


Figure 4.5: Verification of DNA for isolates 10,11,15,26 and 27. The bands on the gel show the respective DNA isolated from the respective strains as given in the key.

KEY: L: Fast DNA Ladder; **10**: 02-221#16; **11**: 02-251#253; **15**: 04-044RT1; **26**: 02-251#16; **27**: 02-251#274

4.4 Quantification of isolated genomic DNA

The results for the spectrophotometric quantification of isolated genomic DNA (gDNA) of all strains are tabulated in Tables 4.1 and 4.2.

Table 4.1: Isolated genomic DNA quantities for the bacterial strains under study.

Assigned identity	Original sample identity	DNA Quantity (µg/ml)
Sample 1	02-158#10	45.00
Sample 2	02-138#6	21.72
Sample 3	02-128*****#3	65.80
Sample 4	02-139#9	32.57
Sample 5	02-209#4	20.40
Sample 6	02-138#3	111.60
Sample 7	02-139#18	95.87
Sample 8	02-118#4	80.93
Sample 9	02-251#1	54.00
Sample 10	02-221#16	9.75
Sample 11	02-251#253	97.21
Sample 12	03-013#19	147.20
Sample 13	02-128*****#1	22.94
Sample 14	02-118#5	29.28
Sample 15	04-044RT1	6.25
Sample 16	02-251#136	43.05
Sample 17	04-015#8	22.38
Sample 18	02-118#8	88.25
Sample 19	02-139#14	114.00
Sample 20	02-203#1	45.56
Sample 21	02-209#2	55.21
Sample 22	02-221#26	88.64
Sample 23	02-231#25	111.37
Sample 24	02-251#10	75.20
Sample 25	02-251#135	77.33
Sample 26	02-251#16	49.75
Sample 27	02-251#274	56.70
Sample 28	02-251#322	120.37
Sample 29	02-251*#36	55.21
Sample 30	04-015#16	79.58

Table 4.2 DNA concentrations for re-extracted bacterial strains

Assigned identity	Original sample identity	DNA Quantity (µg/ml)
Sample 10	02-221#16	22.35
Sample 11	02-251#253	105.72
Sample 15	04-044RT1	27.80
Sample 26	02-251#16	62.57
Sample 27	02-251#274	22.40

4.5 16S rRNA gene amplification

After running the PCR for the amplification of the 16S rRNA gene, the agarose gel pictures that were generated can be seen in Figures 4.6 and 4.7.

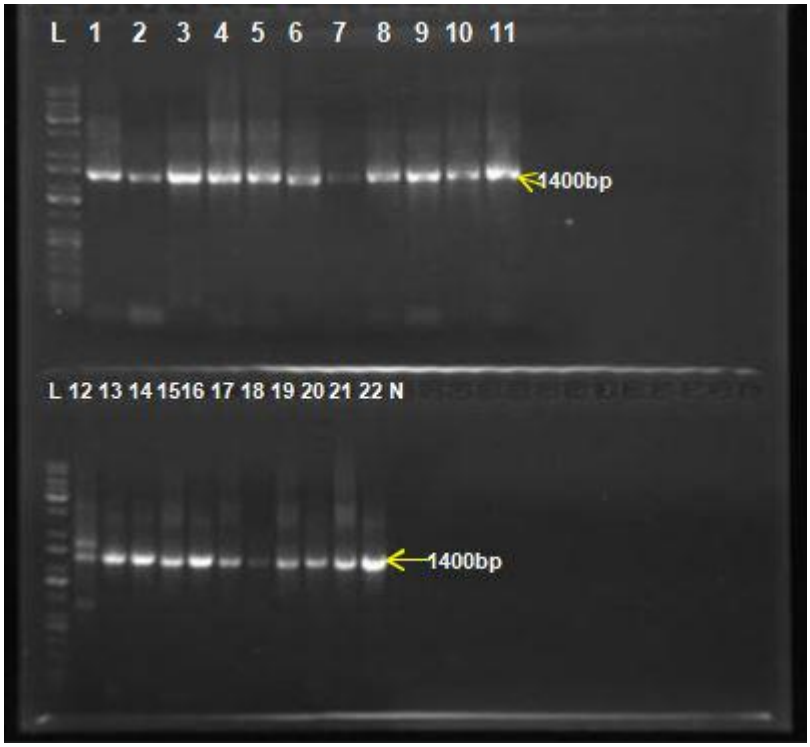


Figure 4.6: The agarose gel picture for amplification of the 16S rRNA gene using F1/R5 primers for samples 1-22. The bands on the gel show the respective 16S rRNA gene amplicons from the respective strains as given in the key.

KEY: L: Fast DNA Ladder; **1**: 02-158#10; **2**: 02-138#6; **3**: 02-128*#3; **4**: 02-139#9; **5**: 02-209#4; **6**: 02-138#3; **7**: 02-139#18; **8**: 02-118#4; **9**: 02-251#1; **10**: 02-221#16; **11**: 02-251#253; **12**: 03-013#19; **13**: 02-128*#1; **14**: 02-118#5; **15**: 04-044RT1; **16**: 02-251#136; **17**: 04-015#8; **18**: 02-118#8; **19**: 02-139#14; **20**: 02-203#1; **21**: 02-209#2; **22**: 02-221#26; **N**: negative control.

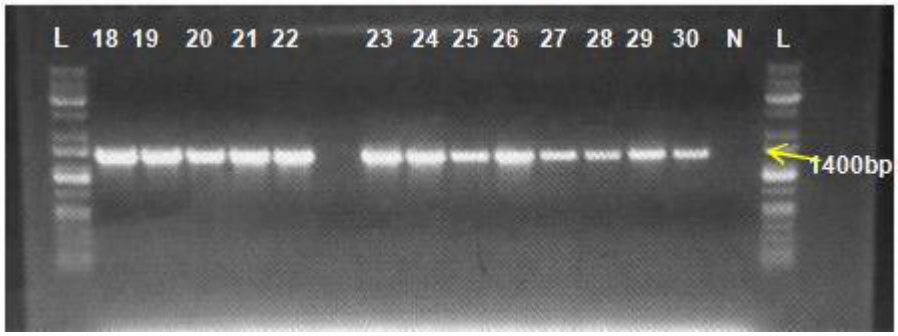


Figure 4.7: The agarose gel picture for amplification of the 16S rRNA gene using F1/R5 primers for samples 18-30. Samples 18-22 were included in this amplification for the second time since they had been amplified already as shown in Fig 4.6. The bands on the gel show the respective 16S rRNA gene amplicons from the respective strains as given in the key.

KEY: **L**: Fast DNA Ladder; **18**: 02-118#8; **19**: 02-139#14; **20**: 02-203#1; **21**: 02-209#2; 22: 02-221#26. **23**: 02-231#25; **24**: 02-251#10; **25**: 02-251#135; **26**: 02-251#16; **27**: 02-251#274; **28**: 02-251#322; **29**: 02-251*#36; **30**: 04-015#16; **N**: Negative control.

All the bands that were observed under UV illumination required size interpretation against the reference FAST DNA ladder shown in Figure 4.8.

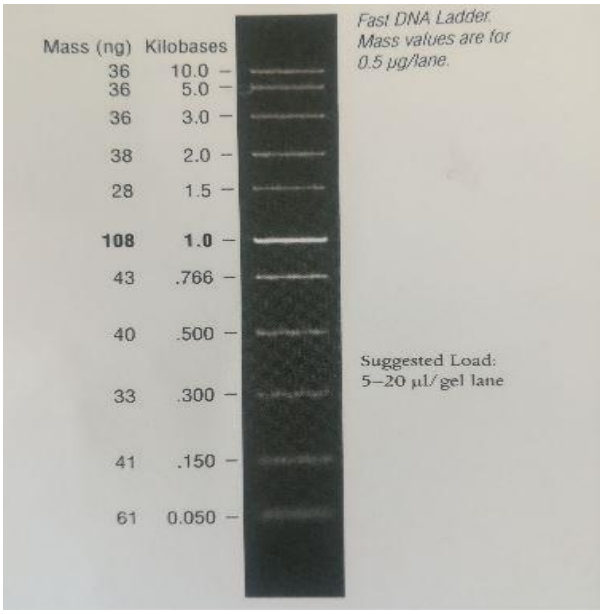


Figure 4.8: An image showing the demarcations, on the Fast DNA ladder (Biolabs), that are used to estimate the size of amplicons [in kilobases (kb)] of amplicons generated.

Sequences from the amplicons generated from 16S rRNA gene PCR identified the strains to belong to the genus *Micromonospora*, with the exception of strains 02-251#136 and 02-251#135 (Table 4.3).

Table 4.3 Top hits of proven similarity-based searches of *Micromonospora* strains against quality-controlled databases of 16S rRNA sequences (EzBioCloud)

Strain No.	Original ID	Hit Taxon name	Hit Strain name	Similarity %	Sequence Length (bp)	GC %
1	02-158#10	*Micromonospora tulbaghiae*	DSM 45142	98.82	1 366	60.1
2	02-138#6	*Micromonospora aurantiaca*	ATCC 27029	100.00	1 351	60.2
3	**02-128*#3**	*Micromonospora aurantiaca*	ATCC 27029	100.00	1 362	60.5
4	02-139#9	*Micromonospora tulbaghiae*	DSM 45142	98.82	1 368	60.1
5	**02-209#4**	*Micromonospora aurantiaca*	ATCC 27029	100.00	1 348	60.2
6	02-138#3	*Micromonospora aurantiaca*	ATCC 27029	100.00	1 350	60.1
7	02-139#18	*Micromonospora tulbaghiae*	DSM 45142	100.00	1 366	60.1
8	02-118#4	*Micromonospora citrea*	DSM 43903	98.52	1 363	60.1
9	02-251#1	*Micromonospora aurantiaca*	ATCC 27029	100.00	1 351	60.2
10	02-221#16	*Micromonospora aurantiaca*	ATCC 27029	100.00	1 362	60.1
11	02-251#253	*Micromonospora tulbaghiae*	DSM 45142	99.78	1 368	60.1
12	03-013#19	*Micromonospora aurantiaca*	ATCC 27029	100.00	1 361	60.3
13	**02-128*#1**	*Micromonospora aurantiaca*	ATCC 27029	100.00	1 361	60.3
14	02-118#5	*Micromonospora tulbaghiae*	DSM 45142	98.82	1 352	60.5
15	04-044RT1	*Micromonospora chokoriensis*	DSM 45160	98.59	1 294	60.0
16	02-251#136	*Jishengella endophytica*	202201	97.19	1 366	60.5
17	04-015#8	*Micromonospora tulbaghiae*	DSM 45142	100.00	1 366	60.1
18	02-118#8	*Micromonospora aurantiaca*	ATCC 27029	100.00	1 351	60.2
19	02-139#14	*Micromonospora tulbaghiae*	DSM 45142	99.78	1 366	60.0
20	02-203#1	*Micromonospora citrea*	DSM 43903	99.41	1 363	60.3
21	02-209#2	*Micromonospora aurantiaca*	ATCC 27029	100.00	1 355	60.1
22	02-221#26	*Micromonospora aurantiaca*	ATCC 27029	100.00	1 361	60.3
23	02-231#25	*Micromonospora tulbaghiae*	DSM 45142	99.41	1 352	60.5
24	02-251#10	*Micromonospora tulbaghiae*	DSM 45142	99.41	1352	60.5
25	02-251#135	*Jishengella zingiberis*	PLAI 1-1	99.93	1366	60.0
26	02-251#16	*Micromonospora aurantiaca*	ATCC 27029	100.00	1348	60.2
27	02-251#274	*Micromonospora aurantiaca*	ATCC 27029	100.00	1348	60.2
28	02-251#322	*Micromonospora citrea*	DSM 43903	99.38	1302	60.3
29	**02-251*#36**	*Micromonospora aurantiaca*	ATCC 27029	100.00	1337	60.1
30	**04-015#16**	*Micromonospora aurantiaca*	ATCC 27029	100.00	1 352	60.2

* ***** The strains in bold and red are the five top strains according to antimicrobial activity performed (as described in the relevant sections).

4.6 Multi-Locus Sequence Analysis (MLSA)

The gel pictures for those PCRs that were positive (as viewed under UV light) in the MLSA study are shown in Figures 4.9 a, b and c.

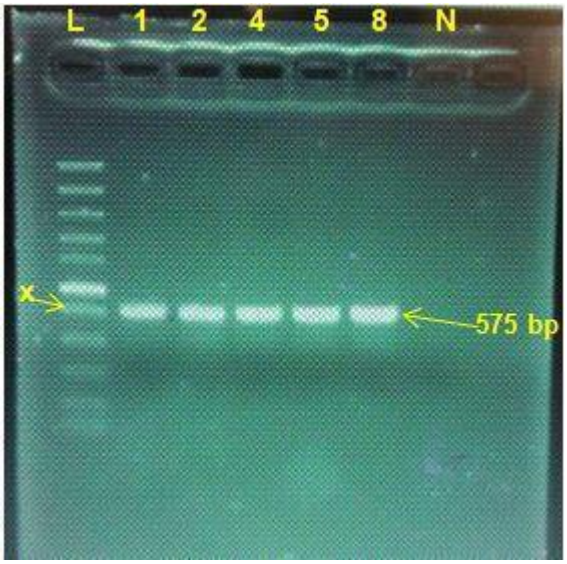


Figure 4.9 a: The agarose gel picture for amplification of the *rpoB* gene using MYCOF^{i} / MYCOR^{i} primers for strains 1, 2, 4, 5 and 8. The bands on the gel show the respective *rpoB* amplicons from the respective strains as given in the key.

KEY: L: Fast DNA Ladder; **1**: 02-128*#1; **2**: 04-015#16; **4**: 02-128*#3; **5**: 02-251*#36; **8**: 02-209#4; **N**: negative control; **X**: 500 bp

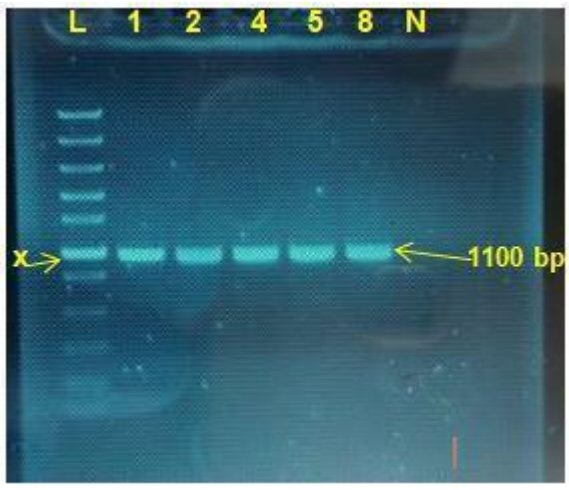


Figure 4.9 b: The agarose gel picture for amplification of the *gyrB* gene using the GYF1 /GYR1B primer pair for strains 1, 2, 4, 5 and 8. The bands on the gel show the respective *gyrB* amplicons from the respective strains as given in the key.

KEY: L: Fast DNA Ladder; **1**: 02-128*#1; **2**: 04-015#16; **4**: 02-128*#3; **5**: 02-251*#36; **8**: 02-209#4; **N**: negative control; **X**: 1 kb

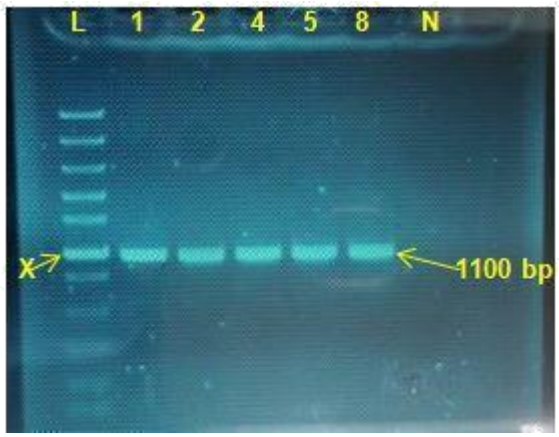


Figure 4.9 c: The agarose gel picture for amplification of the *gyrB* gene using the GYF3 /GYR3B primer pair for strains 1, 2, 4, 5 and 8. The bands on the gel show the respective *gyrB* amplicons from the respective strains as given in the key.

KEY: L: Fast DNA Ladder; **1**: 02-128*#1; **2**: 04-015#16; **4**: 02-128*#3; **5**: 02-251*#36; **8**: 02-209#4; **N**: negative control; **X**: 1 kb

4.7 Phylogenetic analysis

The four phylogenetic trees (for the genes *gyrB*, *rpoB* and 16S rRNA genes, as well as the concatenated tree) that were constructed using the MEGA X programme are given in Figures 4.10, 4.11, 4.12 and 4.13. The tree algorithm presented in all these figures is the neighbour-joining algorithm. The maximum likelihood and minimum evolution tree algorithms for all the phylogenetic trees given in Figures 4.10 to 4.13 are presented in Annexures A (16 S rRNA gene phylogenetic trees), B (*gyrB* phylogenetic trees), C (*rpoB* phylogenetic trees) and D (Concatenated sequences phylogenetic trees) .

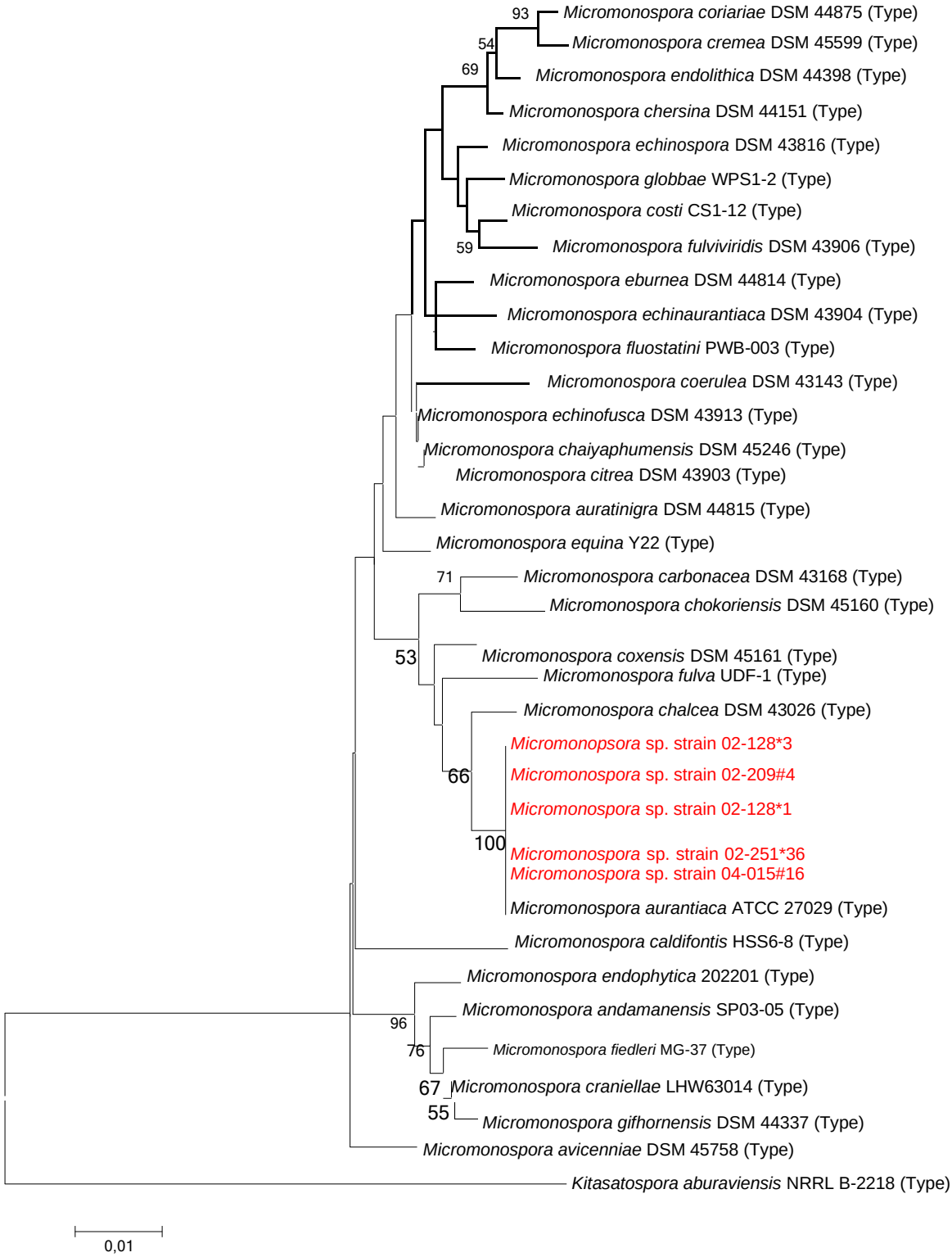


Figure 4.10: The phylogenetic relationships between the 16S rRNA gene sequences of the *Micromonospora* strains 02-128*3, 02-209#4, 02-128*1, 02-251*36, 04-015#16 with 30 other type strains of other *Micromonospora* species are shown in the Neighbour-joining tree (Saitou & Nei, 1987). Strains 02-128*3, 02-209#4, 02-128*1, 02-251*36 and 04-015#16 are shown in red. The *Kitasatospora aburaviensis* NRRL B-2218 was used as an outgroup. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 36 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were 1338 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

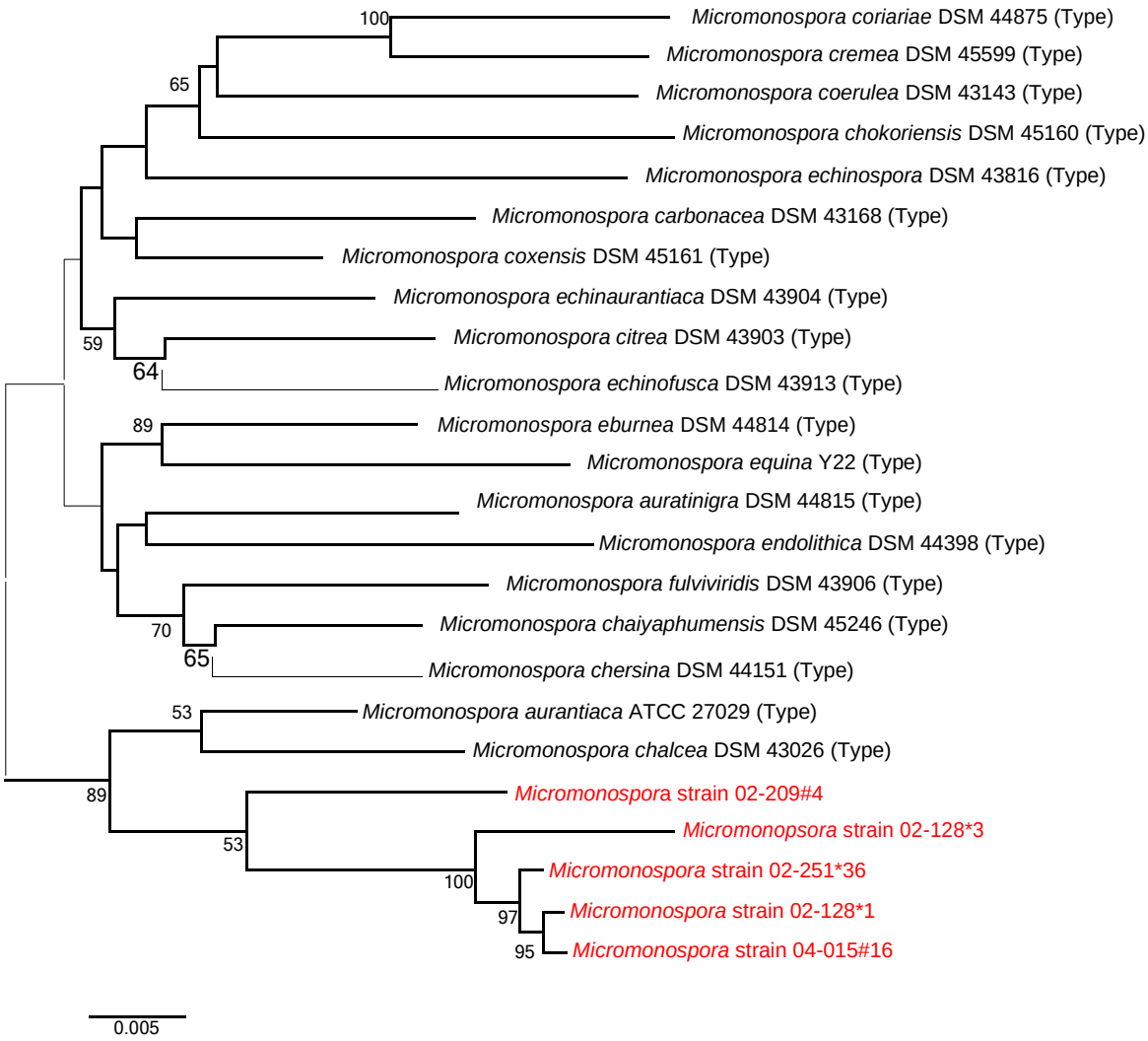


Figure 4.11: A Neighbour-joining phylogenetic tree (Saitou & Nei, 1987), is shown between the MLSA derived composite sequences of the 5 *Micromonospora* strains (02-128*3, 02-209#4, 02-128*1, 02-251*36, 04-015#16) and Type strains of other *Micromonospora* genus. During the construction of this particular tree, the 5 *Micromonospora* strains were presented as concatenated sequences obtained by combining their individual 16S sequences, *gyr*B and *rpo*B gene sequences from the MLSA study. The same order of sequence assembly was maintained for all the strains. Strains 02-128*3, 02-209#4, 02-128*1, 02-251*36 and 04-015#16 are shown in red. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 24 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 2837 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

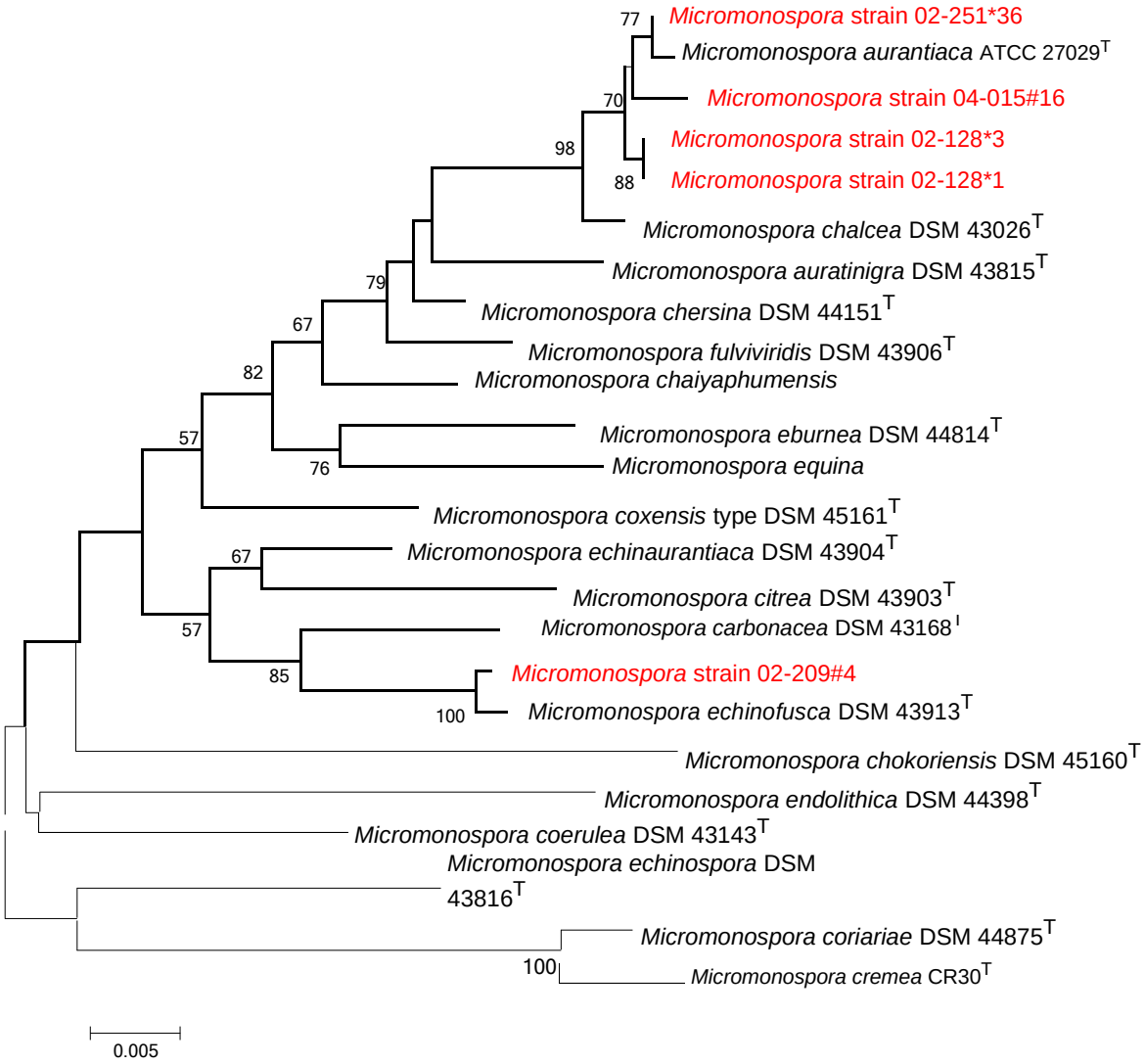


Figure 4.12: An unrooted Neighbour-joining phylogenetic tree (Saitou & Nei, 1987), is shown between the *rpo*B gene sequences of the 5 *Micromonospora* strains (02-128*3, 02-209#4, 02-128*1, 02-251*36, 04-015#16) and strains of other *Micromonospora* genus. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 24 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 602 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

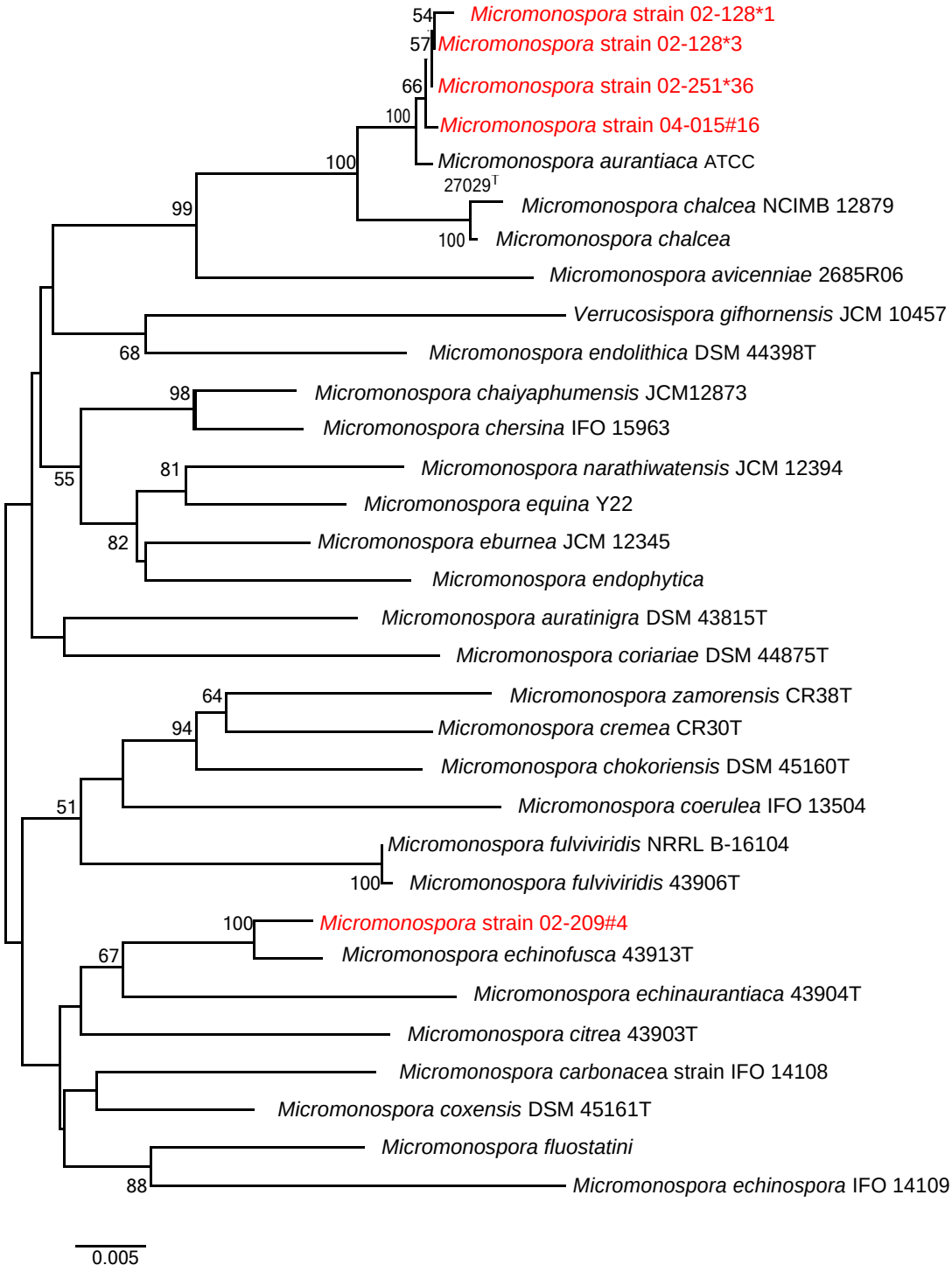


Figure 4.13 An unrooted Neighbour-joining phylogenetic tree (Saitou & Nei, 1987), is shown between the *gyrB* gene sequences of the 5 *Micromonospora* strains (02-128*3, 02-209#4, 02-128*1, 02-251*36, 04-015#16) and strains of other *Micromonospora* genus. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 32 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 987 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

4.8 antiSMASH genomic information

The antiSMASH analysis to predict the BGCs present in the genomes of published *Micromonospora* species was carried out and the outcome is presented in the info graphics presented in this section. For each species, the sequential arrangement of the predicted BGCs is given and below that, identities of the actual BGCs predicted are given. A key to define the necessary BGC, which are provided in short form, is provided at the end of the section.

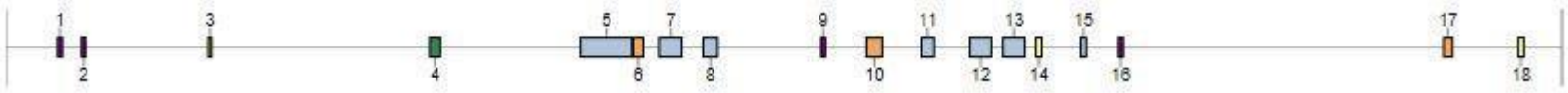
Micromonospora auratinigra

1)NRPS,T1PKS__2)NRPS-L__3)Ter__4)T2PKS__5)Thio__6)Oligo,PKS-L,T2PKS__7)T1PKS__8)NRPS,T1PKS__9)T1PKS__10)Ter,Bac__11)Ter__12)T3PKS__13)Ter__14)Ter__15)NAGGN

Micromonospora coriariae

1)Ter__2)Ter__3)T3PKS__4)Ter__5)Bac,Ter__6)Sid__7)T2PKS__8)Ary__9)Ter__10)NRPS,T1PKS11)Thio,LAP

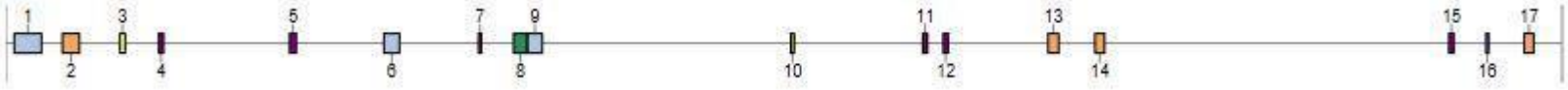
Micromonospora aurantiaca



1)Ter__2)Ter__3)NAGGN__4)NRPS__5)NRPS,T1PKS__6)T1PKS__7)Sid,NRPS,T1PKS,PKS-L__8)NRPS,T1PKS__9)Ter__10)T2PKS__11)NRPS,Oli,Ter__12)PKS-L,NRPS,T-PKS__

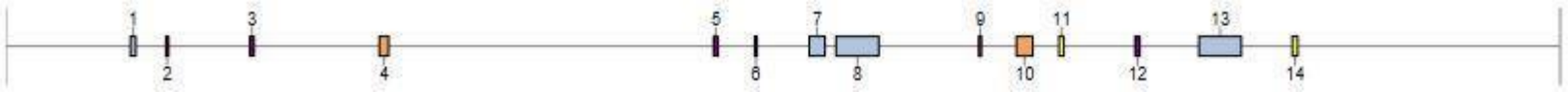
13) Oli, T2PKS__14) Lan__15) Ter, Bac__16) Ter__17) T3PKS__18) Lan

Micromonospora chersina



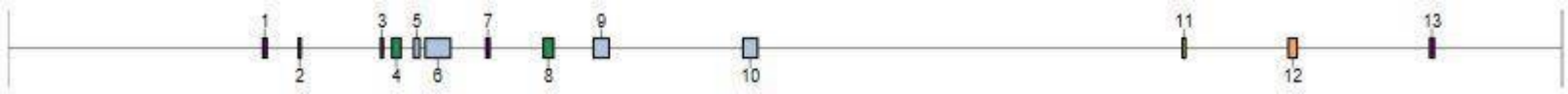
1)T3PKS,NRPS__2)T2PKS__3)Lan__4)Ter__5)Bet__6)NRPS,T1PKS__7)Sid__8)NRPS__9)NRPS,T1PKS__10)NAGGN__11)Ter__12)Ter__13)T1PKS__14)T3PKS__15)Ter__16)Bac__17)PKS-L

Micromonospora chokoriensis



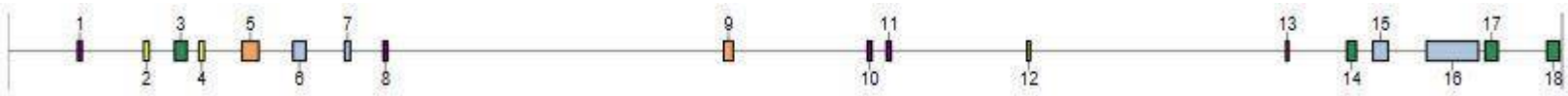
1)LAP,Bac__2)Sid__3)Ter__4)T3PKS__5)Ter__6)Bac__7)NRPS,T1PKS,Ter__8)T1PKS,Thi,LAP,T2PKS__9)Sid__10)T2PKS__11)Lan__12)Ter__13)T1PKS,Beta__14)Lan

Micromonospora echinaurantiaca



1)Ter__2)Bac__3)Sid__4)NRPS-L__5)Thi,LAP__6)NRPS__7)Ter__8)NRPS__9)T2PKS,Aryl__10)NRPS,T1PKS__11)NAGGN__12)T3PKS__13)Ter

Micromonospora coxensis



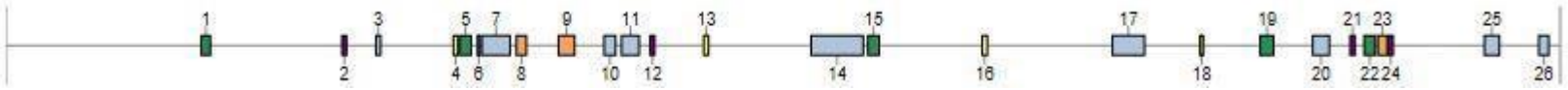
1)Ter__2)Lan__3)NRPS__4)Lan__5)T2PKS__6)NRPS,T1PKS__7)Ter,Bac__8)Ter__9)T3PKS__10)Ter__11)Ter__12)NAGGN__13)Sid__14)NRPS-L__15)NRPS,T1PKS__16)NRPS,T1PKS__

17) NRPS__18) NRPS

Micromonospora inositola

1)NAGGN__2)Ter__3)Ter__4)T3PKS__5)Ter__6)Bac,Ter__7)T2PKS__8)Ter__9)Beta

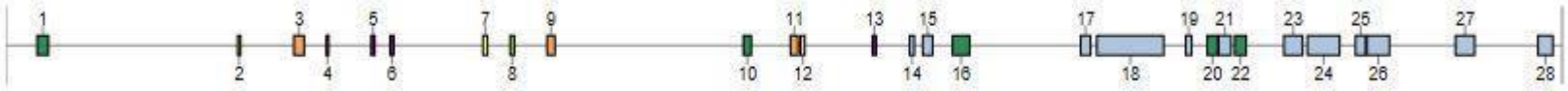
Micromonospora echinofusca



1)NRPS-L__2)Ter__3)Bac,Ter__4)Lan__5)NRPS__6)Bac__7)NRPS,Lan__8)T1PKS__9)T2PKS__10)NRPS__11)Beta,NRPS__12)Ter__13)Lan__14)T1PKS,NRPS,Side__15)NRPS__16)Lan__

17)NRPS,T1PKS,LAP,NRPS-L__18)NAGGN__19)NRPS__20)T1PKS,NRPS__21)Ter__22)NRPS__23)T1PKS__24)Ter__25)NRPS, T1PKS__26)T3PKS,Thi,LAP

Micromonospora echinospora

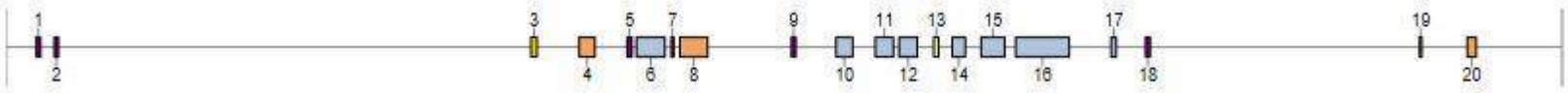


1)NRPS__2)NAGGN__3)T1PKS__4)Sid__5)Ter__6)Ter__7)Lan__8)Amg__9)T3PKS__10)NRPS-L__11)T3PKS__12)Ind__13)Ter__14)Bac,Ter__15)NRPS,T1PKS__16)NRPS__

17)T1PKS__18)NRPS,T1PKS,nuc__19)Lan,Bac__20)NRPS__21)T1PKS,hglE-KS,Bac__22)NRPS__23)NRPS,T2PKS__24)NRPS,T1PKS,Beta,Lass,PKS-L__25)NRPS,T3PKS__

26) T3PKS, T1PKS, NRPS__27) NRPS, T1PKS__28) T2PKS, PKS-L, T1PKS.

Micromonospora eburnea



1)Ter__2)Ter__3)Thio__4)T2PKS__5)Ter__6)T1PKS,NRPS__7)Sid__8)T1PKS__9)Ter__10)NRPS,T1PKS__11)T1PKS,NRPS,__12)Lan,NRPS__13)Lan__14)NRPS,T1PKS__

15)Lan,T1PKS,PKS-L__16)Ladd,PKS-L,T1PKS,Oligo,NRPS,Amg__17)Ter,Bac__18)Ter__19)Ect__20)T3PKS

KEY

NRPS-L: NRPS-like

PKS-L: PKS-like

Lan: Lanthipeptide

Ter: Terpene

Bac: Bacteriocin

Beta: Betalactone

Thio: Thiopeptide

Sid: Siderophore

Ect: ectoine

Oligo: Oligosaccharide

Aryl: Arylpolyene

Amg: Amglyccycl

Ind: Indole

nuc: nucleoside

Lass: Lassopeptide

t-PKS: transAT-PKS

Lad: Ladderane

Ect: ectoine

4.8.1 PCR screening for Biosynthetic Gene Clusters (BGCs)

Only two out of the 30 strains tested were negative for the amplification of the Type II PKS gene. However, all PCR screening for BGCs required for the production of polyene antibiotics, polyether ionophores, glycopeptides, ansamycins, beta-lactams, aminoglycosides and Type 1 PKS were negative, even after performing gradient PCR and the addition of BSA or DMSO. The positive amplification of the Type II PKS gene can be seen in Figure 4.14.

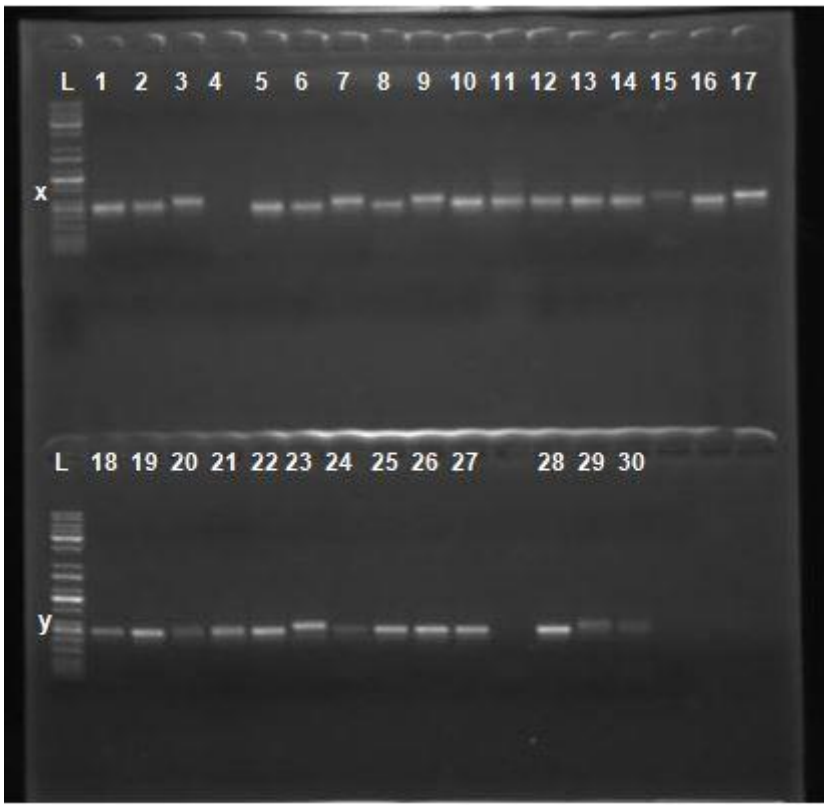


Figure 4.14: The agarose gel picture for amplification of the Type II PKS gene using the ARO-PKS-F/ARO-PKS-R primer pair for samples 1-30.The bands on the gel show the respective Ketosynthase α and Ketosynthase β amplicons from the respective strains as given in the key.

KEY: L: Fast DNA Ladder; **1**: 02-158#10; **2**: 02-138#6; **3**: 02-128*#3; **4**: 02-139#9; **5**: 02-209#4; **6**: 02-138#3; **7**: 02-139#18; **8**: 02-118#4; **9**: 02-251#1; **10**: 02-221#16; **11**: 02-251#253; **12**: 03-013#19; **13**: 02-128*#1; **14**: 02-118#5; **15**: 04-044RT1; **16**:02-251#136; **17**: 04-015#8; **18**: 02-118#8; **19**: 02-139#14; **20**: 02-203#1; **21**: 02-209#2; **22**: 02-221#26. **23**: 02-231#25; **24**: 02-251#10; **25**: 02-251#135; **26**: 02-251#16; **27**: 02-251#274; **28**: 02-251#322; **29**: 02-251*#36; **30**: 04-015#16

4.8.2 Analysis of BGC primer sets and known *Micromonospora* strains

Analysis of BGC primer sets against known *Micromonospora* strains was an exercise done to establish primer knowledge gaps. As a starting point, the BGCs that were targeted by primer sets used in the study were searched for within the set of BGCs that were predicted within known *Micromonospora* species in antiSMASH. From this analysis, it was observed that the BGCs in our primer sets that were predicted in antiSMASH analysis were Type I and Type II PKS. This information would become handy in interpreting the outcome of the PCR-based screen.

4.8.3 Alignments for primer design

The sections used for primer design for lanthipeptides [Figure 4.15 a) i) and 4.15 a) ii)] and bacteriocins [Figure 4.15 b) i) and 4.15 b) ii)] were found within the alignments of the sequences that were downloaded from the genome sequences that were analysed via antiSMASH.

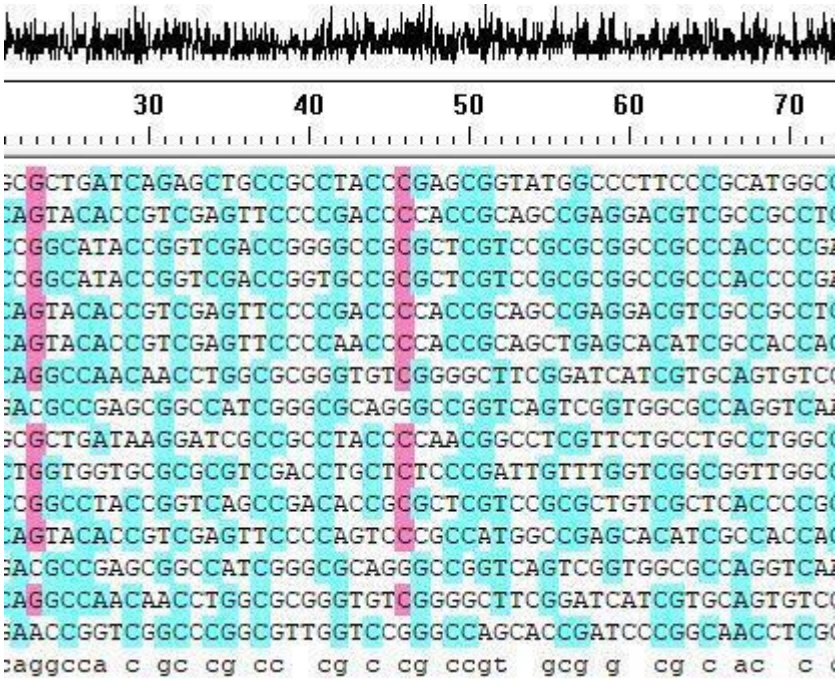


Figure 4.15 a) i): Multiple-sequence alignment extract (DNAMAN) that was used as the basis for lanthipeptide forward primer design. Positions 21-39 was the exact location of the sequence used.

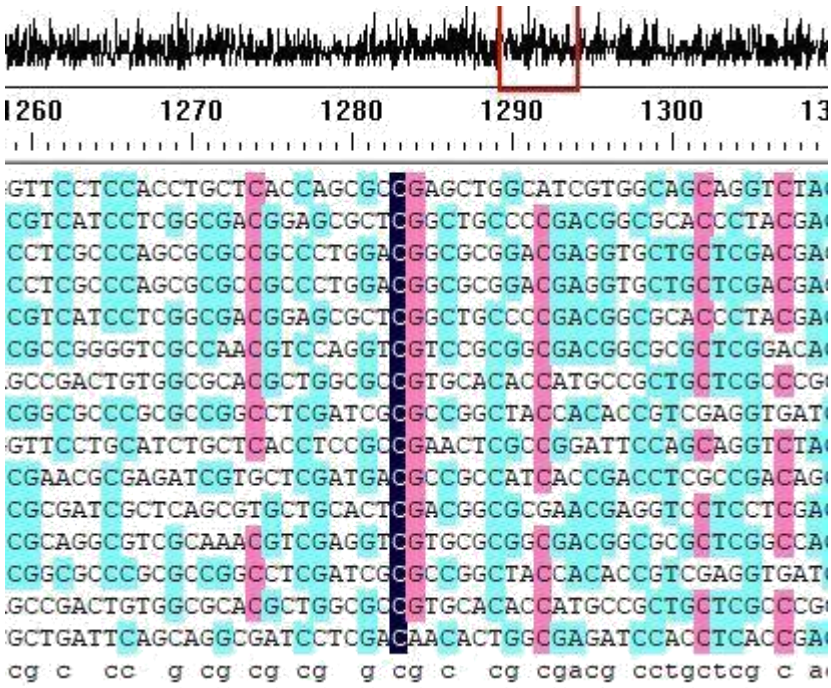


Figure 4.15 a) ii): Multiple-sequence alignment (DNAMAN) that was used as the basis for Lanthipeptide reverse primer design. The exact location of the section selected was from position 1289 to 1307.

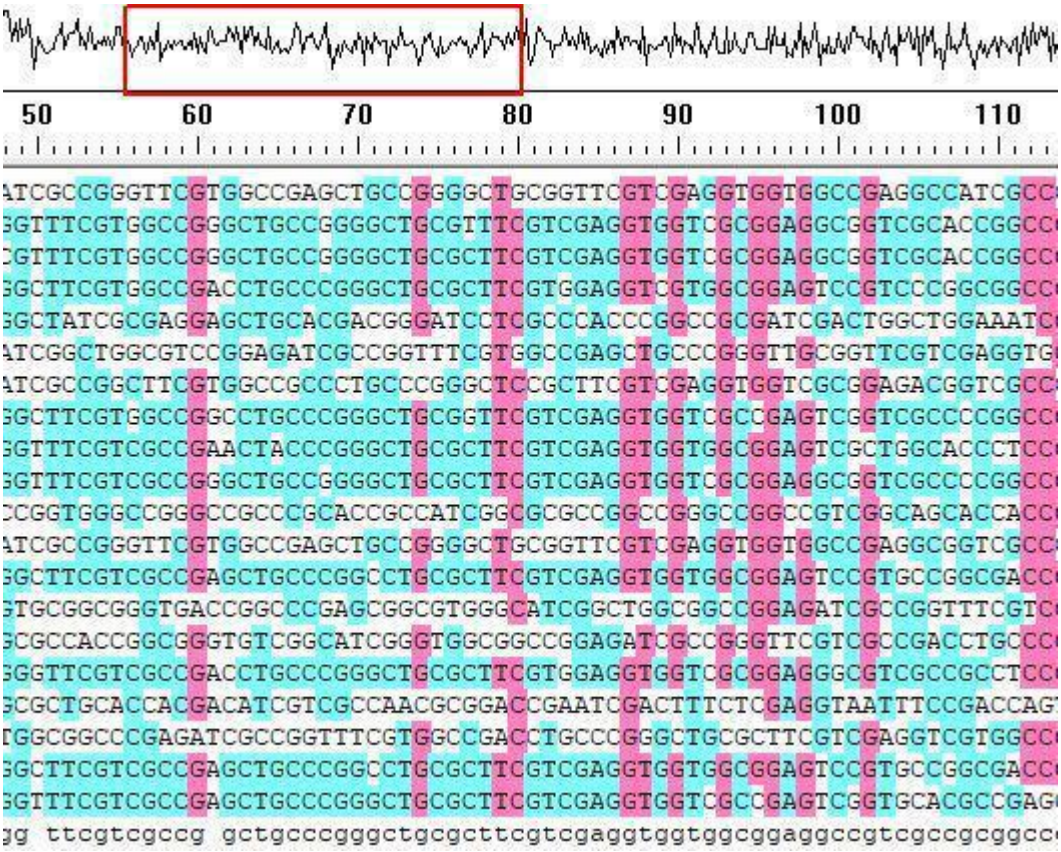


Figure 4.15 b) i): Multiple-sequence alignment extract (DNAMAN) that was used as the basis for bacteriocin forward primer design. Position 85-103 was the exact location of the sequence used.

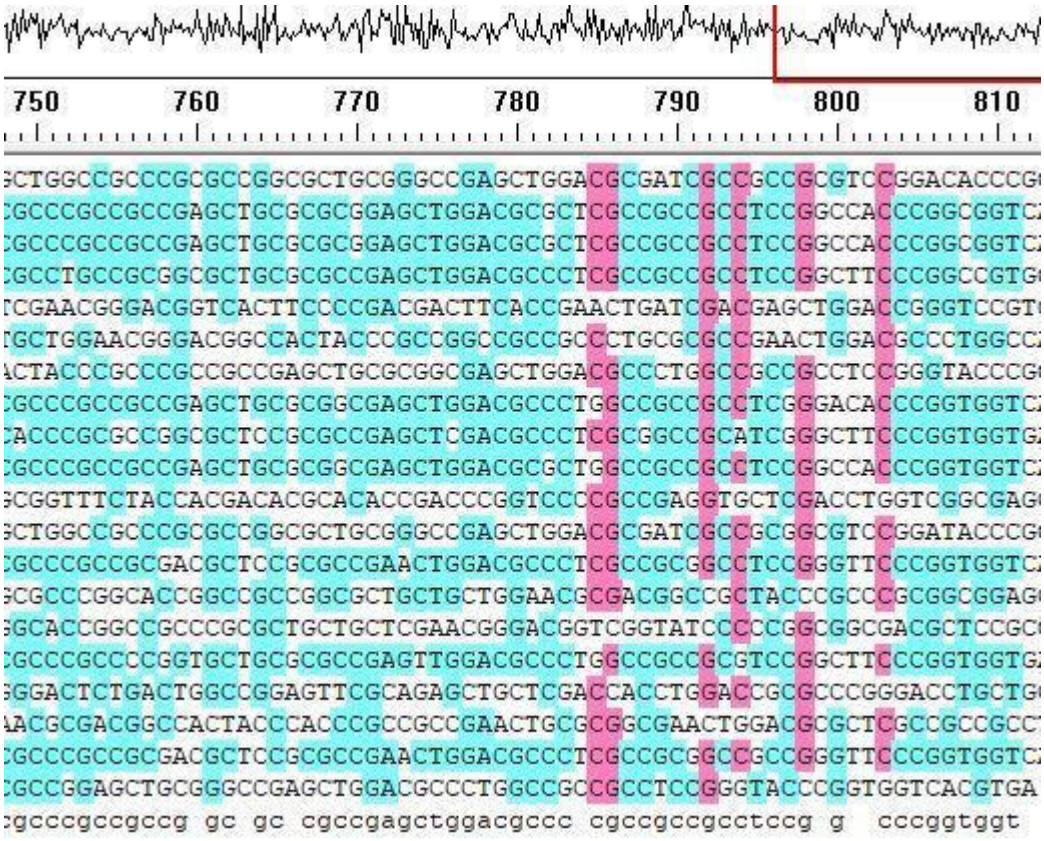


Figure 4.15 b) ii): Multiple sequence alignment extract (DNAMAN) that was used as the basis for bacteriocin reverse primer design. Position 767-785 was the exact location of the sequence used.

4.9 Primer design outcome and oligo-analysis

After the design of the bacteriocin (BAC-F/BAC-R) and lanthipeptide (LAN-F/LAN-R) primers based on the multiple-sequence alignments, analysis of these primer pairs was performed. The comprehensive results of the design and analysis of the oligonucleotides, which were supplied to inqaba biotec^{TM} for oligo-synthesis, are given in Table 4.4.

The synthesis reports for the designed primers were supplied by Inqaba Biotech. The synthesis reports for the bacteriocin and lanthipeptide primer pairs are attached as Annexures E and F respectively.

Table 4.4 Primer design outcome and analysis

Primer	Sequence	Length /bp	GC Content %	Melting T/^{o}C	Molecular weight /g/mole	Expected amplicon sizes/bp
BAC-F	5'-CTGCGCTTCGTCGAGGTC-3'	18	66.70	58.7	5482.6	400
BAC-R	5'-CATCAGGGGCAGCGCAAG-3'	18	66.70	59.7	5558.7	
LAN-F	5'-TACCIGCIGGAGACCCTG-3'	18	61.1 %	61.3 ºC	5495.6	850
LAN-R	5'-CTCGTAGIGGGCGTIGIG-3'	18	61.1 %	62.2 ºC	5607.6	

4.10 Verification of targeted gene amplification

Both primer sets (BAC-F/BAC-R and LAN-F/LAN-R) did not yield any product upon testing them on genomic DNA. Gradient PCR and DMSO enhancement of the PCRs were then employed for both primer sets. The lanthipeptide primer pair did not give any sign of product amplification even after optimisation. However, a positive product amplification result (420bp-500bp) of the bacteriocin primer pair, as observed upon performing agarose gel electrophoresis, is given in Figure 4.16.

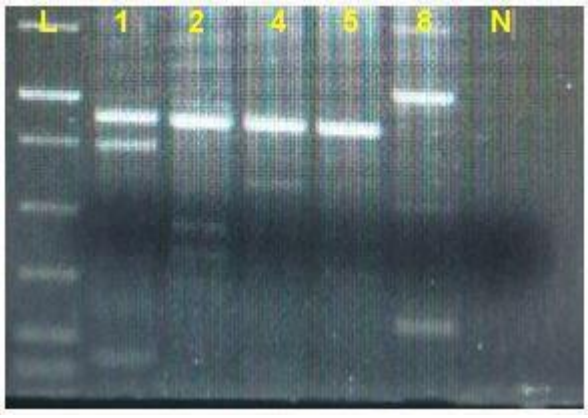


Figure 4.16: The agarose gel picture for the optimised amplification of the bacteriocin gene cluster using the designed BAC-F/BAC-R primer pair for strains 1, 2, 4, 5 and 8. The genomic DNA was used as PCR template in this case.

KEY: L: Fast DNA Ladder; **1**: 02-128*#1; **2**: 04-015#16; **4**: 02-128*#3; **5**: 02-251*#36; **8**: 02-209#4; **N**: negative control

4.10.1 Sequencing of Bacteriocin amplicons

After processing of the raw sequence data generated from the sequencing of the positive bacteriocin gene cluster amplicons, the data was uploaded into BLAST. The results for the top sequences that were seen to produce significant alignments are presented in Table 4.5b.

Table 4.5 Sequences producing significant alignments after blastx searches of bacteriocin sequences

Strain identity	**Top sequences producing significant alignments.**
Strain 1 (02-128*#1)	Dienelactone hydrolase [*Micromonospora* sp. CNZ309]
Strain 2 (04-015#16)	Daunorubicin resistance protein DrrA family ABC transporter ATP-binding protein [*Micromonospora*]
Strain 4 (02-128*#3)	Daunorubicin resistance protein DrrA family ABC transporter ATP-binding protein [*Micromonospora*]
Strain 5 (02-251*#36)	ATP-binding cassette domain-containing protein [*Micromonospora* sp. B006]

4.11 Antimicrobial activity studies

The overlay studies carried out to investigate antimicrobial activity were performed on solid agar media. The antimicrobial activity of only those strains that showed activity was determined by taking the following measurements: colony diameter, diameter of inhibition zone and the area of inhibition zone (Figure 4.17 and Figure 4.18).

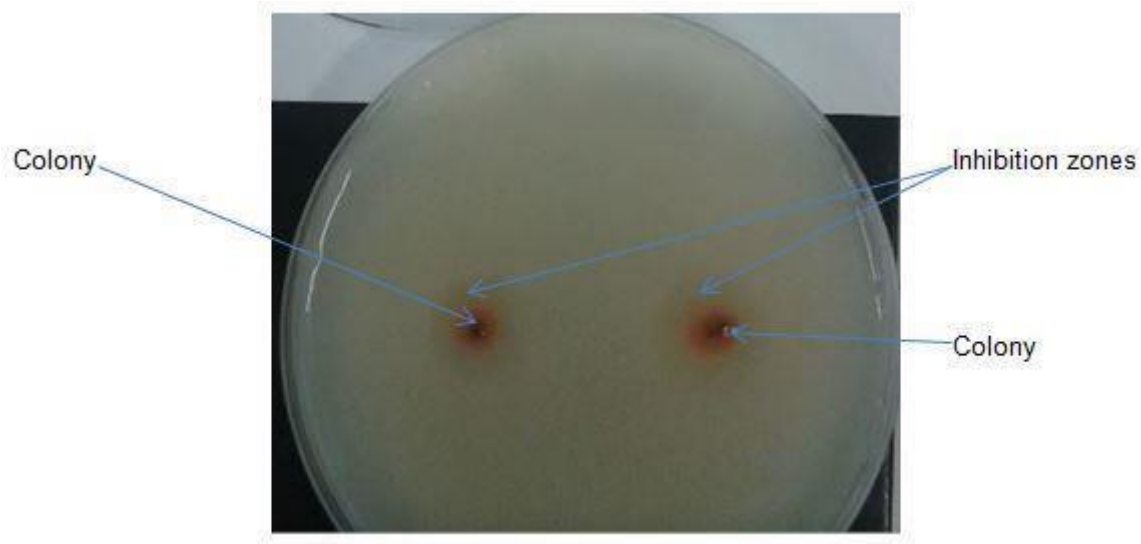


Figure 4.17: An SGG agar plate for strain 02-251#253 showing two zones of inhibition around the bacterial colonies after stab inoculation, incubation and overlaying with *Escherichia coli* ATCC 25922.

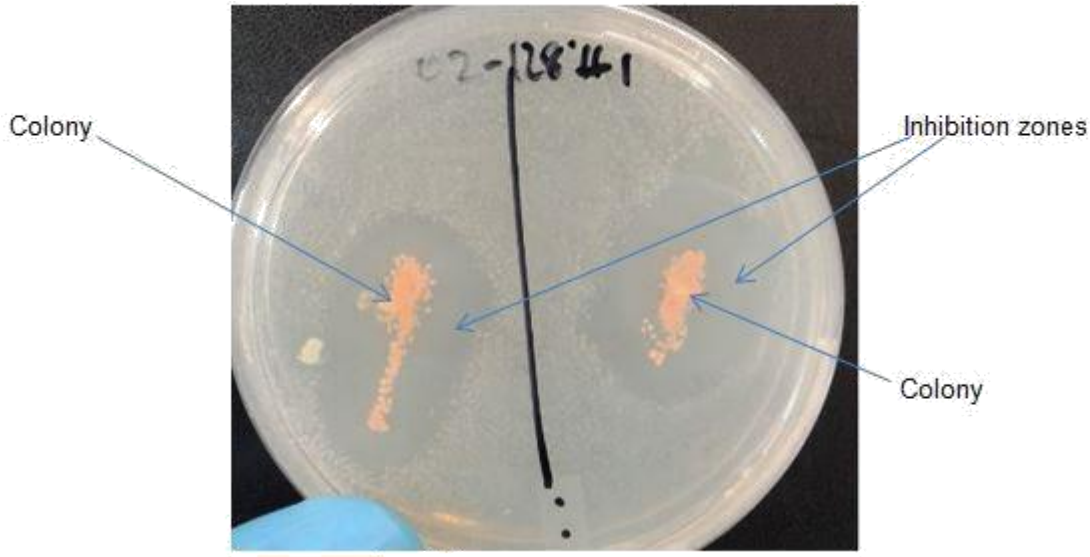


Figure 4.18: A 172 F agar plate for strain 02-128*#1 showing the zones of inhibition and bacterial colonies after stab inoculation, incubation and overlaying with *E. coli* ATCC 25922.

The results obtained for the different solid media overlay studies and the three test strains, are summarised in Tables 4.6-4.8. Notably, only two strains (04-015#16 and 02-118#8) showed activity against the Gram-negative *E. coli* ATCC 25922 strain only. Five strains (02-138#3, 02-221#16, 02-138#3, 02-139#18 and 02-251#322) only showed activity against the Gram-positive *Bacillus cereus* ATCC 10876 strain, while strain 02-118#5 is unique in that it only showed activity against *Candida albicans* ATCC 24433.

Most importantly, four strains (02-128*#3, 02-251*#36, 02-139#18 and 02-251#274) depicted activity against all three test strains, thus Gram-positive and -negative bacteria as well as a yeast. Overall, strains 02-128*#1 (against *E. coli* ATCC 25922) and 02-128*#3 (against *B. cereus* ATCC 10876) showed the highest activity and this was both in media with and without artificial seawater (ASW), respectively.

Table 4.6 shows those *Micromonospora* strains that showed activity against the *E. coli* ATCC 25922 strain. Out of the 30 *Micromonospora* strains under investigation, twelve strains showed bioactivity against *E. coli* ATCC 25922. The range of activity observed was from weak to moderate activity. Strain 02-128*#1 showed moderate activity whilst the rest of the strains depicted weak antibacterial activity against the ATCC 25922 strain.

The findings of the antibacterial activity of our *Micromonospora* strains against *B. cereus* ATCC 10876 are shown in Tables 4.7a and 4.7b. Out of the 30 strains under study, a total of twelve were recorded as having some form of activity against *B. cereus.* Out of the twenty-eight *Micromonospora* colonies that showed some form of activity, twelve showed presence of aerial mycelium, before overlaying with test strains, on visual inspection.

Table 4.6 Activity of *Micromonospora* strains against *E. coli* ATCC 25922

Strain	Medium	Mycelium	Colony diameter (mm)	Diameter of inhibition zone(mm)	Area of inhibition zone (mm^2)
02-128*#1	172F With ASW	Present	15,5	41	1130,99
04-015#16	172F With ASW	Present	6	30,5	701,99
02-209*#6	172F	Present	13,5	32,5	686,09
02-128*#3	SGG	Present	5,5	25,5	486,70
02-251*#36	172F With ASW	Present	4,5	23,5	417,62
02-251*#36	172F	Present	8,5	24,5	414,48
04-015#16	SGG	Nil	5	23	395,64
02-118#4	172F With ASW	Present	7,5	23,5	389,36
02-139#18	172F With ASW	Present	7	23	376,80
04-015#16	SGG With ASW	Nil	6,5	22	346,77
02-251#274	172F With ASW	Present	6,5	20,5	296,73
02-251#274	172F	Present	9,5	21,5	292,02
02-251#1	172F With ASW	Present	8	19	233,15
02-251#1	172F	Present	8,5	19	226,67
02-128*#1	172F	Nil	11,5	20	210,18
02-118#4	172F	Present	10,5	19	196,84
02-118#8	172F With ASW	Nil	3,5	13,5	133,45

Table 4.7 a Activity of *Micromonospora* strains against *B. cereus* ATCC 10876

Strain	Medium	Mycelium	Colony diameter (mm)	Diameter of inhibition zone(mm)	Area of inhibition zone (mm^2)
02-128*#3	SGG	Nil	4,5	39	1178,09
02-128*#3	172F With ASW	Nil	5,5	34	883,71
02-209#4	SGG With ASW	Nil	5,5	27,5	569,91
02-251#274	172F With ASW	Present	5	26	511,04
02-138#3	SGG With ASW	Nil	6	25,5	482,19
02-221#16	SGG With ASW	Nil	5,5	24	428,41
02-251#1	SGG With ASW	Present	6,5	21	313,02
02-221#16	SGG	Nil	5	20,5	310,27
02-251#253	SGG With ASW	Present	5	20	294,38
02-128*#1	172F With ASW	Nil	3,5	19	273,77
02-138#6	SGG With ASW	Nil	5	17,5	220,78
02-128*#1	172F	Nil	9	18,5	205,08
02-251#253	172F With ASW	Present	5	16,5	194,09
02-139#18	172F	Present	16,5	21	132,47

Table 4.7 b Activity of *Micromonospora* strains against *B. cereus* ATCC 10876

Strain	Medium	Mycelium	Colony diameter (mm)	Diameter of inhibition zone(mm)	Area of inhibition zone (mm^2)
02-209#4	172F With ASW	Nil	4,5	13	116,77
02-128*#3	172F	Nil	5	13	113,04
02-221#16	172F With ASW	Nil	3,5	12,5	113,04
02-251#1	172F With ASW	Nil	5,5	13	108,92
02-251#253	172F	Present	7,5	13,5	98,91
02-138#3	172F With ASW	Present	4,5	12	97,14
02-139#18	SGG With ASW	Present	3	11,5	96,75
02-139#18	SGG	Present	3,5	11,5	94,20
02-251#1	172F	Present	5	11,5	84,19
02-251#322	172F	Present	4	11	82,43
02-251*#36	SGG With ASW	Nil	6	11,5	75,56
02-138#6	172F With ASW	Nil	4	10,5	73,99
02-139#18	172F With ASW	Present	4,5	10,5	70,65
02-251*#36	172F	Nil	6,5	8,5	23,55

Table 4.8 Activity of *Micromonospora* strains against *C. albicans* ATCC 24433

Strain	Medium	Mycelium	Colony diameter (mm)	Diameter of inhibition zone (mm)	Area of inhibition zone (mm^2)
02-128*#3	SGG With ASW	Nil	4,5	24	436,26
02-251#253	172F	Nil	3	19,5	291,43
02-251#274	172F	Nil	3,5	19	273,77
02-128*#3	172F	Nil	4,5	19	267,49
02-209#4	SGG	Nil	9,5	18,5	197,82
02-128*#3	172F With ASW	Nil	4,5	16	185,06
02-118#4	172F	Nil	10,5	18,5	182,12
02-139#18	SGG	Nil	7	16,5	175,25
02-139#9	172F	Nil	3	14,5	157,98
02-128*#3	SGG	Nil	5	14	134,24
02-139#18	172F With ASW	Nil	7,5	15	132,47
02-118#5	172F	Nil	5	11,5	84,19
02-118#4	SGG With ASW	Nil	6,5	11,5	70,65
02-138#6	172F	Nil	3,5	10	68,88
02-251*#36	172F With ASW	Nil	4	9	51,03
02-251#274	SGG	Nil	3,5	8,5	47,10

4.12 Bioautography

For this part of the work, the strains were cultured in 172F liquid media. Uninoculated sterile media was used throughout as a negative control. Figure 4.19 shows a typical image of the TLC plate upon which bioautography against *E. coli* ATCC 25922 was performed. Figure 4.20 shows a heatmap chart, which is a representation of an actual TLC plate (Fig 4.20), for the bioautography against *C. albicans* ATCC 24433.

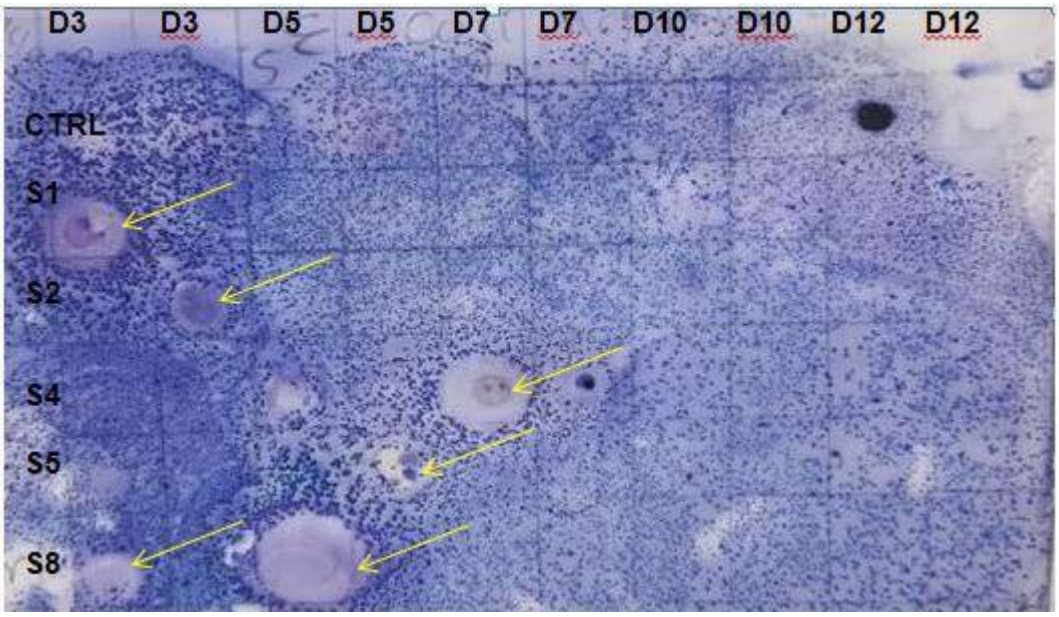


Figure 4.19: A TLC plate showing the bioautography profiles of the five *Micromonospora* strains under study against *E.coli* ATCC 25922. The *Micromonospora* extracts used in bioautography had been obtained from liquid cultures cultivated over a 12-day period.

KEY: D3- Extracts from 3-day incubation; **D5**- Extracts from 5-day incubation; **D7**-Extracts from 7-day incubation; **D10**- Extracts from 10-day incubation; **D12**- Extracts from 12-day incubation.

S1: 02-128*#1; **S2**: 04-015#16; **S4**: 02-128*#3; **S5**: 02-251*#36; **S8**: 02-209#4; **Ctrl**: Media Control; **Yellow arrows:** Typical areas where antibiotic compounds kill test bacteria.

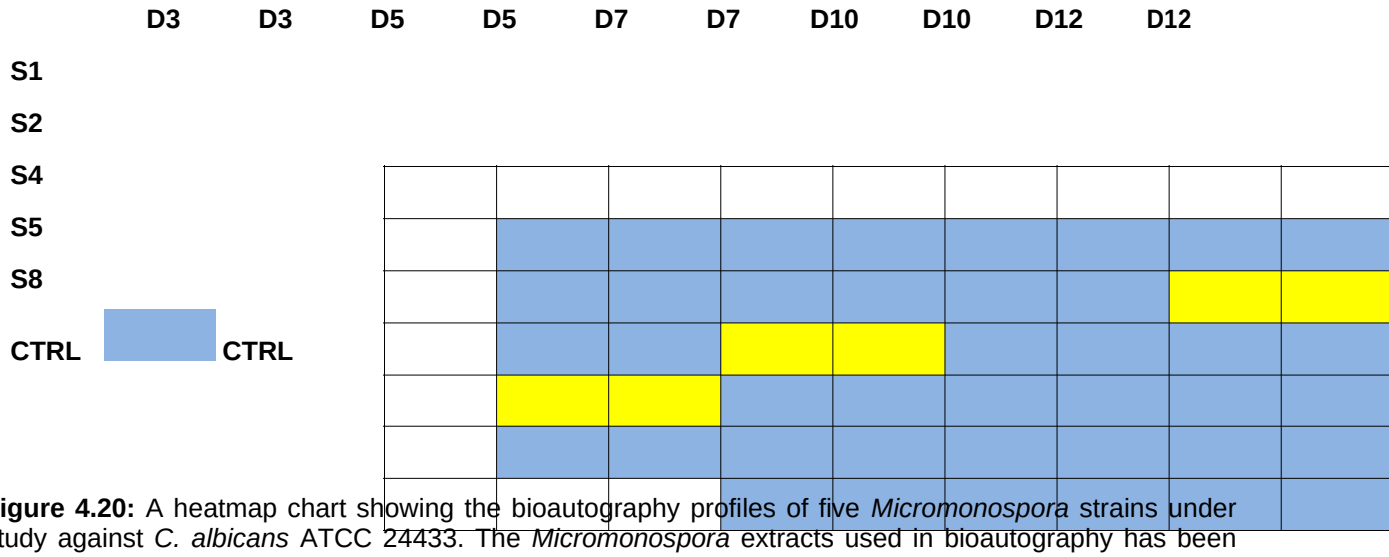


Figure 4.20: A heatmap chart showing the bioautography profiles of five *Micromonospora* strains under study against *C. albicans* ATCC 24433. The *Micromonospora* extracts used in bioautography has been obtained from liquid cultures cultivated over a 12-day period.

KEY: D3- Extracts from 3-day incubation; **D5**- Extracts from 5-day incubation; **D7**-Extracts from 7-day incubation; **D10**- Extracts from 10-day incubation; **D12**- Extracts from 12-day incubation.

White spots on the TLC plate signifying typical areas where antibiotic compounds kill test bacteria.

Purple spots on the TLC plate.

S1: 02-128*#1; **S2**: 04-015#16; **S4**: 02-128*#3; **S5**: 02-251*#36; **S8**: 02-209#4; **Ctrl**: Media Control

To determine whether the type of antibiotic extraction method plays a role in the bioactivity observed (Figures 4.21 and 4.22), strains were cultivated for their optimal time periods as determined from results reported above. Optimal cultivation periods were specific to the test strains used in the experiment above. For bioactivity against *C. albicans* ATCC 24433, strains 1 and 8 were both cultivated for 12 days. Strains 2, 4 and 5 were cultivated for 10 days, 5 days and 3 days, respectively. For bioactivity against *E. coli* ATCC 25922, strains 1, 2 and 8 were cultivated for 3 days and strains 4 and 5 were cultivated for 7 days and 5 days respectively.

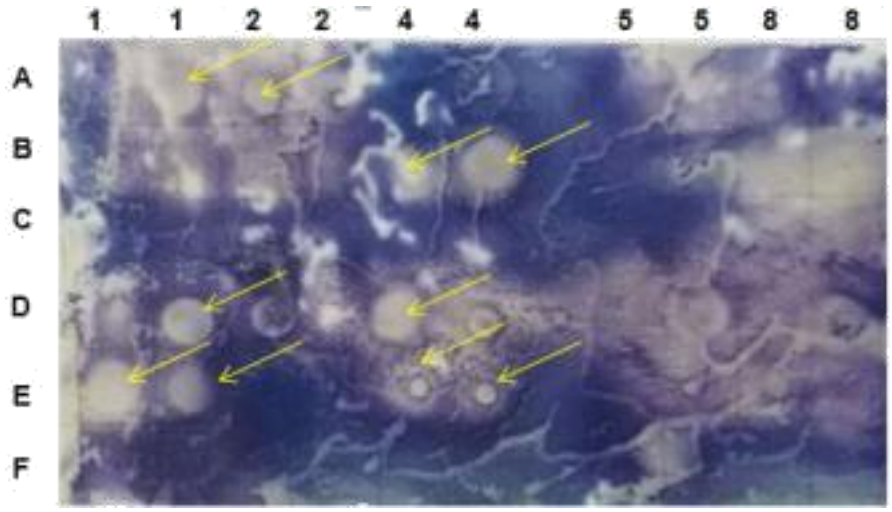


Figure 4.21: A TLC plate showing the bioautography profiles of five *Micromonospora* strains under study against *C. albicans* ATCC 24433. The bioautography profiles of the strains represent activities of extracts obtained by different extraction techniques.

KEY: A-Extracts from whole-cell extraction; **B**- DIAION treatment extracts; **C**-Cell extracts; **D**-Culture filtrate extracts; **E**-Extracts after dialysis; **F**-Media control

1: 02-128*#1; **2**: 04-015#16; **4**: 02-128*#3; **5**: 02-251*#36; **8**: 02-209#4; **Yellow arrows:** Typical areas where antibiotic compounds kill test bacteria.

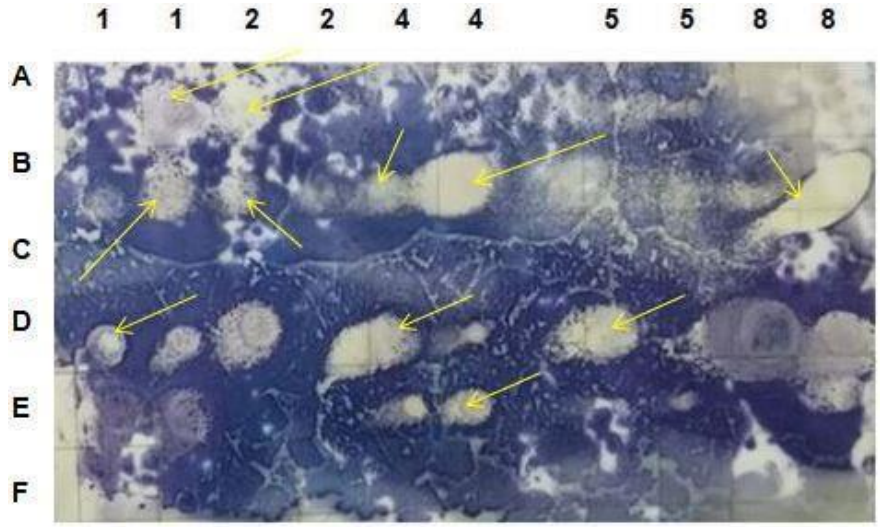


Figure 4.22: A TLC plate showing the bioautography profiles of five *Micromonospora* strains under study against *E. coli* ATCC 25922. The bioautography profiles shown are of the strains that were isolated using extracts obtained by different extraction techniques.

KEY: A-Extracts from whole cell extraction; **B**- DIAION treatment extracts; **C**-Cell extracts; **D**-Culture filtrate extracts; **E**-Extracts after dialysis; **F**-Media control

1: 02-128*#1; **2**: 04-015#16; **4**: 02-128*#3; **5**: 02-251*#36; **8**: 02-209#4; **Yellow arrows:** Typical areas where antibiotic compounds kill test bacteria.

It is important to note that after performing bioactivity analysis using multiple extraction techniques, the filtration technique, particularly the filtrates, was observed to give the best activity results.

This Chapter focused on presenting all the results obtained in this study comprehensively. It is important to interrogate these findings in a manner that seeks to make reference to what has been reported in other studies on the current subject matter. Chapter 5, therefore, seeks to fulfil this aim and in the process possibly singling out new insights that can be highlighted from the study, as well as providing scientific arguments from which conclusions will be drawn in Chapter 6.

CHAPTER 5

DISCUSSION OF FINDINGS

5.1 Introduction

The search for novel antibiotics from various bioactive microbes has become a topical research area in recent times, in the wake of the scourge of drug resistant infections such as multi-drug resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) amongst many others (Carro *et al.*, 2019). Research interest around the biosynthetic potential of the Gram-positive, filamentous, spore-producing microorganisms of the family *Micromonosporaceae* has grown of late due to their known biosynthetic potential. For instance, it is an incontrovertible assertion from many sources that after streptomycetes, *Micromonosporaceae* portray the greatest specialised bioactivity amongst the Actinobacteria (Habbu *et al.,* 2016; Penesyan *et al.,* 2015; Zhang *et al.,* 2005). This has led to the phenomenal increase in the description of novel species of the genus *Micromonospora* from as little as 14 in 2000 to 84 at the time of writing (http://www.bacterio.net/micromonospora.html; Carro *et al.*, 2012; Kasai *et al.*, 2000; Parte, 2018).

The family *Micromonosporaceae* falls under the phylum Actinobacteria and its type genus, as proposed by Ørskov, is *Micromonospora* (Anzai *et al*., 2012; Carro *et al.*, 2012; Everest & Meyers, 2013; Igarashi *et al*., 2011). Microorganisms of this genus are diversely distributed over a wide range of natural habitats on terrestrial environments and marine environments. The last half decade has seen the isolation and description of over 20 new *Micromonospora* species, the majority having been isolated from mangrove environments (Wang *et al.*, 2019). The mangrove environments are characteristic of abnormally elevated levels of saline, moisture, wind as well as osmotic pressures (Jiang *et al*., 2013). Such strenuous and harsh environments have a strong influence on how the microbial population habitant in these areas evolves for the purposes of adapting ecologically. This quest for environmental adaptability is the reason why the metabolic pathways of *Micromonosporaceae* progressively evolve to unique sophistication levels (Jiang *et al.*, Xu, 2011). Consequently, *Micromonosporaceae* have been well documented as producers of diverse and unique novel bioactive metabolites (Wang *et al*., 2019).

Examples of commercially available antibiotic compounds that justify *Micromonosporaceae* as biotechnologically important microorganisms include the aminoglycoside antibiotics: sisomicin, gentamicin and sagamicin developed from *Micromonospora inyonensis*, *Micromonospora echinospora* (formerly *Micromonospora purpurea*) and *Micromonospora sagamiensis* respectively (Kasai *et al*., 2000; Kyeremeh *et al.*, 2014; Weinstein *et al.*, 1963; Weinstein *et al*.,

1970). *Micromonospora griseorubida* and *Micromonospora nigra* are also noted in literature as sources of two macrolide antibiotics mycinamicin and megalomicin, respectively (Carro *et al.*, 2018). Other examples of antibiotics from *Micromonosporaceae* include calicheamicin, netamicin and telomycin (Zhao *et al.*, 2017).

Recently, novel marine-sourced bioactive metabolites that have been isolated from *Micromonosporaceae* include the compound rifamycin S, produced by *Micromonospora rifamycinica* AM105, which shows efficacy against methicillin-resistant *Staphylococcus aureus* (MRSA) strains. Another example is butremycin, which is sourced from *Micromonospora* sp. k310 and potent against *S. aureus* (Wang *et al.*, 2019). Besides antibiotic compounds, *Micromonosporaceae* are also reputable sources of anti-tumour compounds such as lupinacidins A and B from *Micromonospora lupini* (Hirsch & Valdes, 2010). This chapter therefore aims to give an interpretation and evaluation of the results obtained in this study, in light of what is known in this particular field of research as well as highlighting any new insights.

5.2 Solid and liquid culturing and Gram staining

One of the main objectives of this study was to perform a molecular screen of the marine *Micromonosporaceae* strains present in the BTB culture collection. This, therefore, meant that laboratory evidence of the existence of viable *Micromonosporaceae* strains in the BTB culture collection would be the starting point of the study. Thereafter, performing the Gram stain on the bacterial strains under study served as an important indicator with regards to their broad Gram-based categorisation and analysis of culture purity. Consequently, the strains under study were cultured and examined on both solid and in liquid media for typical growth patterns and morphological characteristics consistent with the genus *Micromonospora* before performing a standard Gram stain.

5.2.1 Solid and liquid media culturing

On solid media, particularly SGG with artificial seawater (ASW) and 172F with ASW, the strains presented as bright orange colonies and in some cases with black spore mass upon maturation. In liquid cultures, the colonies ranged from bright orange pellets to typically brownish, reddish and black pellets similar to patterns reported by Kroppenstedt *et al.* (2005). Some solid agar media plates also showed these range of colony colours. This different range of colony colours can be best explained by the different sporulation stages that *Micromonosporaceae* species undergo upon culturing (Ichiwaki *et al*., 2017). As also highlighted by Trujillo *et al*. (2010), *Micromonospora* colonies in our study generally became deeper in colour as incubation prolonged due to progressive spore production.

However, it is important to highlight that the identification of *Micromonosporaceae* species based on colony morphology only is insufficient and inadequate. This is because upon culturing, these microorganisms are capable of forming indistinct colony patterns that mimic those of microorganisms of the order *Actinomycetales*. However, given the fact that the strains were stored as frozen stock cultures at -80^{o}C prior to commencement of this study, there was undoubtedly sufficient laboratory evidence of viable strains, as shown in Figure 4.1. The fundamental question being, were these strains indeed from *Micromonosporaceae* species as suggested by the title of our study?

In a molecular-based study such as this one, the need for extraction of a good quality DNA yield from bacterial cells can never be overemphasised. Henceforth, as a starting point, it was important to have convincing growth of the strains in liquid cultures. Microorganisms of the genus *Micromonospora* and other genera in the family *Micromonosporaceae* are noted in literature as generally slow growers (Carro *et al*., 2012; Cross, 1981; Kirby & Meyers, 2010). Although this corroborated with what was observed in our study, it goes without doubt that the quantity of growth (at least 0.5 ml cell mass per strain) we observed in liquid cultures was going to be sufficient in yielding a sufficient genomic DNA quantity upon extraction. In fact, it was observed that incubating the cultures for 2-3 days longer than the prescribed timeframes for both solid and liquid media under the same conditions improved yield. These observations can also be explained by the bacterial growth curve shown in Figure 5.1.

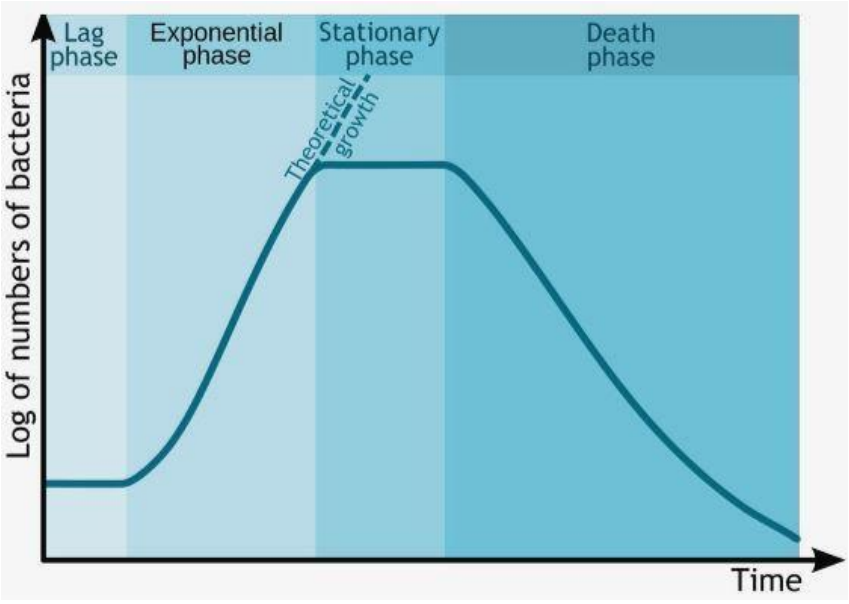


Figure 5.1: A bacterial growth curve showing the four phases of bacterial growth within colonies (Rolfe *et al.*, 2012). The growth curve typically outlines the progression of growth of bacterial colonies in culture media as incubation time progresses.

It was generally observed that under optimal culture conditions, the strains followed a typical growth curve pattern. The first 2-3 days of incubation of liquid cultures did not result in any noticeable macroscopic changes in terms of both colony count and discolouration of the liquid media. This would be the lag phase of the liquid cultures. As the incubation period progressed over 4-10 days, distinct numerous large orange pellets rapidly increased in the liquid cultures. This phase is the exponential phase shown in Figure 5.1. It is characteristic of rapid metabolism and exponential consumption of culture media nutrients by cultured bacteria, thus leading to the macroscopically visible increase of colonies and discolouration of the agar media from clear to brownish-orange (Rolfe *et al*., 2012).

Employing a slight increment in incubation period to 12-13 days ensured an even better DNA yield since this equated to the stretching of the exponential phase to full capacity to ensure a comprehensive cell mass yield. The death phase on the curve signifies a period of limited nutrients due to increased competition for survival as growth diminishes gradually to a halt. However, the growth of strains in liquid media cultures was sufficient (more than 0.5 ml cell mass could be harvested) for successful DNA extraction.

5.2.2 Gram stain

It is noted in literature that microorganisms of the genus *Micromonospora*, being members of the order *Actinomycetales,* are Gram-positive in nature (Chaudhary *et al.*, 2013; Sharma *et al.*, 2014). Further to this, *Micromonosporaceae* are known to exhibit numerous filaments and spores upon observing their Gram stain as highlighted by Das *et al*. (2008). It was, therefore, imperative in this study to perform the Gram stain to observe whether the outcome was consistent with literature. In fact, the classification of microorganisms is a multifaceted process that is better and more accurately done by considering both genotypic and phenotypic traits, thus polyphasic taxonomy (Chuny & Rainey, 2014).

The Gram stain also served as a technique to ascertain the purity of the liquid cultures in this study, as mentioned earlier. In the event that two *Micromonospora* strains co-existed in a single colony, this would be picked up at 16S rRNA gene sequence analysis, whereby chromatograms generated as sequencing output would reveal a lot of background sequencing noise. Consistent with the Gram stain findings of *Micromonospora cremea* sp. nov. and *Micromonospora zamorensis* sp. nov. as described by Carro *et al.* (2012), all 30 strains in this study were observed to be Gram-positive. Some of the isolates such as strain 02-251#1 (Figure 4.2 B) showed spores under oil immersion (100x magnification), while many isolates such as 02-203#1 showed numerous filaments with isolated spores (Figure 4.2 A).

The visualisation of Gram-positive isolates with multiple filaments and spores was consistent with what other studies have reported (Carro *et al.*, 2012; Everest & Meyers, 2013; Hisch & Valdes, 2009; Kirby & Le Roes-Hill, 2009). However, the Gram stain as a sole technique is not adequate and conclusive in conferring any identity to a particular genus of microorganisms such as *Micromonospora.* Other supporting confirmatory tests that could be carried out include determining whether the isolates have the characteristic DAP isomer (*meso*-diaminopimelic acid) and whole-cell sugars (xylose and arabinose) for the genus (Thawai *et al*., 2018). Further to this, the *Micromonospora* strains could have also been characterised by physiological and biochemical methods as described by Chantongcome *et al*. (2009). These approaches were, however, not pursued for strain characterisation in this study. This is because these chemotaxonomic methods are outdated and the newer DNA-based methods, such as 16S rRNA gene sequencing and MLSA, are superior and have better strain resolution.

5.3 Verification and quantification of isolated DNA

5.3.1 Verification of isolated DNA

After successful culturing of the strains under study in liquid cultures, DNA isolation was performed as outlined in section 3.4.1 using the method described by Mandel and Marmur (1968). Two factors that were going to be of paramount importance in the study are the quality and quantity of the extracted DNA from the *Micromonospora* strains (Weber *et al.*, 2017). In a molecular based research study involving extraction of DNA from bacterial cells, the ability to separate DNA fragments is of uttermost importance. Sambrook and Russell (2001) highlighted agarose gel electrophoresis (AGE) as the most efficient laboratory technique to achieve such separation. Lee *et al.* (2012) corroborated with this notion and further highlighted that DNA fragments of up to 25kb are separable by AGE.

Figure 4.3 shows an agarose gel picture of genomic DNA extracted from *Micromonospora* strains 1 to 15 under UV light. With the exception of strains 10 (02-221#16) and 15 (04-044RT1), all other lanes on this gel show successful DNA extraction as depicted by the distinct brightly illuminated genomic-DNA (gDNA) bands under UV light. The illumination of these gDNA bands under UV is due to the intercalation of ethidium bromide in the gel.

Strain 11 (02-251#253) showed a smeared band on agarose gel under UV light. This does not entirely signify failure in extraction, but rather extracted DNA of poor quality. Such smearing can be caused by various shortcomings, chief amongst them degradation of gDNA by nucleases, elevated salt concentrations within samples and excess sample to loading dye ratio (Kirkpatrick, 1991). From the gel shown in Figure 4.3, it meant that strains 10, 11 and 15 had to undergo

gDNA extraction again. It was, however, also an option to re-run the same DNA samples for strain 11 in order to figure out whether the reason for smearing on agarose was pre-extraction or post-extraction related.

The gel picture in Figure 4.4 shows the gDNA of strains 16 to 30 under UV light after electrophoresis. Strains 26 and 27 also showed smeared DNA product and had their DNA re-extracted. After performing repeat DNA extractions on samples 10, 11, 15, 26 and 27, an agarose gel picture was run and captured, as shown in Figure 4.5. In this gel picture, all 5 strains showed distinct DNA bands under UV light signifying successful DNA extraction. It is however notable that strain 27 still gave a faint band on agarose, likely due to a possible low DNA concentration.

5.3.2 Quantification of isolated genomic DNA

Quantification of the extracted gDNA was performed using the Genova Life Science spectrophotometer and the results are shown in Tables 4.1 (original extraction) and 4.2 (re-extractions). On this particular instrument, an extracted DNA quantity of at least 20 µg/ml is regarded as sufficient for further use in PCRs (Bibby Scientific, 2017). As shown in Table 4.1, the DNA quantities for the majority of the strains were above the required 20 µg/ml level. The notable exceptions were strains 10, 11, 15, 26 and 27. DNA quantities of samples 10 and 15 corroborate with the faint DNA bands, as shown on the agarose gel in Figure 4.3. Samples 11, 26 and 27 did not necessarily have low DNA quantities as also shown in Table 4.2; instead, their DNA smeared on electrophoresis hence the decision to re-isolate the gDNA.

Figure 4.5 shows successful DNA extraction as signified by the brightly illuminated distinct bands on agarose, which corroborates with DNA quantities shown in Table 4.2. Sample 27's DNA quantity of 22.40 µg/ml, was deemed sufficient for further analysis as per the manufacturer's instructions (Bibby Scientific, 2017).

5.4 16 S rRNA gene analysis

The objectives of this research study were based on the precept that the microorganisms under study were of the genus *Micromonospora*, family *Micromonosporaceae.* Despite partly elucidating the morphological characteristics of the strains under study, the morphological features observed under a light microscope in section 5.2.2 do not provide conclusive evidence of the genus's identity of the microorganisms. Therefore, it became imperative to amplify the 16S rRNA gene for all the strains and analyse the sequences thereof.

5.4.1 16S rRNA gene sequencing

The study of phylogeny and taxonomy in bacterial species is a process in which the use of the 16S rRNA gene sequencing is of uttermost importance (Carro *et al*., 2017; Case *et al.*, 2006; Janda & Abbot, 2007). The importance of the 16S rRNA gene is centred upon the fact that it contains hypervariable regions that are species-specific, hence important in the identification of bacterial strains. The 16S rRNA gene is, therefore, well conserved. The primer pair used for gene sequencing of the 16S rRNA gene ought to be universal in nature to enable them to match the highly conserved regions of the gene (Janda & Abbot, 2007).

Fundamentally, it is important to note that the entire technique of 16S rRNA gene sequencing and analysis finds its basis in the detection of polymorphisms within the 16S rRNA genes of closely related species. The focal points of such detection within the 16S rRNA genes are the hypervariable regions (Carro *et al.*, 2017). The use of the universal F1/R5 16S rRNA primers described by Cook and Meyers (2003) allowed us to be able to successfully amplify the near full length 16S rRNA genes of the 30 strains under study and identify the genera to which they belonged.

Figure 4.6 shows an agarose gel image for the 16S rRNA gene amplification for strains 1 to 22, as well as the Fast DNA ladders. All strains show positive 16S rRNA PCR product as signified by the illuminating DNA bands on the gel. Using the FAST DNA ladder as a fragment size reference, the established DNA bands for samples 1 to 22 were estimated to be 1400 bp (Thanaboripat *et al*., 2015). Kirby and Le Roes-Hill (2009) highlighted that a sequence of at least 1350 bp is sufficient when describing novel species. This meant that, depending on the percentage similarity of the strains under study in comparison to published strains, strain novelty was a possibility.

Figure 4.7 shows an agarose gel image for samples 18-30. All strains on this agarose gel image showed a positive 16S rRNA PCR product which also had an estimated band size of 1400 bp as referenced to the FAST DNA ladder shown in Figure 4.8. The next important stage after amplifying the 16S rRNA gene was to sequence the gene for all strains, and analyse the sequences generated.

5.4.2 16S rRNA gene sequence output analysis

A vast amount of inferences can be made from sequence data derived from the sequencing of the 16S rRNA gene. Many credible bioinformatics tools have been used by taxonomists to objectively identify novel bacterial species based on the 16S rRNA gene (Chun *et al.*, 2007). However, in this study, we used the EzTaxon gene database as the preferred database to

analyse the 16S rRNA gene sequences of the strains under study. The choice of EzTaxon over common public databases such as Genbank, was based on the fact that EzTaxon only contains sequence data that is peer-reviewed for type strains, thus providing more reliable filtered sequences (Clayton *et al.*, 1995; Mellmann, 2003).

As shown in Table 4.3, 28 of the 30 strains (93.3%) in our study were found to belong to the genus *Micromonospora*. Two of the 30 strains (6.7%) under study were found to belong to the *Jishengella* genus. However, it should be noted that the genus *Jishengella* belongs to the family *Micromonosporaceae* and the morphological features of this genus resembles that of *Micromonospora* (Thawai *et al.*, 2018; Xie *et al.,* 2011). This, therefore, validated the culture-based morphological findings and the Gram stain findings in sections 5.2.1 and 5.2.2 respectively, which pointed out to typical *Micromonosporaceae* characteristics amongst all the strains under study. Consequently, all 30 strains in our study were found to belong to the family *Micromonosporaceae* according to sequence data generated from 16S rRNA gene sequencing and analysed by EzTaxon.

The closest published strains similar to the strains under study are also shown in Table 4.3. From the table, it can be observed that the similarity of the 16S rRNA sequences between our strains and their established closest known counterparts ranges between 97.19% and 100%. The highest similarity of 100% was seen between 15 of the strains (02-138#6, 02-128*****#3, 02-209#4, 02-138#3, 02-251#1, 02-221#16, 03-013#19, 02-128*****#1, 02-118#8, 02-209#2, 02-221#26, 02-251#16, 02-251#274, 02-251*#36, 04-015#16) and the strain *Micromonospora aurantiaca* ATCC 27029^{T}. The 16S rRNA gene sequence of one strain under study (02-139#18) was also found to have a 100% similarity with that of the strain *Micromonospora tulbaghiae* DSM 45142^{T}. The strain under study that showed the lowest match of its 16S rRNA gene sequence with that of its closest match (*Jishengella endophytica* 202201) was 02-251#136 with a match of 97.19% and may represent a novel species.

The description and reporting of any species, as novel, requires a particular 16S rRNA gene sequence of a strain to be at least less than 97.5% similar to its closest neighbour in a sequence alignment (Kirby & Le Roes-Hill, 2009). A gene sequence-similarity value above the 97.5% cut-off would require the performance of DNA-DNA hybridisation (DDH) and/or genome sequencing before a species is concluded to be novel. Only five of the 30 *Micromonosporaceae* strains shown in Table 4.3 were included in a phylogenetic tree, together with validly published type strains of the genus, as described in section 3.6, mainly because these strains served as the basis for extended antimicrobial activity testing.

It is notable that all the strains in Table 4.3, except strain 02-251#136, depict gene sequence-similarity values over the 97.5% cut-off for novel sequence description. This implies that only strain 02-251#136 would qualify as a candidate for novelty designation without necessarily undergoing DDH and/or genome sequencing (Kirby & Le Roes-Hill, 2009). However, the uniqueness of a particular strain cannot be solely argued around the basis of its 16S rRNA gene sequence being less than 97.5% similar to its closest phylogenetic neighbour. The uniqueness should be beyond reasonable doubt phenotypically, genotypically and biochemically, hence polyphasic-based novelty.

It can, therefore, be noted that 16S rRNA gene sequencing and analysis becomes handicapped at the species level (Yoon *et al*., 2017). In other words, we cannot use the information in Table 4.3 as exclusive evidence of gene sequence-similarity with existing type strains even in cases whereby their 16S rRNA gene sequences have a 100% match. The same can be said about decisions on strain novelty based on 16S rRNA gene sequence similarities with closest phylogenetic neighbours. Therefore, the use of 16S rRNA sequence-sequence comparisons is merely a way of identifying bacterial strains that fall into similar genera without necessarily giving information of their interspecies relatedness (Kim, 2014; Quast *et al*., 2013)

Table 4.3 also shows a column where sequence lengths for all the strains' 16S rRNA gene were recorded. The sequence lengths ranged from 1294 bp to 1368 bp with a calculated median of 1353 bp. On average, the sequence length of the 16S rRNA gene is 1400 bp (Ichiwaki, 2017). The range of the 16S rRNA sequences obtained in our study is close to the known average. It is important to note that this slight deviation is possibly encountered during editing of sequences in Chromas Lite (Version 2.6) programme (Technelysium), post-sequencing. The sequences tend to get slightly shorter due to base eliminations in cases of ambiguity and mismatch, and this is carried over to sequence assembly.

Micromonosporaceae are known to have a high GC content in their genomes (Atlas, 1997; Ventura *et al*., 2007). A high genomic GC content would typically be 60% and above (Doroghazi & Metcalf, 2013). Table 4.3 shows that for all our strains, the GC content of the sequenced 16S rRNA gene was slightly above 60% with an observed variation within the 0% - 0.5% range. It should, however, be noted that the high GC content that is referenced in literature as a typical characteristic of the *Micromonosporaceae* family, is total genomic GC content as opposed to 16S rRNA GC content. This is because the 16S rRNA, being a highly conserved gene, has a GC content that remains fairly constant in terms of size, a concept which corroborates with our findings as shown in Table 4.3. The GC content of strains in our study suggests that all of them

had a high GC content, consistent with what is known about the *Micromonospora* genus (Carro *et al.*, 2012; Maldonado & Quintana, 2015; Trujilo *et al*., 2014).

5.5 Phylogenomic analysis

After establishing the genus that our 30 strains were classified under, five of the strains that were chosen for further study based on their bioactivity had selected sequences analysed through the use of phylogenetic trees to explore their evolutionary relatedness. This was achieved by constructing phylogenetic trees based on 16S rRNA gene sequences and the genes sequences targeted in the MLSA study, outcome of which is discussed in this section.

5.5.1 16S rRNA phylogeny

16S rRNA gene sequencing performed in this study aided in identifying the strains under study to genus level. In order to get better insight into the evolutionary relatedness of the five *Micromonospora* strains selected for further study and type strains of the *Micromonospora* genus, the 16S rRNA sequences were incorporated into a Neighbour-joining phylogeny tree presented in Figure 4.10. This tree is supported by the maximum likelihood and minimum evolution tree algorithms presented in Annexure 1. From the phylogenetic tree, it was observed that all five strains were deduced to be closely related to the strain *Micromonospora aurantiaca* ATCC 27029^T.

5.5.1.1 The significance of *Micromonospora aurantiaca*

The representative genome for the strain *M. aurantiaca,* as deposited into GenBank is that of its type strain *M. aurantiaca* ATCC 27029^T. Six other strains of this species have also been described and these are *M. aurantiaca* 110B, *M. aurantiaca* DSM 45487, *M. aurantiaca* L5, *M. aurantiaca* NRRL B-2673, *M. aurantiaca* RV43 and *M. aurantiaca* WMMB 235 (Wang *et al*., 2019).

The strain *M. aurantiaca* 110B was isolated by Wang and colleagues from China's Fujian province (Wang *et al.*, 2019). From this strain, three novel glycosides were structurally elucidated post isolation before investigating their bioactivity against the hepatocarcinoma causing HepG2 cell line, the lung tumour-causing A549 cell line and the colon tumour cell line, HCT116 (ibid). Further to this, the bioactivity of the compounds against the test strains *C. albicans,* MRSA and *E. coli* was investigated. Although average cytotoxic activity was reported for the three compounds, no significant bioactivity was reported against the bacterial test strains and fungi. In this study, antiSMASH analysis was performed for the genomes of the six published *M. aurantiaca* strains (Table 5.1).

Fourteen gene cluster types were observed in the antiSMASH results amongst all the *M. aurantiaca* strains as shown in Table 5.1. The predicted BGCs were found to be similar amongst all strains except the arylpolyene, transAT PKS-like and Betalactone BGCs that were predicted only in some strains. This information would be handy in consideration of gaps in primer knowledge in the BTB culture collection, whereby the choice for targets for primer design would be amongst those BGCs found within all strains. However, the issue of relevancy of the chosen BGC as determined by literature would also be a factor in this regard.

Table 5.1 Comparison of BGCs predicted by antiSMASH in the different strains of *M. aurantiaca*

BGCs predicted	*M. aurantiaca* strains					
	110B (2018)	**DSM 45487**	**L5**	**NRRL B-2673**	**RV43**	**WMMB 235**
NRPS	✓	✓	✓	✓	✓	✓
T3PKS	✓	✓	✓	✓	✓	✓
Terpene	✓	✓	✓	✓	✓	✓
Lanthipeptide	✓	✓	✓	✓	✓	✓
Siderophore	✓	✓	✓	✓	✓	✓
Oligosaccharide	✓	✓	✓	✓	✓	✓
TransAT-PKS-like	**X**	**x**	**X**	✓	✓	**x**
NAGGN	✓	✓	✓	✓	✓	✓
T2PKS	✓	✓	✓	✓	✓	✓
T1PKS	✓	✓	✓	✓	✓	✓
PKS-like	✓	✓	✓	✓	✓	✓
Bacteriocin	✓	✓	✓	✓	✓	✓
Arylpolyene	**x**	**x**	**X**	**X**	✓	**x**
TransAT-PKS	✓	✓	✓	✓	✓	✓
Betalactone	✓	✓	✓	**X**	**x**	**x**

Key: ✓ BGC predicted within the strain's genome
x BGC not predicted within the strain's genome

The evolutionary relationships established between our five strains and *M. aurantiaca* ATCC 27029^T as per phylogenomic studies warranted the analysis of BGC distribution within the genome of *M. aurantiaca.* antiSMASH analysis of the genome revealed the presence of a diverse array of BGCs spanning over 18 coding regions dominated by gene clusters for terpenes, NRPS, T1PKS, siderophore, bacteriocin and lanthipeptide. This finding gives a good insight into the possible biosynthetic capabilities of the five strains which were found to be closely related to the *M. aurantiaca* ATCC 27029^T strain. Given that limited studies are looking at the antimicrobial compounds produced by this strain, it subsequently means any compound potentially isolated from any of its closely related five *Micromonospora* strains would likely be novel (Carro *et al.*, 2018). Above all, the variation of BGCs observed amongst various strains consequently means that if these *Micromonosporaceae* species are isolated from different marine samples, there is an increased chance of diversity of BGCs that can be accessed.

Local alignment between each of the five *Micromonospora* strains' 16S rRNA gene and that of *M. aurantiaca* ATCC 27029^T was performed in DNAMAN and in all cases sequence similarities between 99% and 100% resulted. This, therefore, meant that it would have been helpful to make use of DNA-DNA hybridisation or genome sequencing in the quest to conclude on the five strains' novelty. Despite not being within the current scope of this study, biochemical tests, particularly physiological characterisation, would also have further elucidated the relationship between each of the five *Micromonospora* strains and their closest evolutionary comparisons; *M. aurantiaca* ATCC 27029^T and *Micromonospora chalcea* DSM 43026^T.

5.5.1.2 The significance of *Micromonospora chalcea*

Micromonospora chalcea is well-known for being the producer of the group of macrolide antibiotics known as juvenimicins as well as everninomicin (Hatano *et al.*, 1976). One of the compounds produced by *M. chalcea*, juvenimicin A_3, was reported to exhibit bioactivity against both Gram-positive and Gram-negative microorganisms, hence broad-spectrum activity (ibid). This was important for our strains as it was a hint that due to similar ancestry, we could possibly observe one or more of our five strains exhibiting broad-spectrum activity similar to *M. chalcea.*

5.5.2 Multi-Locus Sequence Analysis study

In order to zoom into the interspecies relationships between the *Micromonospora* species more effectively, performing MLSA studies of housekeeping genes is recommended (Carro *et al.*, 2018). MLSA analysis is advantageous in that it results in the construction of more robust trees (higher bootstrap values) and better resolution (longer branch lengths). The MLSA in our study was performed as outlined by Carro *et al.* (2012) and the housekeeping genes that were targeted included *recA, atpD, rpoB* and *gyrB.* After optimisation of all PCR reactions, at least the *rpoB* and *gyrB* genes successfully amplified and their Neighbour-joining phylogenetic trees were successfully constructed as given in Figures 4.12 and 4.13, respectively. These are supported by the Maximum likelihood and Minimum evolution tree algorithms presented in Annexure 1. Fundamentally, the successful amplification of the *rpoB* and *gyrB* genes was a positive finding since these two have been reported as useful markers in interspecies delineation within the *Micromonospora* genus (Carro *et al*., 2012). In fact, Hirsch and Valdes (2009) alluded to the fact that because of its higher evolutionary rate than the 16S rRNA gene, the *gyrB* gene is a better option in elucidating interspecies relatedness than the 16 S rRNA gene.

As shown in Figure 4.13, the phylogenetic tree incorporating *gyrB* sequences confirmed the ancestral relationships between strains 02-128*3, 04-015#16, 02-128*1 and 02-251*36 with the type strain *M. aurantiaca* ATCC 27029^T. This is similar to the evolutionary relationships between

these strains and *M. aurantiaca* ATCC 27029^T, established in the 16S rRNA phylogenetic tree presented in Figure 4.10. However, strain 02-209#4 positioned as more closely related to the type strain *Micromonospora echinofusca* 43913^T than *M. aurantiaca* ATCC 27029^T. The difference in the topology of the phylogenetic trees between the gyrB and 16S rRNA sequences is an occurrence that is not unusual and has been reported before (Carro *et al*., 2012; Kirby & Meyers, 2010).

Figure 4.12 shows the phylogenetic tree that was constructed based on *rpoB* sequences. In general, this phylogenetic tree confirms the close evolutionary relationships between all *Micromonospora* strains and *M. aurantiaca* ATCC 27029^T, which corroborates with the evolutionary relationships presented in the 16S rRNA phylogenetic tree. The only exception is strain 02-209#4 which is observed to group more closely with *Micromonospora echinofusca* 43913^T thus confirming the evolutionary relationships also noted in the *gyrB* phylogenetic tree. The concatenated phylogenetic tree presented in Figure 4.11 shows that the five strains which were identical in the 16S rRNA phylogenetic tree begin to differentiate. This observation subsequently means that the inclusion of additional housekeeping genes in phylogenetic analysis has a capability of resolving intra-strain relationships. The tree was more robust, as shown by longer branch lengths.

In order to avoid any ambiguity with regards to the interspecies relatedness of the five strains that appear very closely related from phylogenetic studies, their genetic diversity could be analysed by performing BOX-PCR and amplified rDNA restriction analysis (ARDRA) (Carro *et al.*, 2012). However, these experiments were not pursued in the current study considering the cost, tediousness, as well as the main objectives of this study.

5.6 PCR screening for Biosynthetic Gene Clusters (BGCs)

Another important objective of this study was to perform a molecular screen amongst the *Micromonospora* strains, in order to investigate the presence of any of eight BGCs within their genomes. This was performed to get insights into the biosynthetic potential of the strains under study with reference to common BGCs. The eight BGCs that were screened for are listed in Table 3.4.

All of the *Micromonospora* strains under study, except sample 4 (strain 02-139#9), showed a positive PCR reaction for the Type II PKS BG. The Type II PKS BGC was targeted by the use of the ARO-PKS-F and ARO-PKS-R primer pair as described by Wood *et al.* (2007). Using the FAST DNA ladder demarcations in Figure 4.8 as a point of reference, the band sizes of the amplicons were roughly 600 bp, consistent with what has been reported in other studies (Das &

Khosla 2009; Zhang *et al.*, 2017). Since the 16S rRNA gene for strain 02-139#9 amplified successfully (Figure 4.5), the failure of amplification of its Type II PKS BGC cannot be attributed to PCR inhibitors (Wawrik *et al*., 2005). Similarly, the sufficiency of the strain's DNA yield could not be singled out as a possible hindrance to the gene cluster amplification. This is because the established DNA quantity for strain 02-139#9 was 32.57 µg/ml (Table 4.1), and this had been deemed sufficient for further analysis. Based on the positive screen of the Type II PKS gene in 29 out of 30 strains under investigation, it is important to interpret this finding from a relevancy point of view. A new question therefore arises; being the only BGC that was found to be positive in the molecular screen, what significance does the presence of the Type II PKS BGC have in the broader aim of the study?

5.6.1 The Type II PKS BGC

In broader terms, polyketide synthases (PKSs) are sophisticated enzymes that are involved in the synthetic pathways that yield biologically potent metabolites (Selvin *et al.*, 2016). These enzymes are subsequently coded for by the Type II PKS BGC, which comprises of the ketosynthase-alpha and ketosynthase-beta genes. Khosla *et al.* (1999) defined PKSs as a group of complex enzymes comprising of multiple domains, which are well known for subsequently producing secondary bioactive compounds known as polyketides.

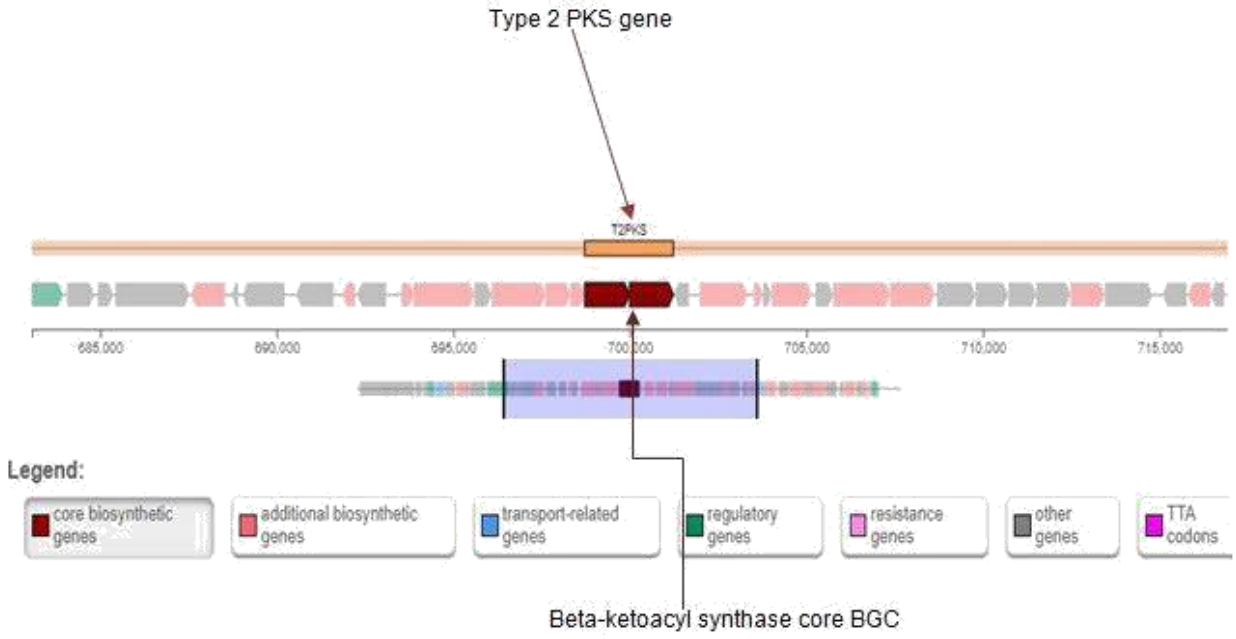


Figure 5.2: An antiSMASH extract showing the genomic region housing the Beta-ketoacyl synthase core biosynthetic genes (locus tag ctg_1 563 and ctg_1 564), which includes ketosynthase alpha and ketosynthase beta genes encoding for the Type II PKS gene in *M. aurantiaca* WMMB 235 ^{T}.

In general, bacterial PKSs exist in three distinct groups that are Type I, Type II and Type III. The Type II PKSs are involved in the biosynthesis of biologically potent compounds such as doxorubicin and tetracycline (Gomez *et al.*, 2013). The biologically potent derivatives of Type II PKS enzymes have a wide range of activity such as antibacterial, antiviral, anti-tumour and enzyme inhibitory activities (Sun *et al*., 2012). In the analysis of the antiSMASH results that were generated for the published *Micromonospora* strains, the Type II BGC was predicted in almost all of the strains that were selected for analysis, an example of which can be seen in Figure 5.2.

Zhang *et al.* (2017) further highlighted the significance of the Type II PKS in the synthesis of bacterial aromatic polyketides such as pentangular polyphenols and anthracyclines amongst many others. The significance of the Type II PKS gene in *Micromonospora* is seen in many examples whereby useful antibiotic compounds have resulted from the secondary metabolites. One good example is the polyketide antibiotic maklamicin, which is produced by *Micromonospora* sp. NBRC 110955, with a chemical structure shown in Figure 5.3 (Igarashi *et al.*, 2011).

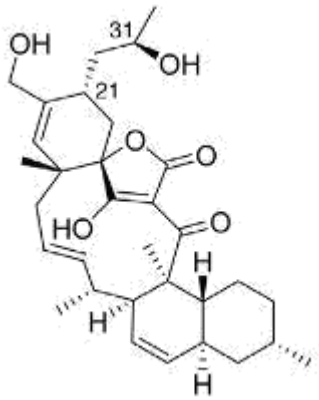


Figure 5.3: A chemical structure of Maklamicin, a spirotetronate antibiotic developed from the actinobacteria *Micromonospora* sp. NBRC 110955. The antibiotic is known to be potent against Gram-positive microorganisms such as *Micrococcus luteus*.

This antibiotic is effective against a vast range of Gram-positive bacteria such as *Micrococcus luteus* (Igarashi *et al.*, 2011). From the bioactivity observed for the five strains against *Bacillus cereus*, it is therefore, possible that this activity observed could be due to the production of Type II PKS compounds. After establishing the relevance of the Type II PKS gene, it can be concluded that 29 of the 30 strains that had a positive screen for this particular gene have great potential of producing other bioactive metabolites, which directly resonates with the aims of this study.

5.6.2 Additional BGCs screened for

The section on molecular screening of BGCs focused on eight BGCs in total. As discussed already, only one out of these eight BGCs screens yielded a positive outcome, significance that has already been outlined. However, it is important to be able to relate the significance of the additional BGCs screened for to what we observed in the antiSMASH analysis of known *Micromonospora* strains. Amongst the eight BGCs that were screened for, only Type 1 PKS and Type II PKS BGCs were predicted in the antiSMASH results of the known *Micromonospora* species analysed. It is important to note that in some instances, a database such as antiSMASH becomes limited as it may predict the absence of certain BGCs within genomes of specific microbes, especially if such genomes are incomplete (Medema *et al.*, 2011). Therefore, to cover this gap, PCR screening methods are used as an alternative despite possible biases associated with these methods. A typical bias with the PCR BGC screening method is that primers may be specific to a particular genus/family and will only amplifying genes from these limited taxa. Many of the primers in our study were designed specifically for streptomycetes as opposed to

Micromonosporaceae. We, therefore, cannot rule out this fact as a causative of negative amplification of the intended BGCs (Bervanakis, 2008; Hwang *et al.*, 2007; Wang *et al.*, 2011; Wood *et al.*, 2007). However, given the effect of horizontal gene transfers (HGT) of biosynthetic genes between different genera and families, it is expected that streptomycete genes will not necessarily be confined to this genus. This was the rationale behind using streptomycete-designed primers in our *Micromonospora* strains.

Nevertheless, it remains imperative to discuss the rationale behind screening of the rest of the BGCs. This is because these BGCs are of immense interest to researchers in novel drug discovery for reasons discussed in sections 5.6.2.1 through to 5.6.2.7.

5.6.2.1 Type I PKS

Alternatively known as Modular Polyketide synthases due to their characteristic orientation of the individual modules, Type I PKSs are also known to produce potent bioactive compounds and they are encoded by the T1PKS gene (Beta-ketoacyl synthase) (Figure 5.4 Image A) (Chen & Du, 2016). Just as their Type II counterparts, Type I polyketides are a matter of research relevancy due to their diverse bioactivity in their application as antibiotics, antiparasitics, immunosuppressants and anti-tumour agents (Fischbach & Walsh 2006). Examples of Type I PKS compounds that are sourced from actinobacteria are Avermectin (Figure 5.4 Image B) which is sourced from *Streptomyces avermitilis* and Calicheamicin (Figure 5.4 Image C) from *Micromonospora calichensis* (Chen & Du, 2016).

Image A)

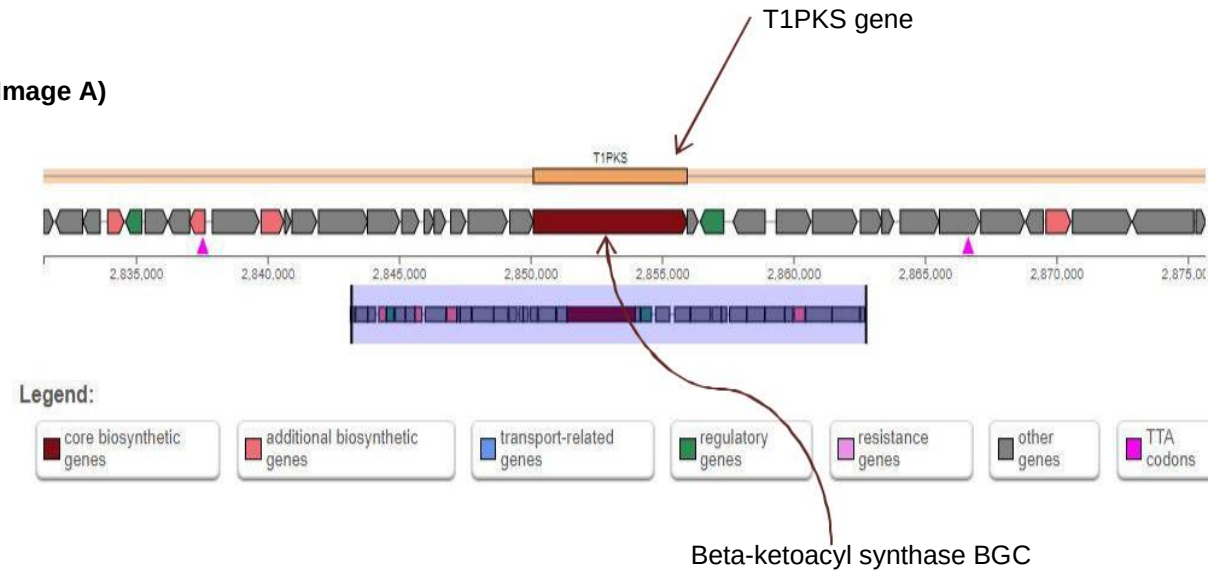
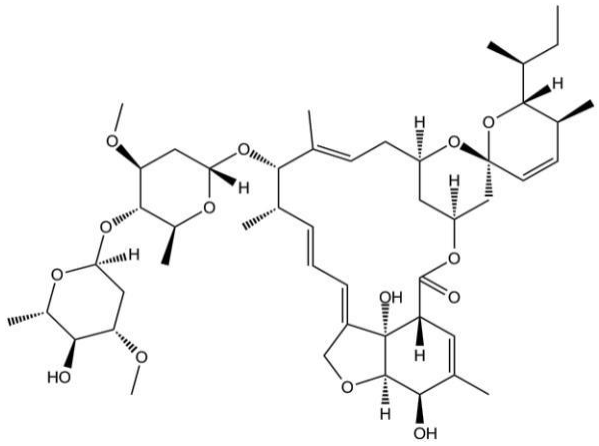
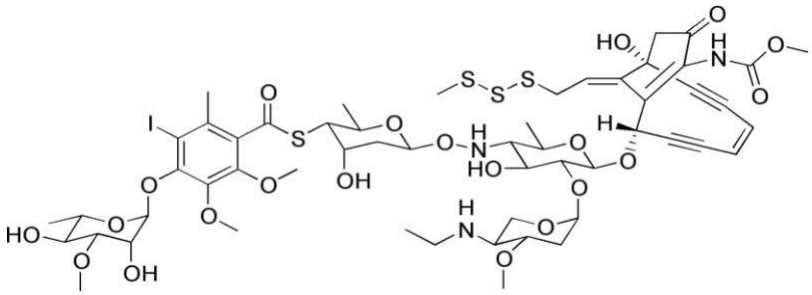


Image B)



Avermectin B1a

Image C)



Calicheamicin

Figure 5.4: Image A) An antiSMASH extract showing the genomic region housing the Beta-ketoacyl synthase core biosynthetic gene encoding the Type I PKS gene in *M. aurantiaca* WMMB 235 ^{T}. All other genes associated with the core BGCs such as transport-related, regulatory, resistance and other genes are also shown in the figure. Image B and Image C: The chemical structures of Avermectin B1a (an anthelmintic and insecticidal agent) and Calicheamicin (an antitumor antibiotic) respectively. These two compounds are typical examples of compounds that have biosynthesis in which the T1PKS gene plays a pivotal role.

In the molecular screen for BGCs, there was no evidence of the presence of the Type I PKS BGC amongst the 30 *Micromonospora* strains screened. It was important to screen for the presence of Type I PKSs since their range of activity covers both fungi and bacteria, thus iterative Type I PKSs and Non-Type I PKSs respectively (Cox, 2007; Hertweck, 2009).

5.6.2.2 Cytochrome P450 hydroxylase

The Cytochrome P450 hydroxylases (P450s) are a group of enzymes that are usually found within specific BCGs in *Micromonospora* species, whereby they play a role in the synthesis of

bioactive metabolites. Two examples of this group of enzymes are MycG and MycCI which play a pivotal role in *Micromonospora griseorubida*'s biochemical pathway for the synthesis of mycinamicin II, a macrolide antibiotic (Anzai *et al*., 2012). It is important to note that the genes that encode for the P450s are embedded within other BGCs. P450s are also known to be associated with polyene antibiotics that are potent antifungal agents (Demain & Sanchez, 2009). From our findings, as shown in Table 4.4, none of our strains contained a BGC that housed the particular genes encoding the Cytochrome P450 hydroxylase.

5.6.2.3 Polyether ionophore

Amongst the vast array of bioactive compounds that are produced by *Actinomycetales,* polyether ionophores stand out as a unique group of compounds synonymous with this order. They are a special type of Type I polyketides with a proven record of broad-spectrum activity (Dutton *et al*., 1995). Polyether ionophores are part of the broader ionophores family that have their bioactivity and antibiotic potency centred upon their ability to use their distinct structural components in enabling them to interact effectively with metal species (Kevin *et al.*, 2016). It was very important in our study to include the screening for the BGC encoding these compounds since they are produced exclusively by actinobacteria (Wang *et al.*, 2011).

5.6.2.4 P450 monooxygenase – glycopeptide

The screen for the BGC encoding for the Cytochrome P450 monooxygenase, involved in glycopeptide production, resulted in a negative result for all 30 strains, as shown in Table 4.4. The inclusion of this gene cluster in the molecular screen was chiefly based on the known activity of the P450 monooxygenase, especially in the biosynthesis of glycopeptide antibiotics. Actinobacteria are known to be the source point of glycopeptide antibiotics, which inhibit pathogenic bacterial cell wall biosynthesis as their mode of action (Beltrametti *et al*., 2007).

Examples of glycopeptide antibiotics in which P450 monooxygenase enzymes play a part in their biosynthesis are Vancomycin and Teicoplanin. Vancomycin is sourced from the actinobacterium *Amycolatopsis orientalis* while teicoplanin, a semisynthetic glycopeptide, is derived from *Actinoplanes teichomyceticus* (de Lalla *et al.*, 1992). These two antibiotics are effective against Gram-positive infections such as those caused by *Enterococcus faecalis* and MRSA (ibid).

5.6.2.5 Ansamycins

Ansamycins are macrolide antibiotics that are derivatives of compounds produced by actinobacteria (Vardanyan & Hruby, 2016). They are well noted in literature as potent biological compounds that display antibacterial, anticancer as well as antiviral properties (ibid).

Ansamycins have a wide spectrum of activity since they have demonstrated efficacy against both Gram-positive and Gram-negative bacterial strains (Floss & Yu, 1999). In our study, we targeted the BGC encoding the 3-amino-5-hydroxybenzoic acid (AHBA) synthase enzyme using the ANSA-F and ANSA-R degenerate primers as described by Wood *et al.* (2007). The screen for the AHBA synthase-encoding BGC was found to be negative amongst all 30 strains under study.

5.6.2.6 β-lactams

β-lactam antibiotics are a group of antibiotics that contain a highly reactive and specialized cyclic amide ring within their molecular structures called the β-lactam ring (Holten & Onusko, 2000). Ever since the discovery of penicillin in 1928, they have been one of the most prescribed groups of antibiotics. Their mode of action is based upon their ability to interfere with the peptidoglycan cell wall synthesis in pathogenic bacteria (Elander, 2003). This mode of action makes them potent mainly against Gram-positive microorganisms as opposed to Gram-negative microorganisms since the former have peptidoglycan as a structurally key component of their cell walls (ibid).

β-lactam antibiotics constitute the majority of commercially available antibiotics and they consist of broader families such as penicillins, cephalosporins and cephamycins, which are produced by actinobacteria species (Liras & Martin, 2006). Our screen for the β-lactam BGC gave a negative result, hence it can be concluded that although there was evidence of bioactive metabolites in 29 of our 30 strains, these certainly did not have characteristics of β-lactams at the molecular level.

5.6.2.7 Aminoglycosides

The BGC encoding for aminoglycosides was also screened for and the result amongst all 30 strains was negative. However, similar to β-lactam antibiotics, the rationale behind screening for aminoglycosides was mainly because they are also derived from actinobacterial species (Krause *et al.*, 2016). Antibiotics of this class have broad-spectrum potency, which is based on their ability to dismantle the protein synthesis machinery within pathogenic bacteria (ibid). Aminoglycoside antibiotics are effective against both Gram-positive and Gram-negative microorganisms such as the *Enterobacteriaceae*, *Yersinia pestis*, *Staphylococcus aureus*, *Mycobacterium* species and *Proteus* species among many others (Waksman *et al*., 2010).

Examples of actinobacteria from which aminoglycoside antibiotics have been isolated include *Streptomyces griseus*, which is famous for being the source microorganism of the first Aminoglycoside, Streptomycin (Dutton *et al.*, 1995). Another justification for including the screen for Aminoglycoside BGC in our study was that gentamicin, a famous and widely used

aminoglycoside antibiotic, is a known compound produced by *Micromonospora purpurea* (Waksman *et al*., 2010). This subsequently meant that a positive screen of the aminoglycoside BGC in any of our *Micromonospora* strains, would also have been of immense interest.

5.7. Primer design outcome and oligo-analysis

The outcome of the primer design process was pertinent for the successful screening of the Bacteriocin and Lanthipeptide BGCs. Borah (2011) highlighted that critical to the success of any PCR is the use of primers of good design and quality. Certain considerations should have been taken into account in designing the primers, as shown in Table 4.5. These include the length of the primers, melting temperature, GC content, annealing temperature and the possibility of formation of secondary structures (Patricia *et al*., 2009).

5.7.1 Lanthipeptide and Bacteriocin genes

Given that many of the BGCs that were predicted within the genomes of *Micromonospora* species analysed were not covered by the PCR-based screening, it was imperative to close this gap by identifying BGCs that could be targeted for primer design. The designed primers would be different from the primer sets used in the PCR-based screening since they would be designed specific to *Micromonosporaceae.* This is the reason why antiSMASH analysis of *Micromonospora* genomes was performed and served as the basis of primer design, as outlined in section 4.8 (Chapter 4). One of the most important questions upon which this research project was founded upon, sought to address whether the primer design exercise contributed to the fulfilment of the main aim of the study, as outlined in the project title.

It is important to note that most of the primer sets that were used in the PCR screen for BGCs were previously designed based on sequences from streptomycete species. This is the most probable reason why there was no amplification of the seven BGCs, which were being targeted within the *Micromonospora* strains. Having established this, a decision to target BGCs for which primers have not been designed for was taken, specifically for the genus *Micromonospora.* This led to the design of primer pairs for targeting lanthipeptide and bacteriocin. It was important to assess how these designed primers would assist in determining the antibiotic biosynthetic potential of the selected marine *Micromonosporaceae* species under study. After designing and synthesising primers to target lanthipeptide and bacteriocin BGCs (specifically Lant_dehydr_C and the DUF692 genes), it was important to investigate the presence of any of these within the genomes of at least five of the *Micromonospora* strains under study.

5.7.1.1 Lanthipeptides

From the information given in the outcome of primer design, it was expected that successful amplification of the lanthipeptide gene would yield bands with a length of about 850 bp upon successful agarose gel electrophoresis. This was, therefore, a starting point in answering the question whether any or all of the *Micromonospora* strains housed the lanthipeptide genes within their genomes. After performing the PCR and running the agarose gel, no bands were observed. Optimisation was attempted from different angles that firstly included repetition of the PCR runs with a fresh set of reagents. The PCR run with the fresh stock of reagents was performed as a gradient PCR with a gradient of 10^{o}C. This also did not yield any result. The reaction was also supplemented in another run with BSA to counteract any accumulated inhibitors as outlined by Lorenz (2012). After all these optimisation studies, it was concluded that there was no evidence of the existence of BGCs encoding for lanthipeptides in our five selected *Micromonospora* strains.

The focus on targeting the lanthipeptide gene in a bid to qualify our *Micromonospora* strains as potentially novel from a bioactivity standpoint, was chiefly due to the reputation of these compounds. Ongey and Neubauer (2016) highlighted that being natural peptide products, lanthipeptides make prospective alternatives to antibiotics currently in the clinical space due to their unique structural composition and accessibility. The lanthipeptide, nisin, discovered by Rogers in 1928, went on to be an important food preservation agent and its FDA approval in 1988 sparked research interest of lanthipeptides amongst generally regarded as safe (GRAS) microbes (McAuliffe *et al.*, 2000). Also, worth noting is the fact that the majority of known lanthipeptides depict antibacterial activity and are sourced from Gram-positive microorganisms hence the pursuit of these compounds in our study (Bierbaum & Sahl, 2009).

5.7.1.2 Bacteriocins

The expected amplicon size from the amplification of the bacteriocin gene was 400 bp as given in Table 4.5. On viewing the agarose gel that was run after amplifying the bacteriocin gene, multiple bands were observed under UV light. There are a couple of inferences that can be made in interpreting the respective agarose gel image given in Figure 4.16. The first observation was the formation of multiple bands of different sizes for each of the five strains. Troubleshooting and optimisation of many aspects of the PCR such as reagent components, annealing temperature and reaction stringency were performed as suggested by Lorenz (2012). However, the best outcome is shown in Figure 4.16.

The multiple bands on the agarose gel were highly likely due to the primers' lack of absolute binding specificity (Lorenz, 2012). Despite such flaws, a considerable number of important

inferences can be drawn from the gel picture. Firstly, our target gene was of an expected amplicon size of 400 bp as established from the primer design analysis. From the image, strains 1, 2, 4 and 5 were observed to produce an amplicon of approximately 400 bp within their genomes, hence concluded to be highly likely positive for the DUF692 Bacteriocin gene (Figure 5.5). This is however not exclusive evidence of the presence of the targeted BGC.

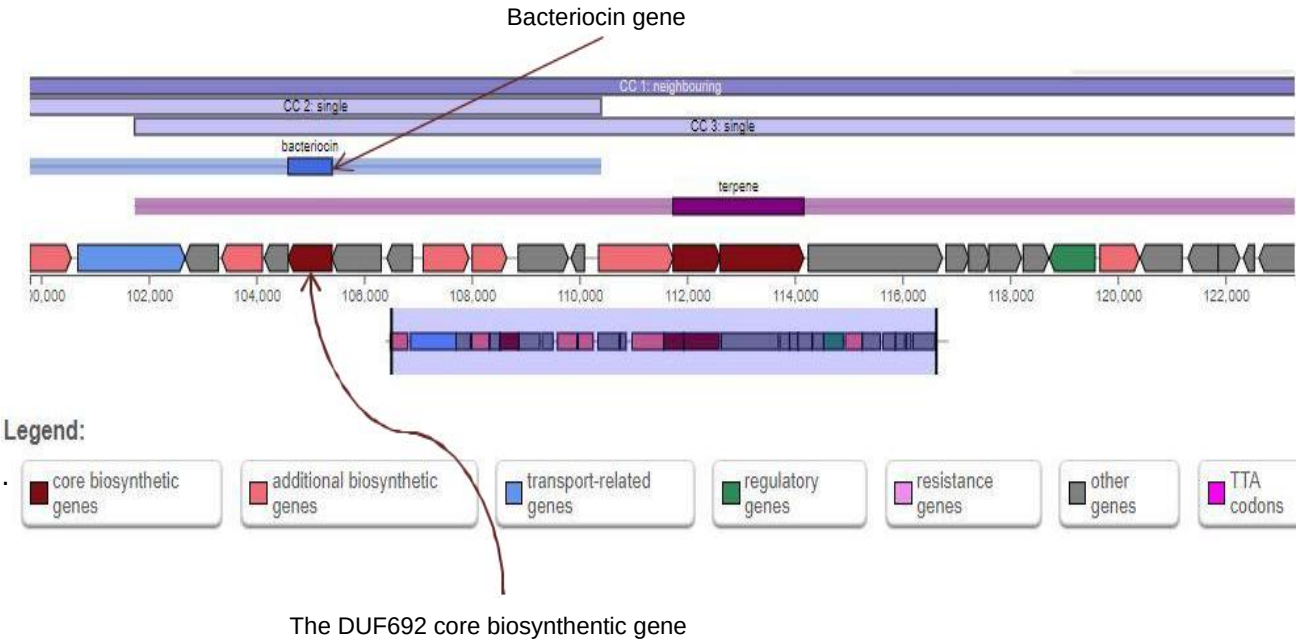


Figure 5.5: An antiSMASH extract showing the genomic region housing the DUF692 gene encoding for bacteriocin in *M. aurantiaca* NRRL B-2673^{T}. The figure shows an example of the location of the Bacteriocin gene within a genomic region, which also houses the Terpene biosynthetic genes. All other genes associated with the core BGCs such as transport-related, regulatory, resistance and other genes are also shown in the figure.

The 400bp-long bands were specifically extracted from the gel by the “gel-cutting” technique and processed for sequencing. This enabled us to determine whether indeed these bands represented genes encoding bacteriocins or related compounds.

5.7.2 Outcomes of the Blastx Bacteriocin sequence enquiries

Cotter *et al.* (2012) defined bacteriocins as bacterial-originating toxins of a peptidic nature that are produced in order to halt the growth of other bacteria. The bacterial strains targeted by bacteriocin compounds are usually closely similar to those which are the sources of these bacteriocins.

It was, therefore, established that some of the *Micromonospora* strains under study likely housed the bacteriocin-encoding BGC. A critical question that arises would focus on the relevance of this finding. The binding specificity of the designed primer pairs was going to be a critical factor in their successful function. It is not uncommon that when gene-specific primer sets are designed, non-specific binding can occur (Borah, 2011). To investigate the specificity of our designed primer pairs, the sequences were first submitted to Primer-BLAST. Detailed primer reports were generated for both the bacteriocin and lanthipeptide primer pairs and from these it was seen that an element of non-specificity was observed. It is important to note that the primer designs had been refined thoroughly, therefore, reaction optimisation was the most suitable avenue to explore to improve primer functionality.

The sequence results given in Table 4.6 were therefore not much of a surprise considering that our investigations had hinted on the possibility of non-specific binding. However, certain measures could be taken to improve optimisation and broaden troubleshooting. Such interventions would include reducing number of cycles in the PCR phases, reducing the extension and annealing times, relooking the annealing temperature as well as adjusting the thermo-cycler ramping speed (Borah, 2011).

5.10 Antimicrobial activity studies

The current study mainly focused on investigating the ability of the 30 *Micromonospora* strains to produce bioactive metabolites. Therefore, it meant that we had to establish laboratory evidence of any form of efficacy of the *Micromonospora* strains against known pathogenic microorganisms. The test strains that were selected for this investigation were *Escherichia coli* ATCC 25922, *Bacillus cereus* ATCC 10876 and *Candida albicans* ATCC 24433. The rationale behind the selection of these strains was to cover as wide a spectrum of activity as possible. With *E. coli* being a Gram-negative bacterium, *B. cereus* being a Gram-positive microorganism and *C. albicans* being a yeast, it meant that the analysis of antimicrobial activity indeed covered a broad range of microorganisms to a reasonable extent. Specifically, in liquid cultures, bioactivity was investigated by performing bioautography as opposed to solid culture overlay studies. This would be beneficial in the analysis of bioactivity for those strains that did not produce metabolites on solid agar. Further to this, the other aim of performing bioautography studies was to establish the specific days on which the *Micromonospora* strains effectively produced their antibiotic compounds. These were going to be useful as optimum incubation periods in performing liquid culture bioactivity analysis using multiple extraction techniques. Bioautography is a more specific bioactivity tool and it was useful in corroborating the analysis of the strains' antibacterial activity by solid agar overlays in our study.

5.10.1 Solid agar overlay studies

The results shown in Tables 4.6, 4.7a, 4.7b, and 4.8 are only for the instances whereby any antimicrobial activity was observed by means of clear zones of inhibition on the solid agar plates after overlaying. The extent of antibacterial activity in this section was determined by paying reference to arbitrary assignment of strength of activity (Table 5.2) as highlighted by Kirby and Le Roes-Hill (2009).

Table 5.2 Arbitrary assignment of strength of antibacterial activity

Antimicrobial activity	Zone area (mm^2)
VW (Very weak)	<100
W (Weak)	100-1000
M (Moderate)	1001-2000
S (Strong)	2001-3000
VS (Very strong)	>3000

5.10.1.1 Activity of *Micromonospora* strains against *E. coli* ATCC 25922

According to Kirby and Le-Roes-Hill (2009), the commencement of development of aerial mycelia on the surfaces of actinobacterial colonies is often associated with antibiotic production. This is consistent with what was observed in the experiment, whereby mycelial growth was recorded in fifteen out of seventeen *Micromonospora* colonies that depicted activity against *E. coli* ATCC 25922.

The *E. coli* ATCC 25922 used in this experiment is the recommended strain because it is susceptible and does not produce endotoxins (Chen *et al.*, 2017). *E. coli is* a known pathogenic causative of ailments such as septicaemia and other related infections, including urinary tract infections (Chen *et al.,* 2017). The observation of bioactivity of the twelve strains against *E. coli* ATCC 25922 suggests that either these strains produced bioactive metabolites that inhibit β-lactamase activity, or they resisted the hydrolysis effect of β-lactamase (Babic *et al.*, 2006). The production of the enzyme β-lactamase by bacteria such as *E. coli* ATCC 25922 leads to the hydrolysis of the β-lactam ring, prior to their binding to a penicillin-binding protein (PBP). This subsequently renders antibiotic compounds completely inactive (Hidayati *et al.*, 2013). Analysis of bioactivity was performed both in solid and liquid cultures.

A very good example of a *Micromonospora* isolate antibiotic that is potent against *E. coli* is gentamicin, which is isolated from *Micromonospora purpurea* (Wagman & Weinsten, 1980). Another antibacterial compound, butremycin, isolated from *Micromonospora* sp. K310 by

Kyeremeh *et al.* (2014) also showed similar bioactivity against the same *E. coli* ATCC 25922 strain that we used in our study.

This background consequently validates the significance of the activity observed amongst the *Micromonospora* strains against the *E. coli* ATCC 25922 strain. It can be concluded, in general, that twelve of the 30 *Micromonospora* strains under study have demonstrable potency against a Gram-negative microorganism such as *E. coli,* with strain 02-128*#1 being the most the active, as shown by the areas of inhibition zone values.

5.10.1.2 Activity of *Micromonospora* strains against *B. cereus* ATCC 10876

Bacillus cereus is a Gram-positive microorganism that is commonly associated with food poisoning in its pathogenic form (Guinebretiere *et al.*, 2013). Its pathogenicity is based on the bacterium's ability to produce toxins which are considerably thermo-resistant, hence causing poisoning in undercooked food such as fried rice (ibid). Being β-lactamase producers, *B. cereus* strains show resistance to β-lactam antibiotic therapy such as that of cephalosporins and Penicillins. Some strains of *B. cereus* also show elements of resistance towards conventional non-β-lactamase antibiotics which builds up progressively from tolerance due to overuse from over-prescription (Chen *et al.*, 2017).

In an attempt to correlate aerial mycelium presence and antimicrobial activity, it was observed that although other isolates were characterised by antimicrobial activity coupled with aerial mycelium production, antibacterial activity was also observed around some colonies where no aerial mycelium was observed. These mycelia-related observations are in sync with the known behaviour of actinobacteria as they produce antibiotics, as highlighted by Kirby and Le Roes-Hill (2009).

With reference to the arbitrary assignment of strength of antibacterial activity (Table 5.2), activity against *B. cereus* ranged from very weak, weak to moderate. The strain that showed the most activity against *B. cereus* ATCC 10876 was strain 02-128*#3 which showed moderate activity with a calculated area of inhibition of 1178mm^2. The antibacterial compound Butremycin, isolated from *Micromonospora* sp. K310 by Kyeremeh *et al.* (2014) was reported to depict bioactivity against the strain *S. aureus* ATCC 25923, a Gram-positive test strain. The microorganism *Micromonospora* sp. K310 is, therefore, a good example of the broad spectrum bioactive ability associated with some strains of this genus since it showed such activity against both Gram-positive and Gram-negative test strains (Wang *et al.*, 2019). Another example of similar bioactivity was reported by Talukdar *et al.* (2016) when they demonstrated the antibacterial activity of *Micromonospora auratinigra* against *Bacillus subtilis*, a Gram-positive

pathogen. Gentamicins are an example of a group of commercial antibiotics on the pharmaceutical market since 1971 with broad-spectrum activity. Ever since the discovery of antibiotics from *Micromonosporaceae,* the gentamicins have proven to be the most popular and they are sourced from *M. echinospora* NRRL 2953 (Boumehira *et al.*, 2016).

5.10.1.3 Activity of *Micromonospora* strains against *Candida albicans* ATCC 24433

The antagonistic activity of *Micromonospora* strains against *C. albicans* was investigated and the results are tabulated in Table 4.8. *C. albicans* is an opportunistic commensal that is a causative agent of the fungal infection candidiasis, in its pathogenic form (Kabir *et al*., 2012). It is noted in literature that about three quarters of women suffer from candidiasis at least once in their lifetime and in immunocompromised patients, the condition presents itself in its most severe form (Ruhnke & Maschmeyer, 2002; Schulze & Sonnenborn, 2009). Although this study chiefly focused on the "antibiotic" biosynthetic potential of *Micromonospora* strains, inclusion of fungi in the investigation of production of bioactive metabolites was justifiable. As reported by Zhao *et al.* (2017), some *Micromonospora* strains such as *Micromonospora parathelypteridis* have potency against fungi. Therefore, to have any of our strains depicting antifungal activity further to antibacterial activity would be an incentive on the aim of our study. This was the basis for investigating antifungal activity of our strains, specifically against a virulent strain of *C. albicans.*

As shown in Table 4.8, a total of ten out of a possible 30 *Micromonospora* strains showed some form of activity against *C. albicans.* By use of the arbitrary assignment of strength of antibacterial activity as given in Table 5.2, the activity observed ranged from very weak to weak. Strains 02-118#5, 02-118#4, 02-138#6 and 02-251*#36 are the ones that had calculated areas of inhibition zones corresponding to very weak activity while the rest of the strains showed weak activity. Even though there was no strain showing activity ranging from moderate through to very strong, the observation that ten *Micromonospora* had some form of antifungal activity was a significant one for our study. Kim *et al.* (1999) reported the isolation and structural elucidation of the antibiotic streptimidone from *Micromonospora coerulea*, which showed antifungal activity against the plant fungi *Didymella bryoniae* and *Magnaporthe grisea*. Two other antibiotics spartanamicin A and B, isolated from *Micromonospora* strain no. MSU-43097, have also been reported to exhibit activity against *C. albicans* as well as *Cryptococcus*, *Aspergillus* and *Cladosporium* species (Nair *et al.*, 1992).

It is important to look at other factors that might have influenced the bioactivity results that were observed. One very pertinent example is the effect of culture media used in investigating antibiotic production by actinobacteria. Kiranmayi *et al.* (2011) corroborated with this notion and

highlighted that cultivation and nutrition significantly influence the synthesis of bioactive metabolites. Carbon and nitrogen sources within media constituents as well as how these are optimised can influence how actinobacteria produce their bioactive metabolites (Souagui *et al.*, 2019). Without media optimisation, we cannot conclude that the bioactivity observed, for example, with the yeast, is the only possible activity possessed by a strain. Zeeck and colleagues conceptualised the one strain many compounds theory (OSMAC) which rightfully postulates that a vast array of secondary metabolites can be sourced from a single microbial strain, but only a subset of these will be produced under specific culture conditions (Bode *et al.*, 2002). Therefore, variation of culture aspects such as carbon and nitrogen source and concentration, temperature of incubation, salinity and aeration could have led to the accessing of more metabolites (ibid).

5.10.2 Bioautography

5.10.2.1 Principle and relevance of bioautography

Interpretation of the TLC plates in bioautography was based on the principle that tetrazolium salts such as MTT turn purple upon reduction by dehydrogenases of thriving cells (metabolically active cells) (Balouiri *et al.*, 2016). The MTT used in bioautography contains formazan dye, a known indicator of bacterial growth. A purple colour on the TLC plate would, therefore, indicate presence of living cells (Kirby & Le Roes-Hill, 2009). In essence, this meant that observation of any spots on the TLC plates with purple discolouration signified failure of the spotted bacterial extracts, to elicit antibiotic activity. An observation of white or cream clear areas on the TLC plates signified the killing of bacterial test strains by the antibiotic compounds produced by the *Micromonospora* strains as no dehydrogenases were produced.

The relevancy of bioautography in investigating the production of antibiotic compounds in our *Micromonospora* liquid cultures was centred upon the technique's efficacy as a bioassay. Such efficacy is primarily attributed to the ability of the technique to localise bioactivity, despite formation of complexities at the molecular level, hence allowing for specificity in targeting the bioactive compounds (Suleiman *et al*., 2010). Sharverdi *et al.* (2007) corroborated with this comment and further pointed out the use of bioautography as a fast, efficient and cost-effective way to isolate only the relevant and bioactive compounds on a TLC plate.

5.10.2.2 Bioactivity against *C. albicans* ATCC 24433

Figure 4.21 shows an image of the TLC plate whereupon the activity of the five *Micromonospora* strains was investigated for production of antibacterial compounds against *C. albicans* ATCC 24433. The isolates that were spotted on the TLC plate had been prepared from serial extracts

obtained over a 12-day incubation period. From the image, all strains showed some form of activity against the test strain as shown by the white spots labelled by the yellow arrows. It is, however, important to note that the activity was not as distinct and clear-cut as compared to bio-activity of these strains against other test strains. No activity was observed amongst the negative controls, thereby validating the experiment. Most importantly, the growth days that the strains produced their antibiotic compounds were noted, as these would play a pivotal role in investigating antibacterial activity using multiple extraction techniques.

5.10.2.3 Bioactivity against *E. coli* ATCC 25922

A clearer and more typical picture of the activity of *Micromonospora* in liquid cultures is given as Figure 4.19, where *E. coli* ATCC 25922 was used as the test strain. Without any ambiguity, the white spots on the TLC plate show that all the strains do show activity against the *E. coli* ATCC 25922 test strain. It is interesting to note that the antibacterial activity shown by these strains was between 3 days and 7 days of incubation. No activity was observed from isolates that were spotted onto the TLC on incubation days 10 and 12.

5.10.2.4 Bioactivity against *B. cereus* ATCC 10876

There was no activity observed amongst the *Micromonospora* strains against the test strain *B. cereus* ATCC 10876 as signified by a TLC plate that did not show any colour change during incubation. However, in section 5.10.2, it was observed and noted that the *Micromonospora* strains show antibacterial activity against the same *B. cereus* strain using the solid agar overlay method. The difference in activity against the same test strain between solid and liquid media can be explained by the fact that metabolite profiles of strains grown in these two media differ due to differences in growth parameters such as aeration.

5.10.3 Multiple extraction techniques: bioactivity against *C. albicans* ATCC 24433

Section 3.9.4 of the methodology chapter covered the use of different extraction techniques in pursuit of accessing antibiotic compounds. As highlighted by Betina (1973), the use of different extraction techniques is a time and cost-effective manner of simultaneously accessing bioactive compounds from liquid cultures. The value of combining these techniques is that gaps are bridged in the isolation of the variety of bioactive compounds, therefore a compound missed by one technique is highly likely to be picked up by some other isolation technique/s (ibid).

Therefore, the use of multiple extraction techniques in our study was necessary in an attempt to cover a wide range of antimicrobial compounds as well as to establish the best extraction technique for each of the individual *Micromonospora* strains under study. TLC as the underlying

technique allowed for the separation of possible antibiotic compound mixtures from the strains (Betina, 1973). In Figure 4.18, the yellow arrows in the image highlight the white zones on the TLC plate, which signify production of bioactive antimicrobial compounds against *C. albicans* ATCC 24433. A summary of the bioactivities observed against *C. albicans* ATCC 2443 is given in Table 5.4. It is important to note that the incubation times for the liquid cultures that were employed before the extracts were accessed by the different extraction techniques found their basis from the experiment outcomes discussed in sections 5.12.2, 5.12.3 and 5.12.4.

Table 5.3 Summary of the bioactivities observed against *C. albicans* ATCC 2443 using different extraction techniques

Strain	**Culture media**	**Day of Growth**	**Extraction method**				
			Whole cell extraction	DIAION treatment	Cell extracts	Culture filtrate	Ammonium sulphate precipitation & dialysis
02-128*#1	172F	12th	Active	Active	Active	Active	Active
04-015#16	172F	10th	Active	Active	No activity	Active	No activity
02-128*#3	172F	5th	Active	Active	No activity	Active	Active
02-251*#36	172F	3rd	Active	No activity	No activity	Active	No activity
02-209#4	172F	12th	Active	Active	No activity	Active	No activity

The TLC plate's results in Figure 4.21 were validated due to the negative result of the controls, that is to say, there was evidence that there were not any bioactive compounds in the liquid culture media before inoculation with *Micromonospora* strains. From Figure 4.21, it can also be concluded that the filtrate obtained via the filtration technique showed the most antifungal potency against the yeast *C. albicans* ATCC 24433. This is because in all five strains, this technique managed to unlock antimicrobial potency as shown by the white zones of clearance on the TLC plate. Treatment of the filtrate obtained from filtration by ethyl acetate produced

purer forms of extracts. These extracts had increased concentration of antibacterial compounds, hence showing significant activity on TLC plates (Valan Arashu *et al.*, 2014).

On the other hand, Figure 4.21 clearly shows that the cells obtained by the filtration technique did not have any active antibiotic compounds as shown by the lack of white zones on the purple background for all the spotted strains. All other extraction techniques such as the whole-cell culture, DIAION treatment and dialysis successfully extracted active compounds from some of the *Micromonospora* strains. In general, as shown in Table 5.4, the most activity was observed in extracts obtained by the ethyl acetate extraction technique (whole-cell culture) as well as the filtration technique. The diverse biological attributes of ethyl acetate as a solvent of extraction, which include average polarity and minimal toxicity towards test strains, makes extraction with ethyl acetate a reliable method for the extraction of both polar and non-polar antibacterial compounds (Valan Arasu *et al.*, 2014).

5.12.6 Multiple extraction techniques: bioactivity against *E. coli* ATCC 25922

Figure 4.22 shows an image of a TLC plate whereupon the bioactivity of antibiotic compounds that were acquired by different extraction techniques was tested against *E. coli* ATCC 25922. As shown on the TLC plate, the controls showed negative results in the row that they were spotted meaning the culture media did not have any antimicrobial properties of its own that could influence the investigation (Waksman *et al*., 2010). This meant that there was compelling evidence to the fact that *Micromonospora*-derived compounds were responsible for the observed antibiotic activity on the TLC plate.

Table 5.4 Summary of the bioactivities observed against *E. coli* ATCC 25922 using different extraction techniques

Strain	Culture media	Day of Growth	Extraction method				
			Whole cell extraction	DIAION treatment	Cell extracts	Culture filtrate	Ammonium sulphate precipitation & dialysis
02-128*#1	172F	12th	Active	Active	No activity	Active	Active
04-015#16	172F	10th	Active	Active	No activity	Active	No activity
02-128*#3	172F	5th	No activity	Active	No activity	Active	Active
02-251*#36	172F	3rd	No activity	Active	No activity	Active	No activity
02-209#4	172F	12th	No activity	Active	No activity	Active	No activity

Similar to the patterns observed in their bioactivity against *C. albicans* ATCC 24433, the extraction by the filtration extraction, particularly the filtrate was the most effective method of accessing the antibiotic compounds produced by the *Micromonospora* species. All strains produced filtrate that evidently contained antibiotic compounds active against *E. coli* ATCC 25922 as shown by the clear white spots against the purple background. Interestingly, the cells derived from the same filtration process did not show any antibiotic activity against the test strain. Antibiotic compounds accessed from strains 1, 4 and 8 by DIAION treatment also showed considerable activity against the test strain. From the TLC image (Figure 4.22), it can also be concluded that the whole-cell culture and dialysis techniques were less effective than the filtration technique in accessing antibiotic compounds in *Micromonospora* strains.

5.12.7 *Micromonospora* strains selected for further study

Based on their bioactivity against the test strains used in the analysis of antimicrobial activity, the five best antibiotic compound-producing strains were found to be 02-128*#1, 04-015*#16, 02-128*#3, 02-251*#36 and 02-209*#6. These strains were further investigated for phylogeny, as shown in section 3.6 of Chapter 3.

Table 5.5 Summary Table of the bioactivities of the *Micromonospora* strains forming the main focus of this study.

Strain	**Area of inhibition zone against test strain (mm^2)**		
	E. coli ATCC 25922	*B. cereus* ATCC 10876	*C. albicans* ATCC 24433
02-128*#1	1130.99	273.77	No activity
04-015*#16	701.99	No activity	No activity
02-128*#3	486.70	1178.09	436.26
02-251*#36	417.62	75.56	51.03
02-209*#6	689.09	No activity	No activity

After discussing the results obtained in this study, it is imperative to see if these have answered to the objectives of the study. In this regard, the last chapter of this thesis will summarise the findings of the study against the individual objectives set and outline concluding remarks. Recommendations for future studies as well as limitations of the current study are also outlined in the next chapter.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Each objective proposed before the commencement of this study was pursued to as reasonable an extent as possible. The pursuit of these objectives generated results outlined in Chapter 4 and subsequently discussed in Chapter 5. From the discussion, a number of conclusions can be drawn.

Objective 1: To assess the viability of the *Micromonosporaceae* strains, stored as frozen cultures, on solid and in liquid cultures.

Conclusion 1: The 30 strains that constituted the subjects of this study were successfully "woken up" to viability from their frozen state (-80^{O}C) on the relevant agar media. Irrefutable evidence of viable *Micromonospora* strains on solid and in liquid cultures was presented in section 4.1 of this study. The culture media used in this study (SGG, SGG with artificial seawater-ASW, 172F and 172F with ASW) was observed to be favourable in bringing the strains to viability as well as growing them, in both their solid and liquid variations.

Objective 2: To identify strains under study up to species level by performing 16S rRNA gene analysis and multi-locus sequence analysis (MLSA).

Conclusion 2: 16S rRNA gene sequencing and analysis led to the conclusion that 28 of the 30 strains under study belong to the genus *Micromonospora* while two belong to the genus *Jishengella.* However, all strains were concluded to belong to the family *Micromonosporaceae.* MLSA studies concluded that the five strains chosen for further analysis could represent novel species, with ancestry very closely related to the *Micromonospora aurantiaca* ATCC 27029^{T} strain. However, tools such as DNA-DNA hybridisation or whole-genome sequencing could be employed to aid in the speciation of the strains hence answering the question on strain novelty.

Objective 3: To perform a molecular screen of the marine *Micromonosporaceae* strains present in the BTB culture collection for selected BGCs.

Conclusion 3: A total of eight BGCs were targeted within the genomes of the 30 *Micromonospora* strains under study. Only one of the eight BGCs, the Type II PKS BGC was positive in 28 of the 30 strains, thus showing antibiotic biosynthetic potential of these strains. This BGC was also predicted in almost all of the 44 validly published *Micromonosporaceae* genomes that were analysed.

Objective 4: To analyse the genome sequences of *Micromonosporaceae* strains to assist with the design of new primer sets.

To identify gaps in the current knowledge around primer sets available for the molecular screening of antibiotic biosynthetic gene clusters and the design of new primer sets.

Conclusion 4: 44 validly published *Micromonosporaceae* type strains were successfully analysed at genomic level to aid in the design of new primer sets. This information was used in conjunction with the current knowledge around primer sets for the molecular screening of the antibiotic BGCs used in this study. Ultimately, two sets of new primer pairs (BAC-F/BAC-R and LAN-F/LAN-R) were successfully designed.

Objective 5: To test all new primer sets designed on the top five *Micromonosporaceae* strains used in this study.

Conclusion 5: The primer sets that were designed were tested, as intended, on the five *Micromonosporaceae* strains to see if they yielded any product. The primer set targeting the bacteriocin BGC gave multiple PCR products on agarose gel electrophoresis signifying non-specific binding of primers. Despite being hopeful, there is need to refine the design of the primers; possibly considering the use of degenerate primer sets to improve on primer function.

Objective 6: To assess the antibacterial activity of strains under study against selected test strains on solid and in liquid media.

Conclusion 6: Antibacterial activity analysis was successfully performed in liquid and on solid media. On solid media analysis, twelve *Micromonosporaceae* strains showed bioactivity, which ranged from weak to moderate against *Escherichia coli* ATCC 25922. Analysis of bioactivity against *Bacillus cereus* ATCC 10876 revealed that twelve strains showed some form of bioactivity that ranged from very weak, weak to moderate. Ten strains showed bioactivity against *Candida albicans* ATCC 24433 and with such activity ranging from very weak to weak.

Bioautography studies conducted for the five best *Micromonosporaceae* strains showed that they all produce antibacterial substances against *C. albicans* ATCC 24433, albeit after different days of incubation. All strains also showed activity against *E. coli* ATCC 25922 after three to seven days of incubation. The filtration technique was found to be the most effective in accessing antibiotic compounds amongst the multiple extraction techniques used.

6.2 Recommendations

The primer sets designed in this research study proved to be promising in targeting the bacteriocin BGC. The other primer set did not yield any product on attempted amplification of the target lanthipeptide BGC within the five *Micromonosporaceae* strains analysed. For the sake of future research, optimisation using other parameters (such as range of $MgCl_2$, other additives such as glycerol, DNA concentration) could be tested. These primer sets would possibly locate the lanthipeptide and bacteriocin BGCs within genomes of many other strains of interest.

6.3 Limitations and highlights of the study

In our PCR-based screening of novel BGCs, we had to have gene sequence information first to enable the designing of primers. This subsequently means that truly novel sequences are highly likely to be missed using such an approach, hence a limitation. In the same vein, primers may only function within a specific genus hence their scope becomes limited. Another major challenge that comes with PCR-based screening is the fact that even if a strain gives amplicons for a particular gene, it does not mean it possesses the entire pathway needed to produce the corresponding compound. In other cases, the entire pathway might be present and still, the compound might not be produced.

Despite the limitations of the study, PCR-based screening (particularly coupled with other genomic methods) has the potential to reveal a plethora of unknown bioactive compounds. Another quite interesting highlight of this study is the fact that so many of the strains studied seem to be phylogenetically related to *Micromonospora aurantiaca*. This microorganism has been shown to exhibit biotechnological potential. The outcomes of this study can therefore also serve as a basis for future studies focused on this interesting group of strains that seem to be associated with different sea sponges and sea squirts collected from the Algoa Bay region.

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ANNEXURE A

16S rRNA gene phylogeny: Maximum likelihood and minimum evolution trees

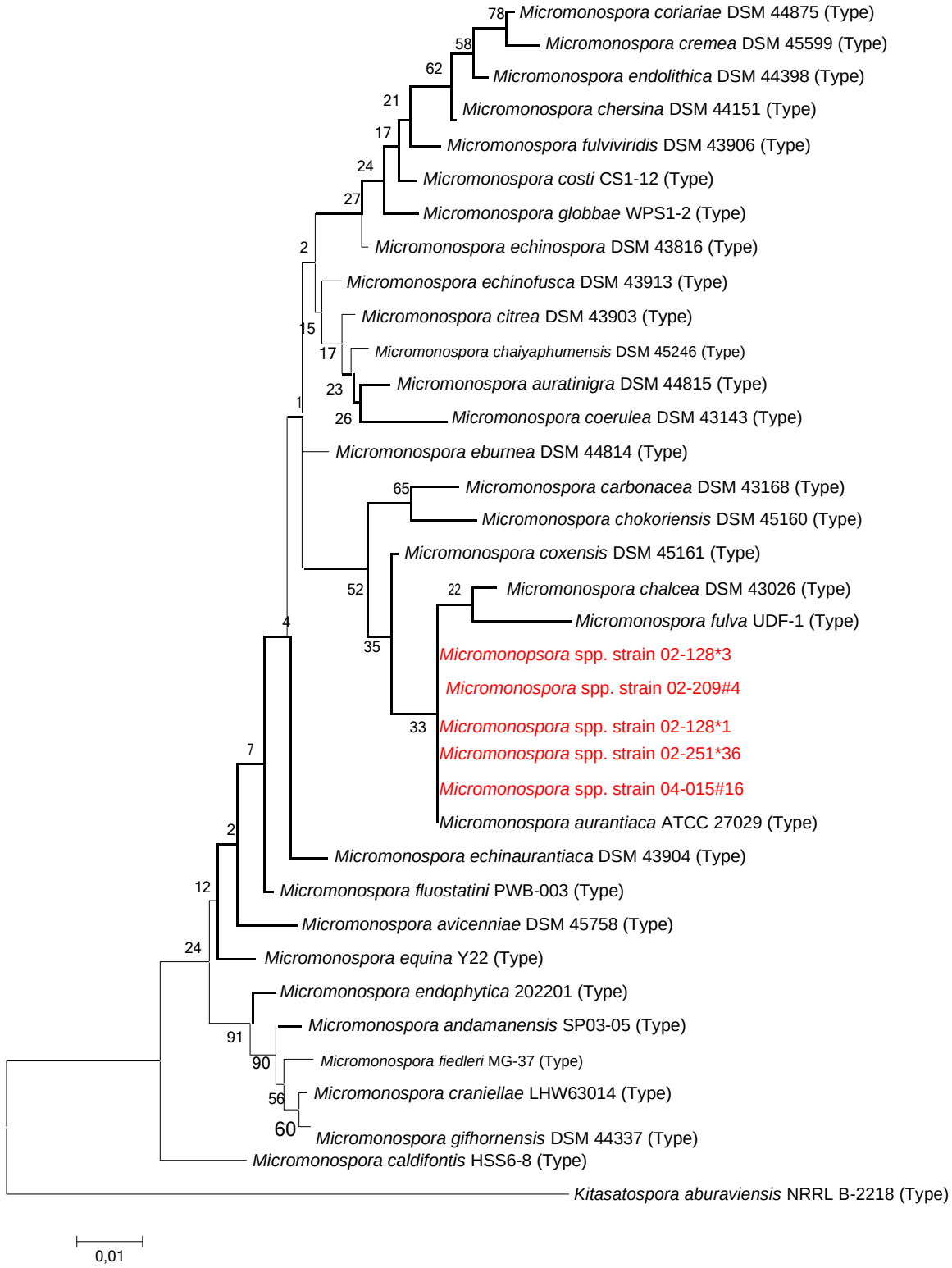

Figure An1: The phylogenetic relationships between the 16S rRNA gene sequences of the *Micromonospora* strains 02-128*3, 02-209#4, 02-128*1, 02-251*36, 04-015#16 with 30 other type strains of other *Micromonospora* species are shown in the Maximum likelihood evolution tree (Saitou & Nei, 1987). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. This analysis involved 36 nucleotide sequences. There were 1338 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

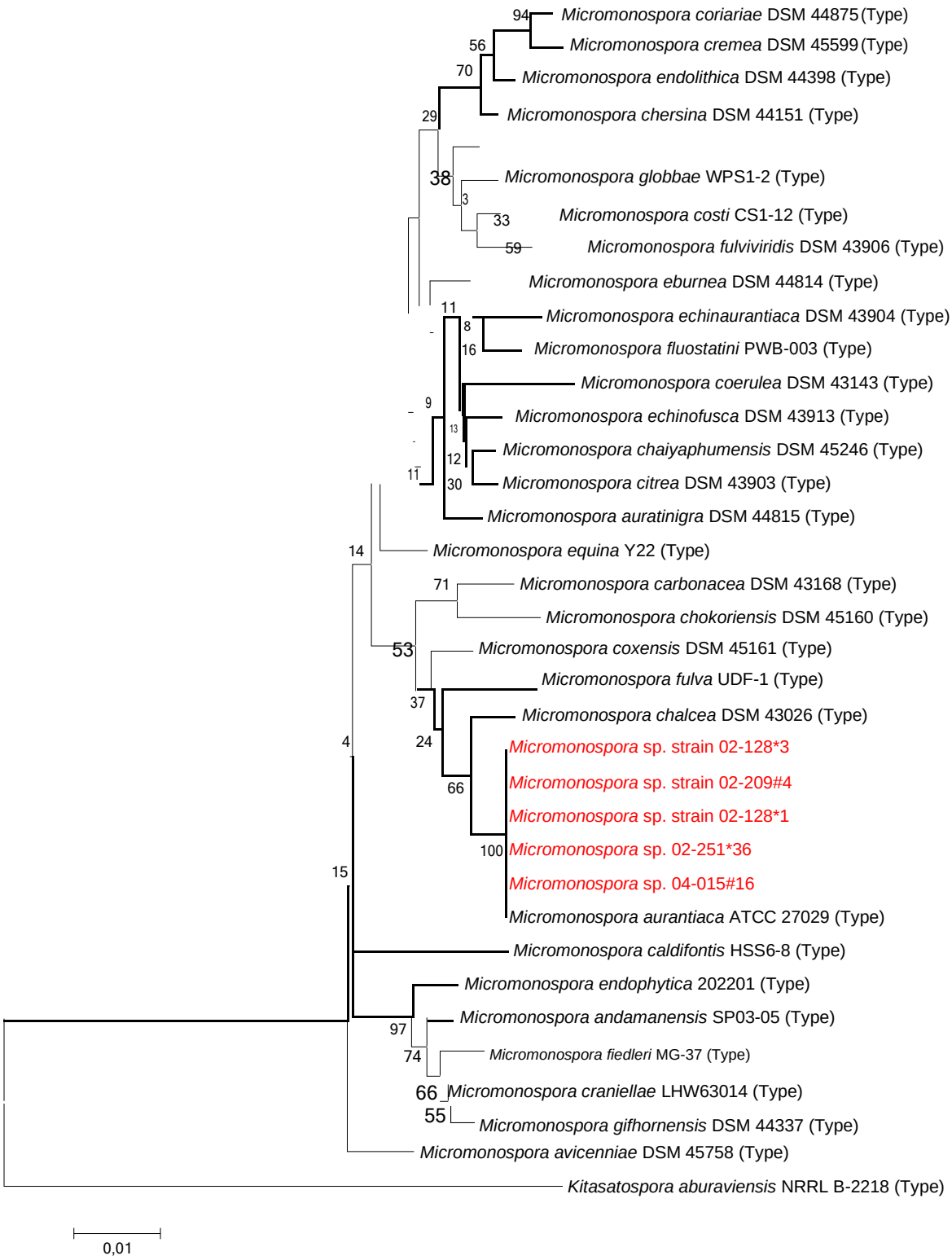

Figure An2: The phylogenetic relationships between the 16S rRNA gene sequences of the *Micromonospora* strains 02-128*3, 02-209#4, 02-128*1, 02-251*36, 04-015#16 with 30 other type strains of other *Micromonospora* species are shown in the Minimum evolution tree (Saitou & Nei, 1987). The tree

is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. This analysis involved 36 nucleotide sequences. There were 1338 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

ANNEXURE B

gyrB phylogeny: Maximum likelihood and minimum evolution trees

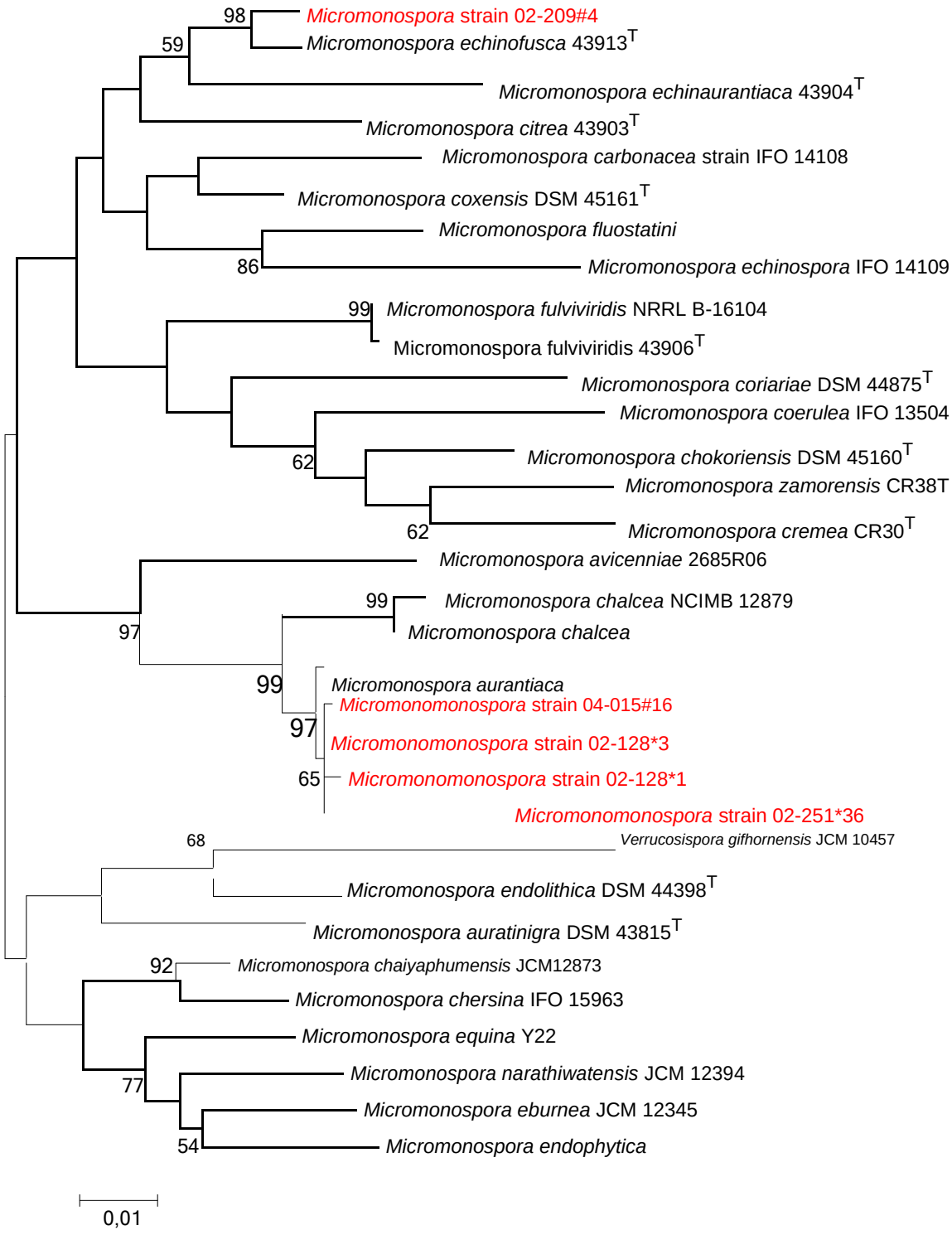

Figure An3: The phylogenetic relationships between the *gyrB* gene sequences of the 5 *Micromonospora* strains (02-128*3, 02-209#4, 02-128*1, 02-251*36, 04-015#16) and strains of other *Micromonospora* species are shown in the Maximum likelihood tree (Saitou & Nei, 1987). The tree is drawn to scale, with

branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. This analysis involved 32 nucleotide sequences. There were 987 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

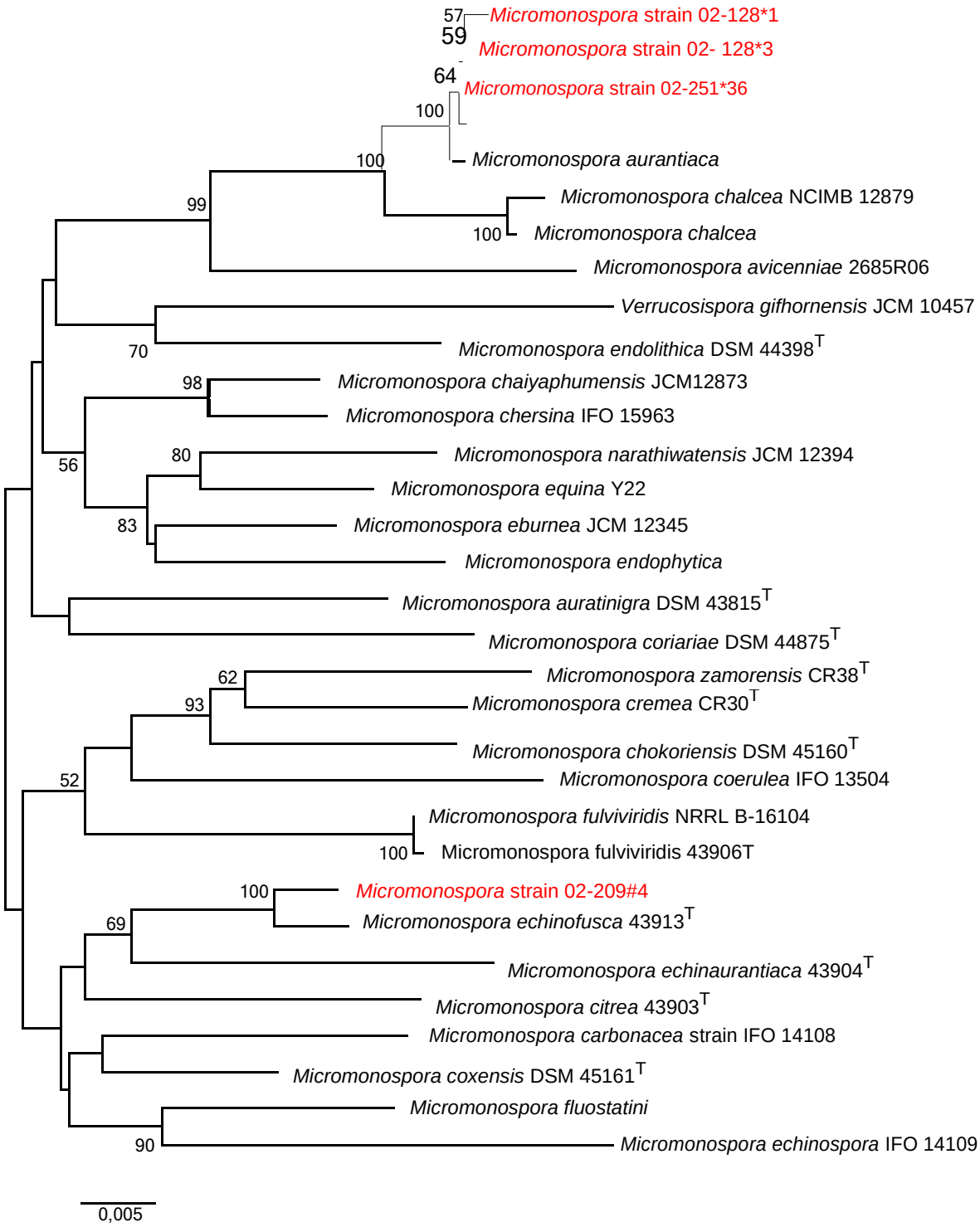

Figure An4: The phylogenetic relationships between the *gyrB* gene sequences of the 5 *Micromonospora* strains (02-128*3, 02-209#4, 02-128*1, 02-251*36, 04-015#16) and strains of other *Micromonospora* species are shown in the Minimum evolution tree (Saitou & Nei, 1987). The tree is drawn to scale, with

branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. This analysis involved 32 nucleotide sequences. There were 987 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

ANNEXURE C

rpoB phylogeny: Maximum likelihood and minimum evolution trees

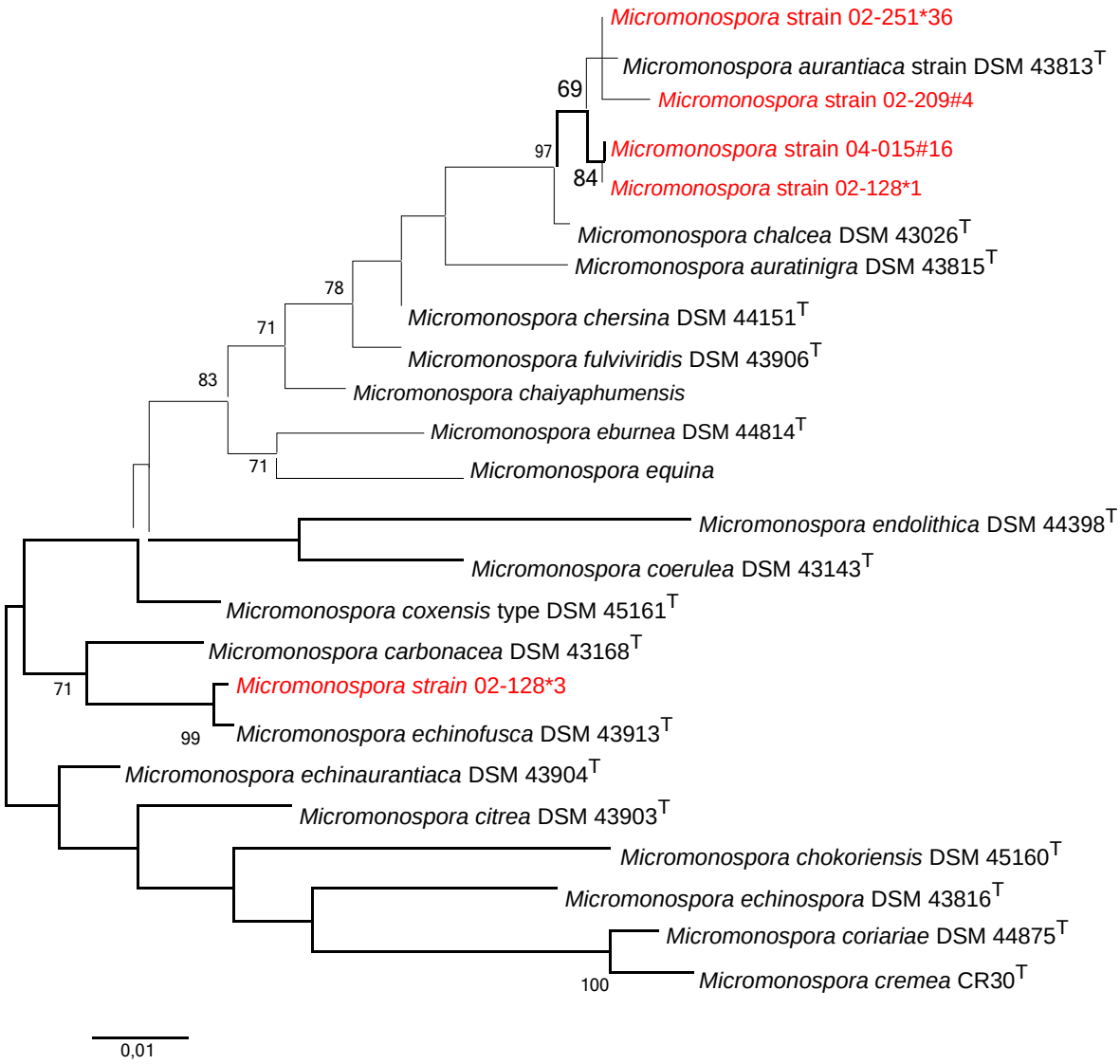

Fig An5: A Maximum-likelihood phylogenetic tree (Saitou & Nei, 1987), is shown between the *rpo*B gene sequences of the 5 *Micromonospora* strains (02-128*3, 02-209#4, 02-128*1, 02-251*36, 04-015#16) and strains of other *Micromonospora* genus. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. This analysis involved 24 nucleotide sequences. There were 602 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

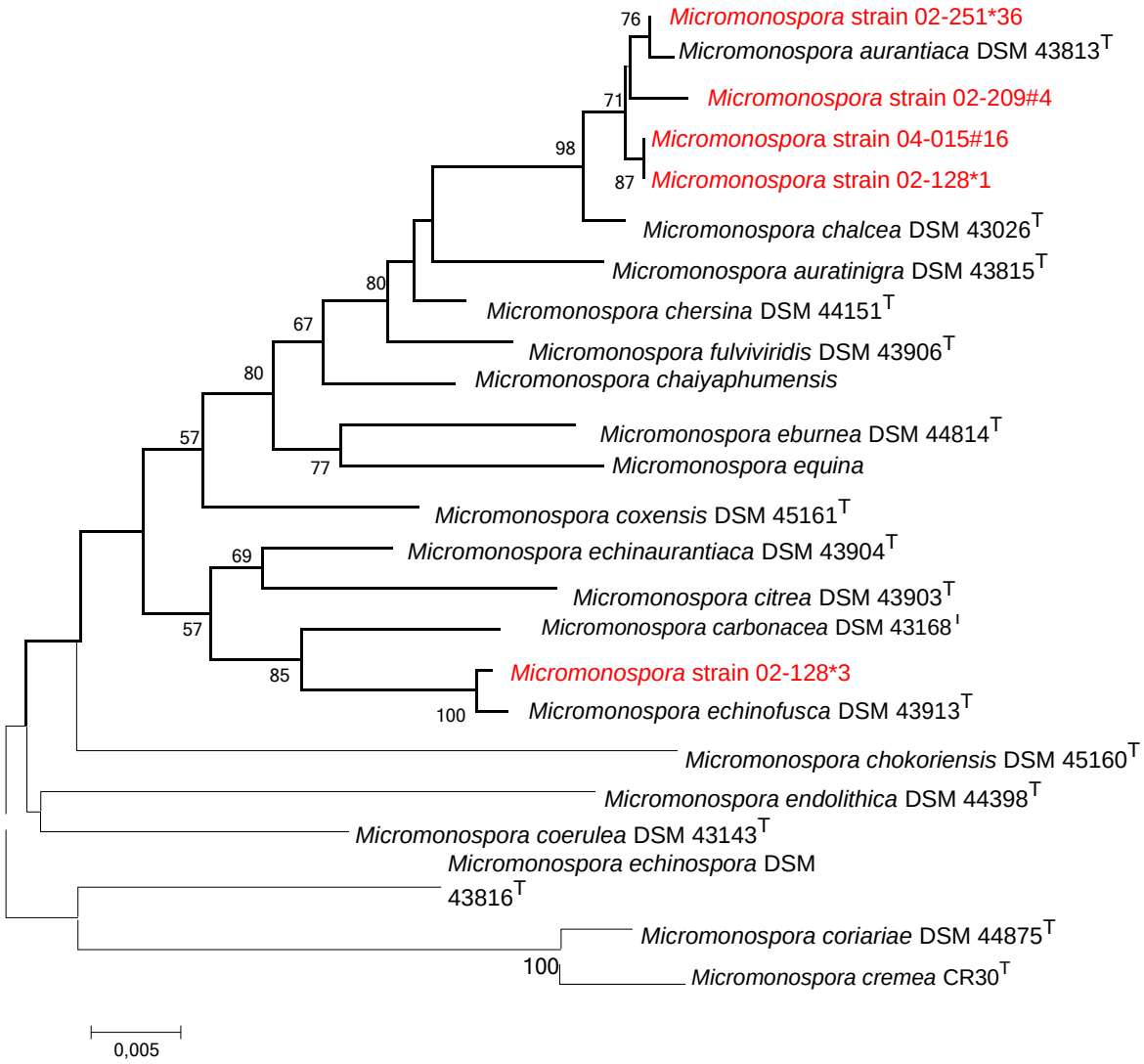

Fig An6: A Minimum evolution phylogenetic tree (Saitou & Nei, 1987), is shown between the *rpo*B gene sequences of the 5 *Micromonospora* strains (02-128*3, 02-209#4, 02-128*1, 02-251*36, 04-015#16) and strains of other *Micromonospora* genus. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. This analysis involved 24 nucleotide sequences. There were 602 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

ANNEXURE D

Concatenated sequences: Maximum likelihood and minimum evolution trees

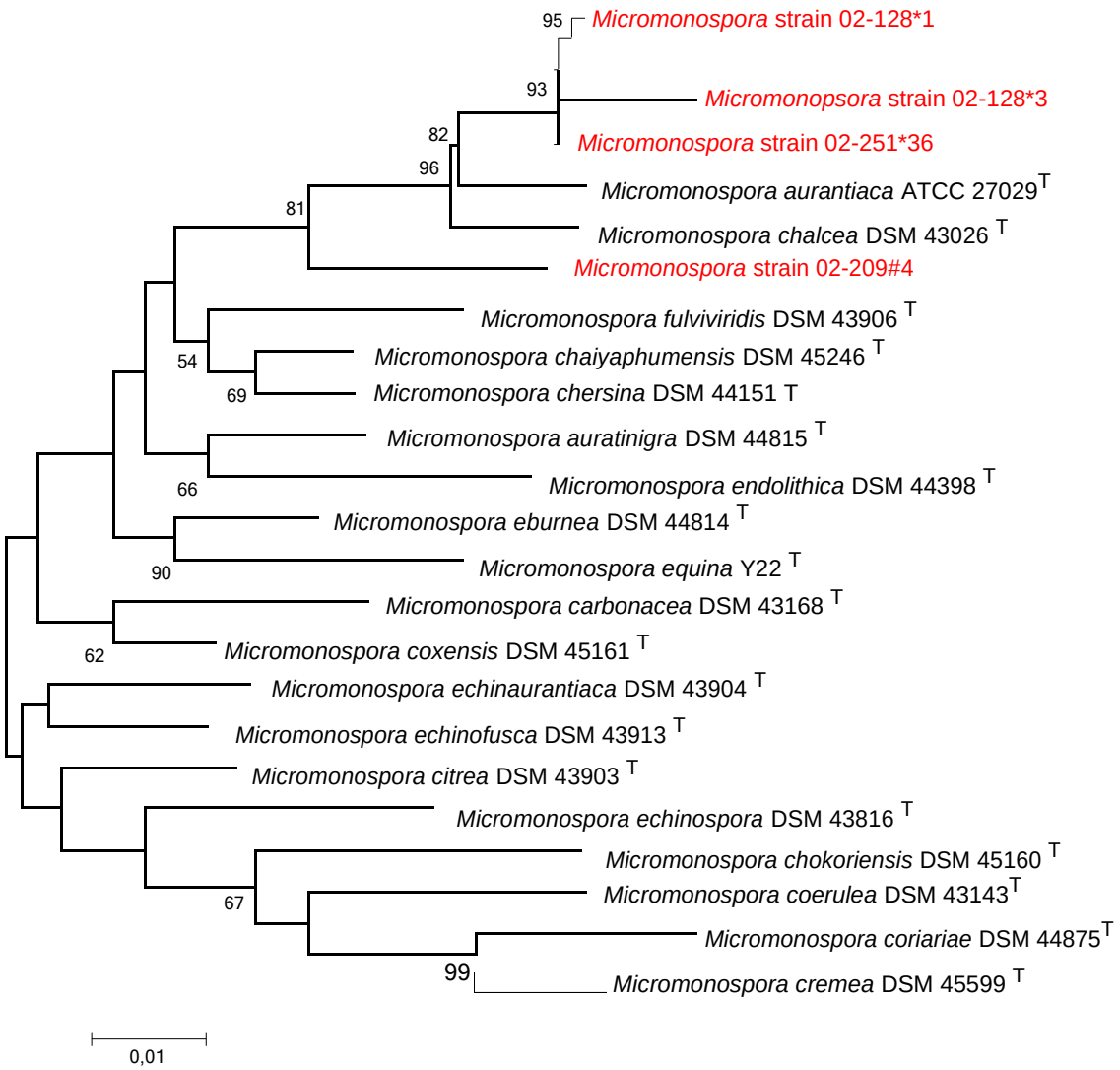

Figure An7: A Maximum likelihood phylogenetic tree (Saitou & Nei, 1987), is shown between the MLSA derived composite sequences of the 5 *Micromonospora* strains (02-128*3, 02-209#4, 02-128*1, 02-251*36, 04-015#16) and Type strains of other *Micromonospora* genus. During the construction of this tree, the 5 *Micromonospora* strains were presented as concatenated sequences obtained by combining their individual 16S sequences, *gyr*B and *rpo*B gene sequences from the MLSA study. The same order of sequence assembly was maintained for all the strains. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 24 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 2837 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

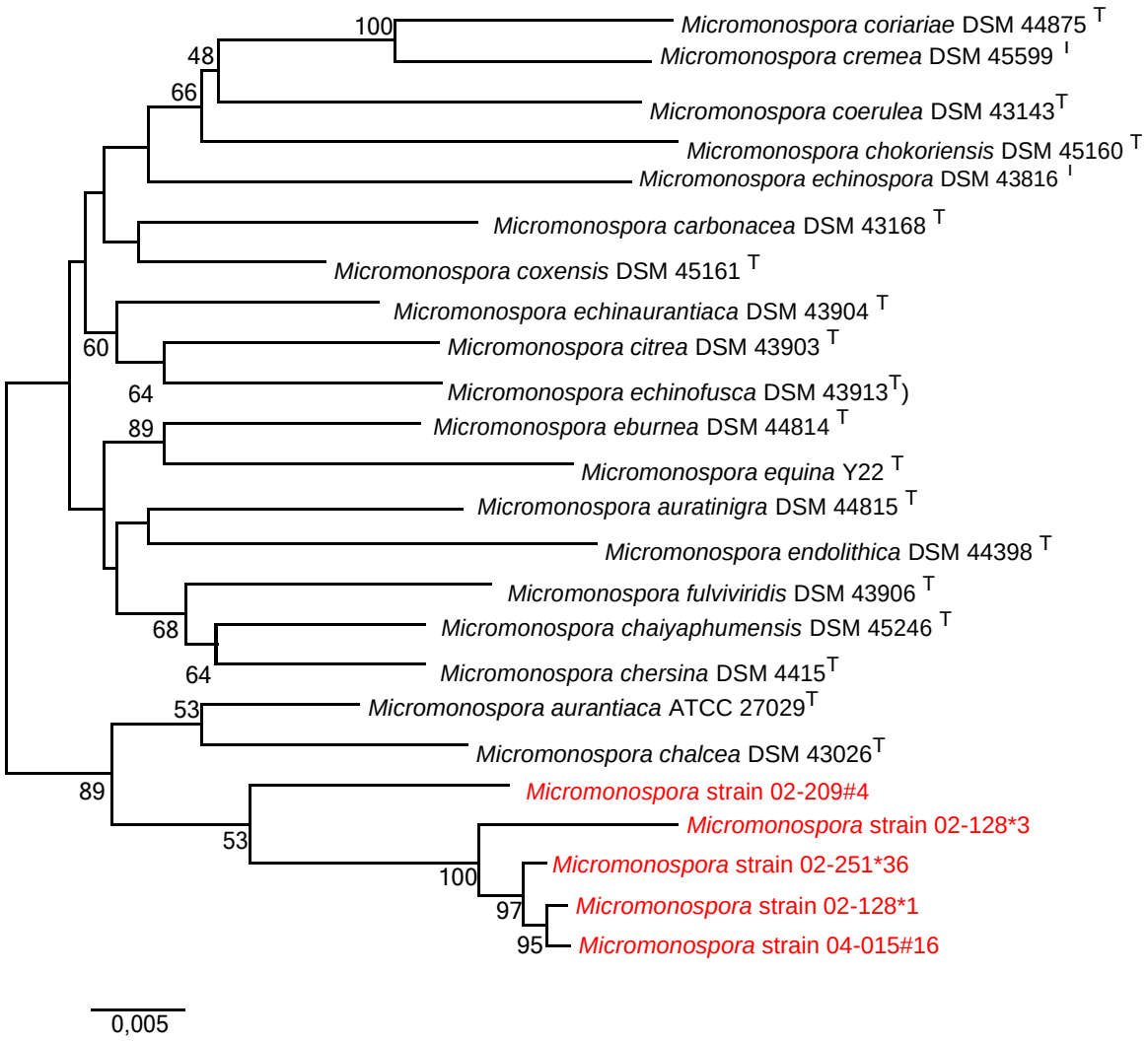

Figure An7: A Minimum evolution phylogenetic tree (Saitou & Nei, 1987), is shown between the MLSA derived composite sequences of the 5 *Micromonospora* strains (02-128*3, 02-209#4, 02-128*1, 02-251*36, 04-015#16) and Type strains of other *Micromonospora* genus. During the construction of this tree, the 5 *Micromonospora* strains were presented as concatenated sequences obtained by combining their individual 16S sequences, *gyr*B and *rpo*B gene sequences from the MLSA study. The same order of sequence assembly was maintained for all the strains. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 24 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 2837 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

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ANNEXURE E: BACTERIOCIN PRIMER SYNTHESIS

Inqaba Biotechnical Industries (Pty) Ltd

Co. Reg. No: 2001/011245/07
VAT No: 4150197251

inqaba biotec™

Prepare

Cape Peninsula University of Technology
(CAP001)
Marilize Le Roes-Hill
Bellville Campus
Institute of Biomedical and Microbial
Biotechnology
Bellville 7535
South Africa
Phone: 27 (0)21 9538499

Thank you for choosing inqaba biotec, Africa's Genomics company, for your oligonucleotide needs.We are the only commercial DNA synthesis facility in Africa and boast over 15 years of experience.Do not hesitate to contact us for technical support. We do also offer a quality portfolio of auxiliary PCR reagents and sequencing services.

Cape Peninsula University of Technology
(CAP001)
Marilize Le Roes-Hill
Bellville Campus
Institute of Biomedical and Microbial
Biotechnology
Bellville 7535
South Africa
Phone: 27 (0)21 9538499

References

Order Number: SA2019/72857 **Oligo Ref #:** 1045451 **Validated On:** **Print Date:** 2019-05-31

Name: BAC-F	**Barcode:** S255D	**Manufacturing Date:**	
Sequence: CTGCGCTTCGTCGAGGTC	**Length:** 18		**PAGE QC Image**
OD: 6.54	**MW min \ max:** 5563.15	**5' Mod:** None	
nmoles: 36.33	**GC % min \ max:** 66	**3' Mod:** None	
Tm min \ max: 54.88	**Purification:** Standard		
For a 100 µM stock solution add 363.32 **µl water or buffer**			
Comments:			

Name: BAC-R	**Barcode:** S255E	**Manufacturing Date:**	
Sequence: CATCAGGGGCAGCGCAAG	**Length:** 18		**PAGE QC Image**
OD: 8.48	**MW min \ max:** 5639.26	**5' Mod:** None	
nmoles: 47.1	**GC % min \ max:** 66	**3' Mod:** None	
Tm min \ max: 54.88	**Purification:** Standard		
For a 100 µM stock solution add 470.99 **µl water or buffer**			
Comments:			

ANNEXURE F: LANTHIPEPTIDE PRIMER SYNTHESIS

inqaba biotec™

Africa's Genomics Company

Inqaba Biotechnical Industries (Pty) Ltd

Co. Reg. No: 2001/011245/07

VAT No: 4150197251

Name: LAN-F	**Barcode:** S255F	**Manufacturing Date:**	
Sequence: TACCIGCIGGAGACCCTG	**Length:** 16		**PAGE QC Image**
OD: 12.38	**MW min \ max:** 5484.17	**5' Mod:** None	
nmoles: 77.4	**GC % min \ max:** 61	**3' Mod:** None	
Tm min \ max: 52.6 \ 57.16	**Purification:** Standard		
For a 100 µM stock solution add 774.01 **µl water or buffer**			
Comments:			

Name: LAN-R	**Barcode:** S2560	**Manufacturing Date:**	
Sequence: CTCGTAGIGGGCGTIGIG	**Length:** 15		**PAGE QC Image**
OD: 8.41	**MW min \ max:** 5550.22	**5' Mod:** None	
nmoles: 56.05	**GC % min \ max:** 61	**3' Mod:** None	
Tm min \ max: 52.6 \ 59.43	**Purification:** Standard		
For a 100 µM stock solution add 560.5 **µl water or buffer**			
Comments:			

RECOMMENDATIONS FOR HANDLING AND STORAGE OF OLIGOS

- Lyophilized oligo pellets might become displaced from the bottom of the tube during shipment. Briefly centrifuge each tube before opening to prevent the loss of the pellet.
- Prepare stock solution of oligos (e.g. 100 uM = 100 pmole per ul) preferably with a sterile buffered solution such as TE (10 mM Tris, pH 7.5 to 8.0, 1 mM EDTA). If sterile distilled water used, make sure that the pH is above 7.0 since acidic solutions favours oligo depurination and subsequent loss of activity.
- Working solutions might be diluted from the stock solution with sterile, nuclease-free water to prevent inhibition of enzymatic reactions (e.g. PCR) by EDTA.
- Store the oligos as concentrated stock solution or lyophilized at -20° C.
- Avoid frequent freeze-thaw cycles by dividing the stock solution into smaller aliquots for long term storage and to prevent accidental contamination.
- Dye-modified oligos are light sensitive and should always be stored in the dark.
- Resuspend modified oligos preferably in a slightly basic solution (i.e., TE at pH 8.0). However, Cy dye modified oligos are best kept at pH 7.0 at -20° C.
- Preferably store the modified oligos as dried aliquots at -20° C.

PRODUCTS FOR RESEARCH USE ONLY!