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Effect of co-culture on the activity profile of marine actinobacteria

by

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Declaration

I, Danielle Dana Mitchell, declare that the contents of this thesis represent my own unaided work, and that the thesis/dissertation has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed

31 July 2024

Date

Abstract

Over the past few years, there has been a dramatic increase in the number of antimicrobial-resistant pathogens. However, this increase has not been matched with the discovery of new antibiotics for the treatment of diseases or infections caused by these antimicrobial-resistant pathogens. In order to address this, marine actinobacterial diversity was explored as a potential source of novel antibiotics. Metabarcoding analysis of sediment samples collected at three subsites, designated as 'Dry', 'Ocean' and 'Rocky', from a tidal pool located within the Table Mountain National Park (TMNP2), reflected a core actinobiome consisting of unculturable and unidentified actinobacteria. In order to access this untapped diversity, actinobacteria were isolated from sediment samples. Initial bioactivity screening of twenty actinobacterial isolates was conducted against a set of ESKAPE pathogens: *Acinetobacter baumannii* ATCC 19606 and ATCC BAA-1605, *Enterococcus faecalis* ATCC 29212 and ATCC 51299, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC BAA-700603, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 29213 and ATCC 33591.

Out of the twenty actinobacterial isolates, eight isolates displayed strong activity against ESKAPE pathogens when cross-streaked on M19, M19 with Red Sea salts (M19+), International *Streptomyces* Project medium 2 (ISP2), and ISP2 with Red Sea salts (ISP2+) media. These isolates were then identified by 16S rRNA sequencing, and it was found that all eight belong to the genus *Streptomyces*. The eight actinobacterial isolates were also cultured as mono-cultures in liquid media to determine their capacity to produce bioactive compounds. A time-based study was performed to determine at which time point (day 1, day 3, day 5, or day 10) the best activity was observed. Extracts of the mono-cultures were prepared, and filter disc assays were performed to determine the activity of the extracts against the ten ESKAPE pathogens. Only two strains, R-30 and R-35, exhibited strong activities against *S. aureus* ATCC 29213 and *A. baumannii* ATCC 19606 when cultured in ISP2. These two strains also showed activity against all ten test strains on solid media.

Strains R-30 and R-35 were therefore chosen for further study, where they were indirectly co-cultured with each other (separated by a membrane filter to ensure non-contact) and directly co-cultured with 'alive' and 'dead' *Mycobacterium aurum* A+. The strains were first grown separately to ensure growth and thereafter added together in one flask to determine if there was a change in the bioactivity of R-30 and R-35. When R-30 and R-35 were co-cultured together, both isolates showed an increase in activity on day 1 (T_1). When co-cultured with 'alive' and 'dead' *M. aurum* A+, only R-35 showed good activity against four test strains. When co-cultured with different concentrations of *M. aurum* A+, the extracts prepared from R-35 showed very good activity against eight of the test strains. Metabolomic analyses via tandem mass spectrometry (MS/MS) fingerprinting were performed on the extracts prepared from the mono-cultures and the co-cultures. It was observed that antimycin was one of the main

secondary metabolites present in the extracts prepared from the indirect co-cultures of R-30 and R-35. MS/MS fingerprinting of extracts prepared from the direct co-cultures of R-35 and *M. aurum* A+, showed the presence of desferrioxamines and N-acetyltyramine. Due to the promising results obtained, whole genome sequencing of strain R-35 was performed to identify potential biosynthetic gene clusters involved in secondary metabolite production under different co-culture conditions. The ability of strain R-35 to produce antimycin and desferrioxamine was confirmed by the antiSMASH results, which indicated the presence of biosynthetic gene clusters that encode for antimycin, desferrioxamine E and desferrioxamine D. The antiSMASH report also indicated the presence of naphthyridinomycin, curamycin, aurantimycin, albaflavenone, himastatin, nigericin, and desferrioxamine B biosynthetic gene clusters, but these compounds were not identified in the MS/MS data analyses. The molecular networking analysis of the metabolomics data also confirmed the presence of multiple unknown secondary metabolites.

The outcome of this study, therefore, confirms that secondary metabolite production is enhanced when two marine actinobacterial isolates are co-cultured together or when they are co-cultured with other bacteria, such as the mycolic acid-producing *M. aurum* A+. Future studies will be aimed at isolating and identifying the compounds responsible for the activity seen in the co-culture experiments and determining if the activity seen is caused by one individual compound class or if it is a group of compounds working together to produce the activity.

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Dedication

I would like to dedicate this to my parents and grandparents. To my parents who have raised me to be a strong and independent woman. Always encouraging me to put my best foot forward and for guiding me in the right direction, always. To my grandparents, who could not be here to see how far I have come and how much I have grown. I know they will always be with me, and that they have been watching over me since the day they have left. I hope that I have made you all proud, and I look forward to making you even more proud in the future.

For Glen & Levona (parents), and my angels – Elaine, Racheal & Abbas (grandparents)

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Abbreviations/Acronyms

ARTS	Antibiotic Resistance Target Seekers
ATCC	American Type Culture Collection
BGC	Biosynthetic Gene Cluster
CAF	Central Analytical Facility
CARD	Comprehensive Antibiotic Resistance Database
CHPC	Centre for High Performance Computing
CSIR	Council for Scientific and Industrial Research
HPLC	High-performance Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
LSF	Liquid State Fermentation
mgDNA	Metagenomic deoxyribonucleic acid
MS/MS	Tandem mass spectrometry
m/z	Mass to charge ratio
NMR	Nuclear Magnetic Resonance
NRPs	Non-ribosomal peptides
NRPS	Non-ribosomal peptide synthetase
OFAT	One factor at a time
OSMAC	One strain many compounds
OTU	Operational taxonomic units
PKs	Polyketides
PKS	Polyketide synthase
RGI	Resistance gene identifiers
rpm	Revolutions per minute
SSF	Solid state fermentation

TLC	Thin-layer chromatography
TMNP2	Table Mountain National Park 2

Glossary

Term	Definition of terms used in this study
Antibiotic	Medicines that inhibit the growth of or destroys microorganisms.
Bioactive compound	A compound having an effect on or causing a reaction or triggers a response from a living system.
Crude extract	Freshly obtained product acquired from the extraction process of the natural substance.
Dereplication	The use of chromatographic and spectroscopic analysis to recognise previously isolated substances in an extract.
Metabarcoding	A targeted sequence-based approach that identifies species from a bulk DNA or environmental DNA sample.
Metataxonomics	The sequencing of marker genes, usually regions of the ribosomal RNA (rRNA) gene that is highly conserved across taxa. Often synonymously used for 'metabarcoding'.
Microbiome	The collection of all microorganisms such as bacteria, fungi, viruses and their genes that are naturally present in a particular environment.
Secondary metabolites	Compounds that are not required for the growth or reproduction of an organism but are produced to confer a selective advantage in the environment.

Chapter 1

Background and problem statement

1.1. Introduction

Antibiotics were accidentally discovered for the first time in 1928 in London by a Bacteriology Professor, Sir Alexander Fleming, at St. Mary's Hospital. The discovery was made while going through agar plates that contained *Staphylococcus,* which is a bacterium that causes sore throat, boils, and abscesses. On the same petri dish, he noticed a fungal contaminant and observed that there was no bacterial growth around the area where the fungus was present. He then isolated the fungus and found that it was a strain of *Penicillium notatum.* Once an extract was obtained, he named the active agent penicillin. With this discovery, he concluded that penicillin has an effect on staphylococci as well as other Gram-positive pathogens (Gaynes, 2017:849).

Antimicrobial resistance (AMR) of bacteria occurs when drugs used to treat bacteria lose their effectiveness against the bacteria. It has been hypothesised that bacteria with AMR will cause the death of millions more people per year (O'Neill, 2014). There are a few ways that resistance can develop; long exposure to the pathogen without treatment, excessive use of antibiotics, and infection being untreatable due to the patient's lack of access to proper medication, to name a few. This could lead to multi-drug resistance (MDR) or untreatable resistance (De Kraker & Lipsitch, 2021:58).

The ESKAPE pathogens, *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Escherichia coli* are the most worrisome pathogens where AMR is concerned. The MDR development of these pathogens is mostly due to the overuse of antibiotics in both agricultural and clinical applications (Haddad et al*.,* 2019:167). The ESKAPE pathogens are the leading cause of nosocomial infections, which involve high morbidity and mortality rates (Santajit & Indrawattana, 2016).

Scientists have relied on natural products as a source of therapeutic agents over the past decades. Different types of microorganisms have been identified as producers of a wide range of bioactive compounds, including antibacterial compounds (Amaning Danquah et al., 2022). Antibacterial compounds are naturally produced by various species of bacteria and fungi, but the most promising class of microorganisms that can produce secondary metabolites are actinobacteria, specifically, actinomycetes (De Simeis & Serra, 2021).

The majority of the earth is covered by the ocean, and it has been estimated that the marine environment is richer in biological diversity than that of tropical rainforests. This theory stems from the conclusion that the marine environment is more 'extreme' than its terrestrial counterpart (Imada et al*.,* 2010:12). Various studies have shown that the marine environment is a rich source of novel antibacterial metabolites produced by actinobacteria (*Actinomycetes*) (Manivasagan et al*.*, 2014:265; De La Hoz-Romo et al., 2022). Metabarcoding of marine environments can provide insights into the actinobacterial communities present, thereby guiding the isolation techniques to be employed for the isolation of potentially novel actinobacterial strains (Ribeiro et al., 2023)

In nature, microorganisms cohabitate. This may lead to the production of antimicrobial compounds from one organism against another to provide them with a competitive edge in a 'hostile' environment where they have to compete for sustainable resources (Yu et al*.,* 2019). For this reason, scientists have tried to mimic the competitive environment through co-culturing actinobacteria with other microorganisms in order to stimulate the production of secondary metabolites that are usually silent when a microorganism is cultured on its own (Tan et al*.*, 2019:18).

In addition to searching for novel metabolites in underexplored environments, determining the potential of actinobacteria to produce novel compounds is a necessity. Thus, the use of genomics has been employed to determine the potential biosynthetic pathways that could be accessed for secondary metabolite production from actinobacteria (Hug et al*.,* 2018). Genome mining is commonly used to analyse DNA sequences to determine the potential of a microorganism to produce compounds of interest (Sekurova et al*.,* 2019). This allows for the exploration of genomic information to target specific genes that might prove useful for metabolite production (Colgrave, 2015:113). The use of genomics and genome mining together allows for the study of more than one gene at a time (Moss et al., 2018:3-4). The most common tool used for genome mining is antiSMASH. This tool is capable of annotating gene clusters that are predicted to produce various antibiotics and other secondary metabolites based on non-ribosomal peptides (NRPs), polyketides (PKs), non-ribosomal peptide synthetases (NRPS), and polyketide synthases (PKS) (Blin et al*.,* 2021: W29, W30; Blin et al*.,* 2023: W47). This information can then be linked to metabolomics data generated from extracts prepared from actinobacterial cultures, whether in mono-culture or co-culture, thereby guiding the researcher toward the optimal conditions required for the production of specific secondary metabolites (van der Hooft et al., 2020).

1.2 Study aim and objectives

The main aim of this study is to induce the production of potentially novel bioactive compounds in marine actinobacteria through the use of a co-culture technique. This was accomplished by the following objectives:

- To determine the actinobacterial diversity associated with marine sediment samples collected from a tidal pool within the Table Mountain National Park as a guide to isolation approaches.
- To selectively isolate marine actinobacteria from the collected marine sediment samples.
- To screen the actinobacterial isolates for bioactivity against selected bacterial strains belonging to the ESKAPE group of pathogens.
- To identify the actinobacterial strains that exhibit bioactivity.
- To determine the effect of co-culture on the production of bioactive compounds by the most promising actinobacterial strains.
- To sequence the genome of the most promising strain and to perform genome mining to determine the presence of promising biosynthetic gene clusters.

Thesis structure: Chapter 2 will provide further background to the study. The following chapters will describe the methods and materials as well as the results and discussion for each experiment carried out in this study. Chapter 3 will show the actinobacterial community profiles of the study site used and the isolation of actinobacteria, Chapter 4 introduces the pre-screening approaches for the detection of antimicrobial activity, and Chapter 5 will address the co-culture experiments performed in order to induce the production of a new set of bioactive compounds, and this will be followed by the conclusions and recommendations in Chapter 6.

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Chapter 2

Literature Review

2.1 Introduction

Since the late 1920s, bio-active compounds, also referred to as antibiotics have flooded the market and found application in a range of industries. During this so-called “Golden era” of antibiotic discovery, thousands of secondary metabolites were discovered (Bérdy, 2012:385). However, since the late 1980s, there has been no new antibiotic class that has entered the pipeline for application in the medical field. This drop in discovery has been linked to various causes, including the exhaustive screening for antibiotics from specific sources to the high costs of screening collections of organisms that produce antibiotics (Zotchev, 2012:168; Zhu et al., 2014:3; Uddin et al*.,* 2021:1753). Since only 1–3% of all antibiotics have been discovered, there is still a high possibility of isolating new antibiotics from bacteria, particularly from actinobacteria (Bérdy, 2012:389; Kumbhar & Watve, 2013:32).

2.2 Antibiotic resistance

In June 2014, the World Health Organisation (WHO) published its first report on global antibiotic resistance surveillance (WHO, 2014). In this report, the increased incidence of antibiotic resistance is noted to be a major burden on health systems and the global economy. Should the current trend continue, it is believed that we will enter a post-antibiotic era where a common infection that was once treatable will lead to death. It is evident and well-recognized that there is an urgent need for new compounds to meet the emergent crisis of antibiotic-resistant microorganisms (Schäberele & Hack, 2014:15).

The main molecular mechanisms through which bacteria develop resistance to antibiotics is through the expression of antibiotic resistance genes (ARG). These mechanisms include reduced permeability and increased efflux, and transfer of resistance genes, to name a few (Zhang et al., 2020). Being regularly exposed to antibiotics leads to antibiotic-resistant microorganisms through mutation and horizontal gene transfer. Genetic information transfer can occur due to mobile elements such as plasmids, integrons, as well as transposons. The use of antibiotics on organisms that have undergone horizontal gene transfer also has a major influence on the occurrence of antibiotic resistance genes in a microbial community (Cheng et al., 2020; Lu et al., 2020; Aleshukina et al., 2022:S12).

2.3 The ESKAPE pathogens

The WHO specifically identified increased antibiotic resistance incidence in strains of *Acinetobacter baumannii* (resistant to carbapenem), *Escherichia coli* (resistant to third-

generation cephalosporins), *Staphylococcus aureus* (methicillin-resistant), *Streptococcus pneumoniae* (resistant to penicillin), and *Klebsiella pneumoniae* (resistant to third-generation cephalosporins) that lead to a higher mortality and morbidity rate (WHO, 2014; Kyriakidis et al*.,* 2021).

2.3.1 *Acinetobacter baumannii*

Acinetobacter baumannii is a Gram-negative pathogen that causes peritonitis, bacteremia, pneumonia, surgical wound infection, meningitis, and urinary tract infections. The increasing mortality rate associated with blood infections and ventilator-associated pneumonia is of great concern. Approximately 500 000 people clinically infected with *A. baumannii* are infected with multi-drug resistant (MDR) strains of the pathogen (Usmani et al., 2021). Recent studies indicated that there are limited bioactive agents that inhibit or reduce clinical infections. As MDR *A. baumannii* strains continue to pose a threat to humans, the urgency for scientists to derive new potential drugs rises.

2.3.2 *Enterobacter cloacae*

Enterobacter cloacae is a Gram-negative bacterium that causes a variety of infections such as bacteremia, septic arthritis, endocarditis, osteomyelitis, and hospital-acquired sepsis (Liu et al*.,* 2018; Dong et al*.,* 2020; Álvarez-Marín et al*.,* 2021:352). The common occurrence of *E. cloacae* in the terrestrial and marine environment continues to gain attraction due to the remarkable adaptability of the pathogen, as well as the high level of antibiotic resistance of the pathogen. The ability of *E. cloacae* to produce AmpC beta-lactamases that hydrolyse beta-lactam antibiotics, such as aminopenicillins and cefazolin, reduces the effectiveness of the antibiotics against this pathogen (Liu et al., 2018:444).

2.3.3 *Escherichia coli*

Escherichia coli is one of the most common foodborne pathogens and can be classified into six different pathotypes: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC). An epidemiology study conducted in Korea reported that the most common *E. coli* outbreak was caused by EPEC at 60.5%, while the least common cases were due to EIEC at 1.5% (Choi et al*.,* 2021).

Escherichia coli also poses a threat to pregnant women. In pregnant women, the most common pathotype is uropathogenic *E. coli* (UPEC). The ability of *E. coli* to colonise different sites in the body is due to its genomic characteristics. The genes expressed, e.g., genes that code for virulence factors, determine how severe an *E. coli* infection will be. Over the past few years, there has been an increase in antibiotic-resistant urinary tract infections. Phylogenetic analysis

has determined that *E. coli* belongs to four major phylogroups (A, B1, B2, and D) that each possess a unique gene profile that distinguishes its particular evolutionary pattern. Group B2 and D are more virulent and found more often in clinical samples, compared to group A and B1, which are generally found at elevated levels in commensal samples (Eghbalpour et al., 2022).

Rifampicin is an antibiotic that is used to inhibit the initiation of transcription in bacteria and has been effectively used in the treatment of *E. coli*, *Mycobacterium tuberculosis*, and staphylococci over the years. However, resistance to this compound has become a great threat to the health of humankind. Mutations in the *rpoB* gene that encodes the β-subunit of RNA polymerase can cause resistance to Rifampicin. This occurrence was first identified in *E. coli*, where scientists discovered mutations in the 81 bp region at codons 507-533. This mutation caused Rifampicin to be ineffective against certain *E. coli* strains (Urusova et al*.,* 2022).

2.3.4 *Enterococcus faecalis*

Enterococcus faecalis is a Gram-positive facultative anaerobic bacterium that commonly causes neonatal sepsis, urinary tract infections, blood infections, and endocarditis and often inhabits the intestinal tract. *Enterococcus faecalis* is responsible for over 90% of enterococcal infections, it is also the third most common cause of infective endocarditis. A yearly study conducted by the Australian Enterococcal Sepsis Outcome Program (AESOP) determined that 33% of the enterococcal population was *E. faecalis.* The horrifying observation that came about was that *E. faecalis* has the ability to develop new drug resistance, and antibiotic-resistant *E. faecalis* has become a severe problem globally. Antibiotics that *E. faecalis* is resistant to include β-lactam antibiotics, teicoplanin, and glycopeptides such as vancomycin, to name a few (Kristich et al., 2014:1-2; Lwin & Bannan, 2020).

2.3.5 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a Gram-negative bacterium that can be isolated from surface waters, soil, plants, as well as the gut of healthy humans and animals. *Klebsiella pneumoniae* can cause infections in the digestive tract (Håkonsholm et al., 2022). In general, the presence of this bacterium in the gut does not pose any threat to an individual, but once the pathogen moves to other parts of the body such as the brain, bladder, bloodstream, lungs, wounds, liver, or eyes, it can cause severe infections. *Klebsiella pneumoniae* is resistant to many antibiotics which causes it to be one of the most dangerous pathogens to humans (Al-Mahfoodh et al*.,* 2021). The WHO considers carbapenem-resistant *K. pneumoniae* (CRKP) and extended-spectrum β-lactam (ESBL) resistant *K. pneumoniae* to be a serious threat to public health. *Klebsiella pneumoniae* is resistant to the primary antibiotic classes, namely cephalosporins,

aminoglycosides, carbapenems, and fosfomycin, which makes these drugs ineffective as treatment methods (Li et al., 2023).

2.3.6 *Staphylococcus aureus*

The Gram-positive bacterium, *S. aureus*, is one of the most prominent causes of bacterial infections in the bloodstream, soft tissue and skin, and the respiratory tract in many developed countries. A quarter of the human population are carriers of *S. aureus,* and it can be found in the nose, groin, throat, gut, and armpit of the human body (Lindsay, 2013:318). Methicillin-resistant *S. aureus* (MRSA) has gained the staphylococcal cassette chromosome *mec* (SCCmec). Infections caused by MRSA are one of the main causes of mortality linked to any single infectious agent (Lindsay, 2013:318). A study conducted by Delaney et al. (2008) in Canada, indicated that out of 1439 patients diagnosed with MRSA, within one year of diagnosis, 314 patients (21.8%) had died. This indicated that MRSA infections are associated with mortality within one year of diagnosis. Over the past fifty years, vancomycin has been the drug of choice when combating MRSA. However, in recent years, vancomycin as a treatment has failed against MRSA and has raised many questions. Although many new antibiotics have been discovered, none has proven superior to vancomycin against MRSA (Pastagia et al., 2012:1072).

2.3.7 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an important opportunistic, Gram-negative bacterium that may cause severe infections in individuals with a compromised immune system, particularly in individuals who suffer from chronic respiratory diseases such as cystic fibrosis. People who suffer from cystic fibrosis infected with *P. aeruginosa* often develop a decrease in pulmonary function and have an increased risk of death. This infection is generally treated with aggressive antibiotics such as nebulized concentrated tobramycin, aminoglycosides, and aztreonam solution for inhalation, which leads to the risk of developing a multiple drug-resistant strain of *P. aeruginosa* (Ehsan & Clancy, 2015:1903-1904; Bonyadi et al*.,* 2022).

The difficulty of treating *P. aeruginosa* is due to the efflux pump mechanism by which the outer membrane pumps toxic components out of the cell, causing antibiotics to be ineffective against the pathogen. This mechanism is present within most of the *P. aeruginosa* strains. With the treatment for *P. aeruginosa* as difficult as it currently is, it has been suggested that anti-virulence compounds, such as quorum sensing inhibitors, biofilm disruptors, and rhamnolipids, are to be used in conjunction with antibiotics as a treatment against multiple drug-resistant mutants (Duplantier et al., 2021; Laborda et al., 2021:129; Bonyadi et al*.,* 2022; Lorusso et al*.,* 2022).

2.3 Sources of antimicrobial compounds

As research continues to unfold, new chemical skeletons are continuously found amongst natural products. This supports the acclamation that nature is an innovative chemist. This is true because, over the last 3.5 billion years, microorganisms have been producing and creating novel products (Bull & Stach, 2007). Natural products, including antimicrobial compounds, have been isolated from a range of sources, including plants, fungi, and bacteria, to name a few (Bérdy, 2012:387).

Over the years, approximately 32500 natural products have originated from microbial sources, with nearly 1000 being sourced from the marine environment. The marine environment is entirely different when compared to the terrestrial environment (mainly because of the different forms of stresses such as salinity, temperature, and pressure), and therefore it has been deduced that organisms found in or near saltwater have different characteristics from their terrestrial relatives (Lam, 2006). It is expected that the native microbial population has adapted to the competitive conditions of the marine environment due to the harsh physical and chemical conditions. As a result of these adaptations, these organisms are able to produce novel molecules that are unique with respect to diversity, as well as structural and functional features when compared to compounds from their terrestrial relatives. This gives them a competitive advantage and allow these bacteria to survive and enhance their existence in this environment (Radjasa & Sabdono, 2003:11; Lam, 2006). Currently, macromolecules that have been isolated from this environment have the potential for commercial drug development. However, none of the compounds that displayed antibacterial properties have entered the market yet (Nalini et al*.,* 2018:697).

2.3.1 Actinobacteria as a source of bio-active compounds

Actinobacteria are Gram-positive, non-motile, and aerobic/microaerobic bacteria with a high G+C mol% in their DNA (Barka et al*.,* 2015:2; Seshadri et al*.*, 2022). Some members of this bacterial group also possess asexual spores (Barka et al., 2015:2). The genome size of actinobacteria can be as small as 0.5 Mbp or as big as 15 Mbp. These organisms display diverse morphological and physical characteristics (Seshadri et al*.*, 2022) and were previously considered as a transitional group between bacteria and fungi, but because of 16S rRNA gene sequencing, are now known as prokaryotic organisms (Veena et al*.,* 2016:584). The phylum *Actinomycetota* Goodfellow 2021 embodies one of the biggest taxonomic components among the major lineages that have recently been approved within the bacterial domain. The genus *Streptomyces* Waksman and Henrici 1943 (Family *Streptomycetaceae*, Order *Kitasatosporales*, Class *Actinomycetes*) is considered to be the dominant group amongst those within the Class *Actinomycetes* and occurs in widespread environments. However, they have been more extensively studied in the terrestrial environment compared to the marine

environment (Bérdy, 2012:390). The actinobacteria are well known for their antibiotic production capabilities where approximately 70% of commercially available antibiotics are derived from *Streptomyces* and *Micromonospora* species. Many of these organisms' secondary metabolites are effective against pathogenic organisms. Since the majority of the actinobacteria were isolated from the terrestrial environment it leaves the marine environment open for exploration. Actinobacteria has only recently (over the past 10-15 years) been isolated from the marine environment, which made it apparent that this environment is a source of novel antibiotics (Manivasagan et al., 2014:173,186; Hassan et al., 2017:35).

2.3.1.1 Bioactive compounds from marine actinobacteria

To date, a wide range of bio-active compounds have been isolated from marine actinobacteria. Abyssomicin C isolated from a *Verrucosispora* sp., Frigocyclinone isolated from *Streptomyces griseus*, Glutingimycin isolated from the marine streptomycete isolate B8652, and Himalomycins isolated from *Streptomyces* spp*.* are a few examples of compounds with antibacterial activity (Maskey et al*.,* 2003; Maskey et al., 2004; Bruntner et al*.,* 2005:347; Bunbamrung et al*.,* 2021) (Figure 2.1).

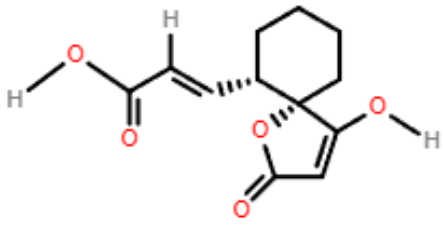
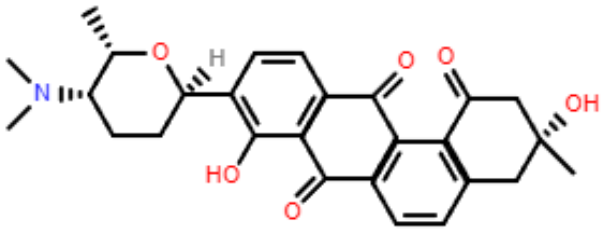
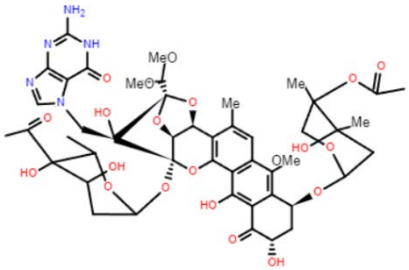
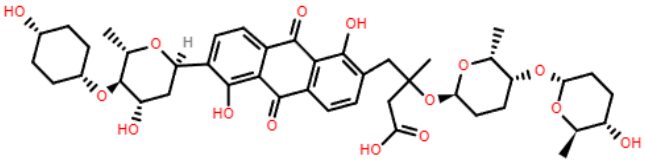
Abyssomicin C  (Bunbamrung et al., 2021)	Frigocyclinone  (Bruntner et al., 2005)
Glutingimycin  (Maskey et al., 2004)	Himalomycins  (Maskey et al., 2003)

Figure 2.1: Examples of antimicrobial compounds extracted from marine actinobacteria (structures were redrawn using ChemSpider elemental draw structure software. https://www.chemspider.com/StructureSearch.aspx)

Aureoverticillactam isolated from *Streptomyces aureoverticillatus*, Chinikomycins isolated from a *Streptomyces* sp., IM-00208 isolated from an *Actinomadura* sp., and Salinosporamide A (NPI-0052) isolated from *Salinispora tropica* are a few examples of compounds that display anticancer activity (Li et al., 2005; Olano et al., 2009:222; Lee & Jeong, 2020; Wang et al., 2021b:2011) (Figure 2.2).

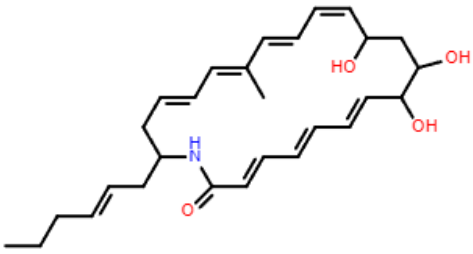
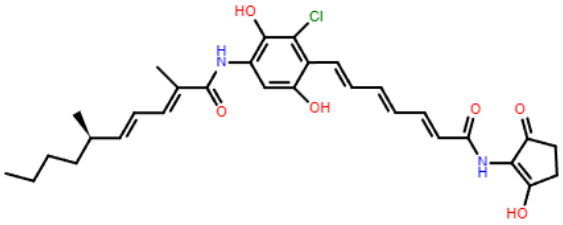
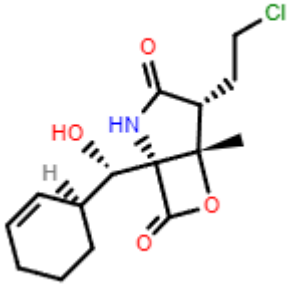
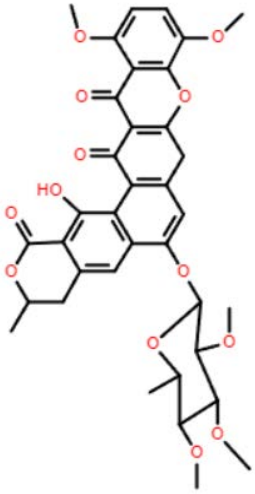
Aureoverticillactam  (Wang et al., 2021b)	Chinikomycin  (Li et al., 2005)
Salinosporamide A (NPI-0052)  (Lee and Jeong, 2020)	IB-00208  (Olano et al., 2009)

Figure 2.2: Examples of anticancer agents extracted from marine actinobacteria structures were redrawn using ChemSpider elemental draw structure software (https://www.chemspider.com/StructureSearch.aspx).

In addition, chloro-dihydroquinones isolated from novel actinobacteria, Diazepinomicin (ECO-4601) isolated from a *Micromonospora* sp., Marinomycins isolated from '*Marinispora'* and Trioxacarcins isolated from *Streptomyces* spp., are four examples of compounds discovered to have both antimicrobial and anticancer activity (Soria-Mercado et al., 2005; Kwon et al., 2006:1623; McAlpine et al., 2008; Švenda et al., 2011:6710) (Figure 2.3). These examples do not represent an exhaustive search of all novel secondary metabolites produced by marine actinobacteria, but merely highlight the great diversity and potential of actinobacteria isolated from the marine environment (Lam, 2006).

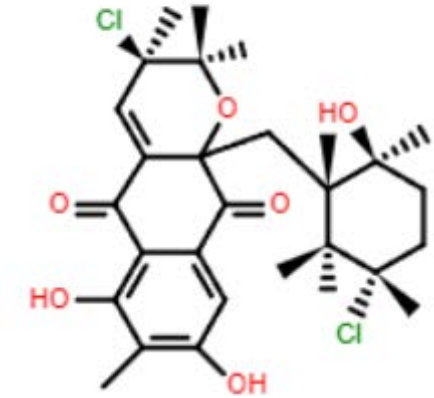
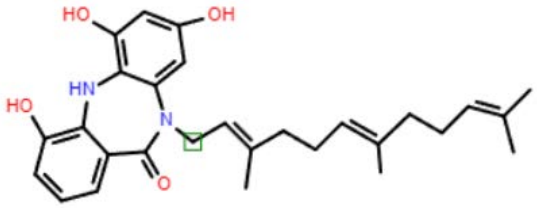
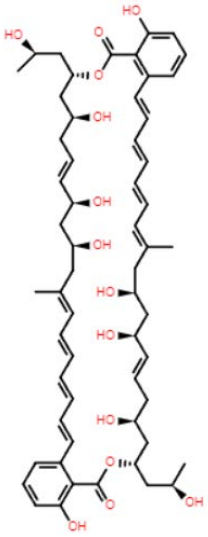
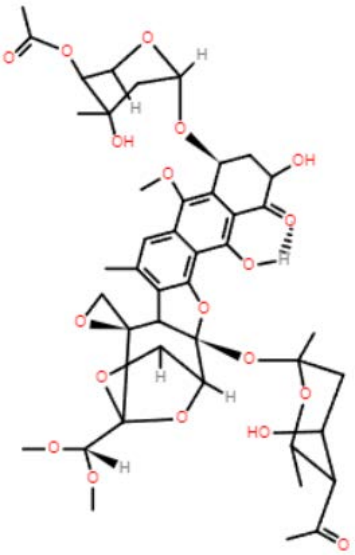
chloro-dihydroquinone  (Soria-Mercado et al., 2005)	Diazepinomicin (ECO-4601  (McAlpine et al., 2008)
Marinomycin  (Kwon et al., 2006:1632)	Trioxacarcin A  (Švenda et al., 2011:6710)

Figure 2.3: Structures of compounds that display both antimicrobial and anticancer properties isolated from marine actinobacteria (structures were redrawn using ChemSpider elemental draw structure software, https://www.chemspider.com/StructureSearch.aspx).

It is clear that marine actinobacteria are a major resource for biotechnology research (Manivasagen et al., 2014b:263). As is evident from the examples provided, marine actinobacteria produce secondary metabolites that possess a wide range of biological activities. These bioactive molecules may serve as a foundation for the synthesis of new curative pharmaceuticals which could reduce the spread of resistant pathogens (Hassan & Shaikh, 2017:47). The search for novel natural products isolated from the marine environment therefore plays an important part in both biomedical research and drug development, as drugs or as lead chemical structures for synthetic drugs. The availability of macromolecules that have been isolated from marine sources will, in the future, be beneficial in many different

applications in the development of pharmaceuticals to combat pathogenic organisms that have gained drug-resistant properties (Nalini et al*.,* 2018:707).

2.3.2 Effect of co-culture on the production of antibiotics

Microorganisms thrive in almost every environment, such as terrestrial, aquatic, and agricultural environments as well as in animal hosts. However, this means that the organisms present in these environmental niches are susceptible to invaders (Mickalide & Kuehn, 2019:521). Co-habitation of microbial species also assists in the evolution of species. This occurs when the characteristics of one organism are beneficial to another, which assists in increasing the ability of the organisms to survive in the environment (Foster & Bell, 2012:1846). Furthermore, the characterisation of the interaction between microorganisms is necessary to understand the function and structure of the microorganisms present in the aquatic ecosystem (Matsui et al*.,* 2000). Co-culturing actinobacteria with other microorganisms, such as bacteria and fungi, can often stimulate the production of secondary metabolites. This is due to the complex interactions and communication between the different organisms during co-habitation and, by extension, in co-culture. These metabolites can have various applications in medicine, agriculture, and industry (Peng et al., 2021:364-371).

Actinobacteria, like many other microorganisms, have the genetic potential to produce a wide array of secondary metabolites. However, under standard laboratory conditions, they may not express all these compounds. Co-culturing can sometimes induce the expression of metabolites encoded by silent or cryptic biosynthetic gene clusters, expanding the chemical diversity accessible from these bacteria (Chen, 2011:594; Scherlach & Hertweck, 2021). These genes are not typically expressed under standard laboratory conditions, even more so for marine actinobacteria (Kumbhar & Watve, 2013:26). Most researchers are in agreement that a deeper understanding of the role of the environment in the production of antibiotics is required to access these unique compounds. This information can be used to manipulate fermentation conditions, thereby inducing their expression (Goodfellow & Fiedler, 2010:121,133-135; Zhu et al., 2014:376). Co-culturing species is an approach used to identify the ability of strains to produce unique bioactive compounds typically not produced under standard growth conditions. When one or more species are included in a culture, the challenge of isolating a single active substance arises (Zhang & Straight, 2019). For a stable co-culture to be obtained, certain requirements need to be met. Firstly, the two strains that are chosen must be able to grow together. Secondly, the growth conditions of the two strains need to be compatible, meaning that the two chosen strains need to be able to grow at the same pH and temperature, and have the same oxygen requirements (Chen, 2011:591). Marine microorganisms are generally more difficult to culture than terrestrial microorganisms due to

co-dependency of marine microorganisms on positive microbial interactions (Romano et al., 2017). Growing strains up alongside one another in this way allows for microorganisms to grow in the same environment and allows chemical interactions to take place. This may then prompt the production of novel compounds that will not typically be produced if grown on their own, thus activating the silent biosynthetic pathways that allow for the production of defensive molecules or mutually beneficial metabolites (Arora et al*.,* 2020). Actinobacteria co-cultures have applications in drug discovery, where they can lead to the identification of novel bioactive compounds. Thus, co-culture of marine actinobacteria has gained a particular interest due to the unique ecological conditions and biodiversity of marine environments (Yu et al. 2019).

2.3.2.1 Mechanisms of Co-culture

A key characteristic of the marine environment that differs from the terrestrial environment, is that the organisms present in the marine environment are all connected by means of the surrounding water. Therefore, it becomes more of a challenge to determine direct cell-cell interactions, as the interactions between the microbial cells that are mediated by the surrounding water or liquid also need to be taken into consideration when co-culturing organisms in a liquid medium (Matsui et al., 2000:109).

I. Indirect contact co-culture

Co-culturing can involve indirect interactions between microorganisms. For instance, one species may modify the growth environment or alter the pH, oxygen levels, or other physical and chemical factors, which can indirectly affect the growth and metabolism of the organisms in a co-culture system (Tshikantwa et al*.,* 2018). A study was conducted between 2015 and 2017 using actinobacteria isolated from marine sponges in co-culture experiments, and the crude extracts obtained from both monoculture and co-culture were tested against Gram-positives (*Bacillus subtilis* and methicillin-sensitive *S. aureus*), a Gram-negative (*E. coli*), and a yeast (*Candida albicans*). Co-culture experiments were performed by using specially designed baffled flasks that were modified to allow two flasks to interconnect and be separated by means of a nylon filter with 0.2 µm pores. This allows for the transfer of molecules secreted by the two different bacterial strains inoculated in the individual flasks (Figure 2.4A). Once screening of the extracts was performed, it was found that extracts obtained where *Micromonospora* strains were co-cultured with a *Rhodococcus* strain showed the largest zone of inhibition. The investigators identified 20 co-cultures that produced novel bioactive compounds or secondary metabolites, with the use of both LC/MS-guided and bioassay-guided detection (Adani et al*.,* 2015:6095; Adani et al*.,* 2017).

II. Direct contact co-culture

Mycolic acid-containing bacteria are most commonly used for direct contact co-culture with actinobacteria (Adnani et al., 2015; Asamizu et al., 2015; Adnani et al., 2017). Experiments were also carried out to assess the mixed culture of 'dead' mycolic acid-containing bacteria with *Streptomyces lividans* (Figure 2.4B). This experiment led to the conclusion that mixed cultures that contained the 'dead' mycolic acid-containing bacteria, conserving the cell shape, had no effect on the production of natural products. This experiment affirmed that adding 'dead' mycolic acid-containing bacteria inhibited the production of specialised metabolites from *S. lividans* (Asamizu et al., 2015). However, another study conducted by Wang et al. (2021a) was performed with *Streptomyces* sp. FXJ1.264 and *Mycobacterium* sp. HX09-1. This experiment compared the extracts of single cultures of the aforementioned bacteria with the co-culture of these strains. The co-culture extracts proved to have a higher yield of secondary metabolites than that of the pure cultures, confirming that co-culturing *Streptomyces* with mycolic acid-containing bacteria has an effect on the secondary metabolites produced.

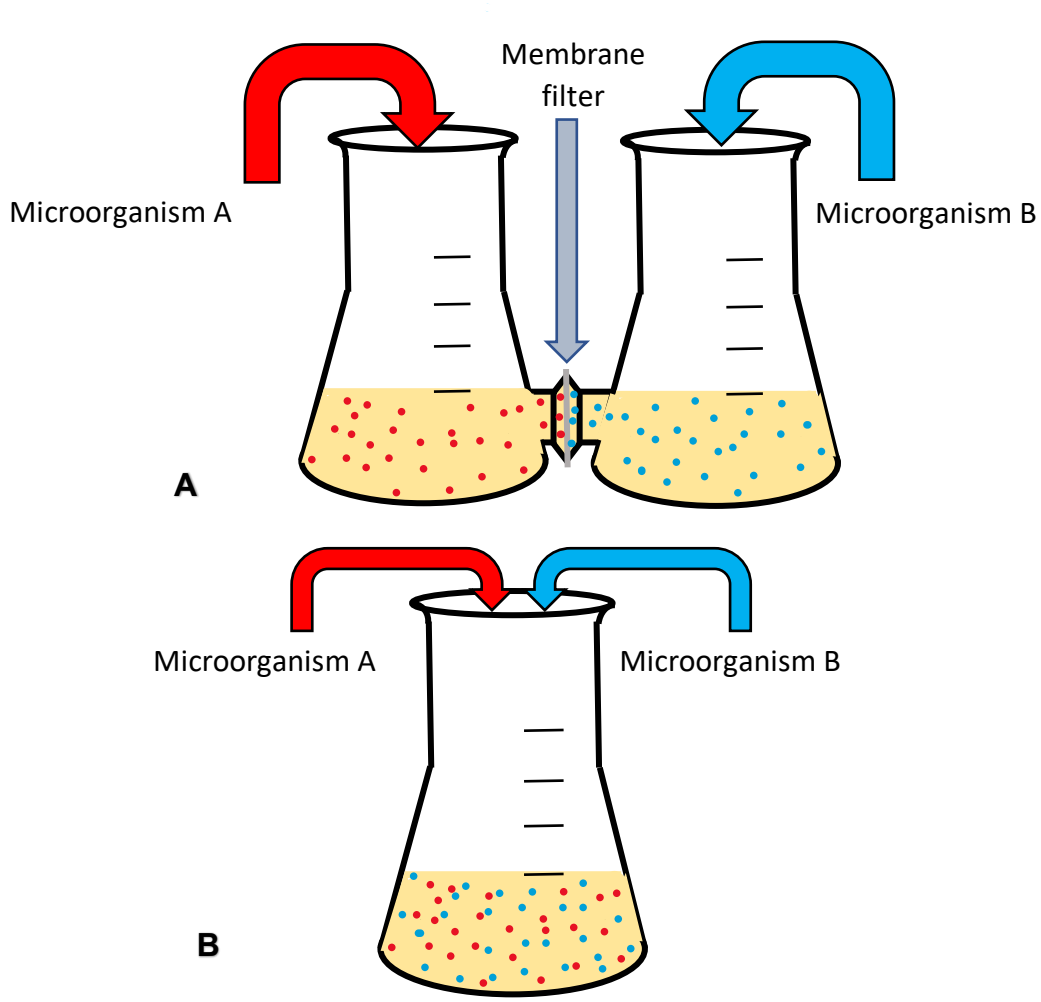


Figure 2.4: A - Indirect contact between microbial species separated by a filter disc to prevent individual organisms from passing through but allowing molecular compounds to pass through; B - Direct contact allows microorganisms to be cultured in the same media with physical contact between the two microorganisms (Kapoore et al., 2022:55)

Overall, actinobacteria co-culture is an exciting area of research that holds promise for the discovery of new bioactive compounds and the exploration of their ecological interactions in various environments. It represents a valuable strategy for unlocking the full potential of these microorganisms for biotechnological and pharmaceutical applications (Jones et al*.,* 2016:56-62).

2.4 Accessing novel bioactivity

Actinobacterial genomes (especially of the genus *Streptomyces*) are larger than most other bacteria and thus have a great ability to synthesise secondary metabolites. The taxonomic diversity covered by these sequenced genomes has revealed that new gene families are discovered that encode for bioactive compounds when more bacterial genomes are sequenced (Wu et al*.*, 2009:1057). Thus, identifying taxonomic diversity within a sampling environment reveals new genetic diversity and, therefore, potentially new biosynthetic capabilities (Goodfellow & Fiedler, 2010:120).

According to Zähner and Fiedler (1995), the main parameters that would influence the production of bioactive compounds from actinobacteria under fermentation conditions include the media composition (carbon and nitrogen sources used), pH, temperature, and time. Optimisation is often a lengthy process, and therefore, standard screening programs will typically start with screening the ability of the isolates to inhibit/kill certain test strains, e.g., standard overlay techniques used in the screening for antimicrobial activity in actinobacteria (Le Roes, 2005). Ideally, the test strains should have known antibiotic-resistance or susceptibility properties (e.g., test strains available from the American Type Culture Collection), thereby acting as a guide as to what the biosynthetic potential of the antibiotic-producing strain could be.

Rapid analyses of bio-active compounds can also be achieved through the use of thin-layer chromatography (TLC) combined with mass spectrometry to determine the bio-active compound fingerprint (metabolome) of strains (Zahn et al., 2001:377). Chemical screens such as high-performance liquid chromatography (HPLC) and TLC can be used in the identification of known classes of compounds or for the allocation of unknown compounds to a specific group at an early stage in a screening programme (Fiedler, 1993). However, for the identification of novel compounds, coupling HPLC with diode array detection, mass spectrometry (MS) and/or nuclear magnetic resonance spectrometry (NMR) is considered to be the more efficient method (Abel et al., 1999:220; Higgs et al., 2001:376). When Liquid chromatography-mass spectrometry (LC-MS)/ NMR is used in conjunction with biological activity assays, new leads can be detected in a very time-effective manner (Lee et al., 1999).

To increase the chances of identifying the secondary metabolites produced and to minimise replication, it is also recommended that the producing strain should be identified to the genus (and even species) level (Genilloud et al., 2011:376). This rational selection of strains by chemical and/or genetic fingerprinting (de-replication) could assist in overcoming the problems of re-isolation of known compounds since previously isolated organisms are excluded from screening programs (Colquhoun et al., 2000; Brandão et al., 2002:77).

2.4.1 Tandem mass spectrometry (MS/MS) fingerprinting

Tandem mass spectrometry or MS/MS fingerprinting, the basis of molecular networking, is a technique used in the field of metabolomics and proteomics to identify and characterise molecules based on their fragmentation patterns in a mass spectrometer (Dettmer et al., 2006:52). This technique involves generating a collection of MS/MS spectra from various molecules in a complex sample. This collection of fragmentation spectra forms a fingerprint-like pattern unique to each molecule or compound present in the sample. By comparing the experimental MS/MS spectra to spectral libraries or databases of known spectra, it is possible to identify and characterise the molecules present in the sample (Consonni et al., 2022).

In a traditional MS experiment, a sample is ionised, and the resulting ions are separated based on their mass-to-charge ratio (m/z) to create a mass spectrum. This shows the abundance of ions at different m/z values. However, this MS data alone may not provide enough information to confidently identify and characterise the individual molecules that are present in a sample (Ho et al., 2003:3). For more detailed information about the composition of the molecules in a sample, an MS/MS experiment is conducted. In this experiment, selected ions from the first mass spectrometer readings are further fragmented, typically by collision-induced dissociation or other fragmentation methods. The resulting fragments are then subjected to a second round of mass analysis to create a fragmentation spectrum or MS/MS spectrum (Neagu et al., 2022). MS/MS fingerprinting is a powerful tool in metabolomics and proteomics research where it is used to identify unknown metabolites, peptides and proteins and to study the metabolic pathways and biological processes associated with them. It enables researchers to gain a deeper understanding of the composition and complexity of biological samples and aids in the discovery of biomarkers, drug targets, and other biologically significant molecules (Gowda & Djukovic, 2014).

A study conducted by Kibret et al. (2018) used LC-MS to compare the profiles of extracts obtained from solid-state fermentation (SSF) and liquid-state fermentation (LSF) of *Streptomyces* spp. From this, the investigators were able to identify both known and unknown secondary metabolites where more novel secondary metabolites were found to be produced during the SSF. Another study showed that the use of MS/MS application coupled with dereplication aided in identifying the functional groups of novel antimicrobial compounds

responsible for bioactivity, the likelihood that each isolate will yield novel compounds, and the source of each metabolite identified (Sebak et al., 2019). Dereplication is a process whereby the chemical profiles of bioactive compounds from new strains are compared with known compounds to identify new molecules (Ito & Masubuchi, 2014:353)

2.4.2 Genome mining

Genome mining is the exploitation of genomic information by the identification of a particular DNA sequence within a specific genotype in order to identify a useful gene from that genotype (Colgrave, 2015:113). A unique sequence of adenine, thymine, guanine, and cytosine is used to describe a complete prokaryotic genome with a minimum of five hundred thousand base pairs for a symbiotic organism and over ten million base pairs for a saprophytic organism (Corre & Challis, 2010). There are many publicly accessible databases that contain many known DNA sequences along with their annotations. Using genome mining alongside genomics, it is possible to study, in-depth, more than one gene at a time. With this in place, new pathways for small-molecule drug discovery, protein-protein interaction characterisation as well as new modes of gene regulation are possible (Moss et al., 2018). Genome mining is one of the main available tools used for the search of novel antimicrobial compounds in more than 2000 sequenced microbial genomes (Poeta et al., 2018).

One of the most well-known and widely used bioinformatics tools for genome mining is “antiSMASH”. This tool was developed to identify and annotate gene clusters responsible for the biosynthesis of various secondary metabolites, including antibiotics in microbial genomes. This has given rise to independent tools such as antibiotic resistance target seekers (ARTS) and biosynthetic gene cluster (BGC) databases (Blin et al*.,* 2021: W29, W30). The primary focus of antiSMASH is on non-ribosomal peptides (NRPs), polyketides (PKs), and other types of secondary metabolites that are often synthesized by large, multi-domain enzymes known as non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS). These secondary metabolites have diverse biological activities and are of significant interest for drug discovery and biotechnological applications (Blin et al*.,* 2021: W30; Blin et al*.,* 2023: W47). The key features and functionalities of antiSMASH include gene cluster prediction that identifies and predicts gene clusters involved in the biosynthesis of secondary metabolites; structural prediction that predicts the possible chemical structure of the identified gene; as well as annotation and comparison which identifies gene clusters and other relevant features also allowing for comparison of gene clusters across different genomes (Medema et al*.,* 2011: W341&W342). As with any genome mining tool, there are limitations. Table 2.1 summarises the advantages and disadvantages of using antiSMASH for the prediction and identification of potential gene clusters – highlighting aspects that should be taken into account when applying this tool in a genomics-based study.

Table 2.1: Advantages and disadvantages of using the antiSMASH tool in the search for novel biosynthetic clusters (Medema et al., 2011: W341&W342; Blin et al., 2019a: 1108; Blin et al., 2019b: W83&W84).

Advantages of antiSMASH	Disadvantages of antiSMASH
• Comprehensive secondary metabolite analysis • Automatic annotation • Structural prediction • Comparative analysis • Drug discovery potential • Integration with other tools • Open access to database • User-friendly interface	• False positives and negatives limited to known gene clusters • Dependency on genome quality – easily influenced by the quality of the genome assemblies used as input data. Low quality fragmented segments may lead to less reliable results. • Overlapping gene clusters (protoclusters) – overlapping genes could lead to difficulties in accurately predicting the boundaries of individual clusters

2.4.3 Metabarcoding

Metagenomics is defined as the genetic analysis of whole genomes found in an environmental sample (Liu et al., 2020:373). Over the past 20 years, metagenomics has led to great developments within our understanding of microbial community structures, especially through the use of metabarcoding (amplicon-based metagenome sequencing). With 99% of the entire bacterial community being unculturable under standard laboratory conditions, metagenomics and metabarcoding allow one to tap into the genomic information of the unculturable (Zepeda Mendoza et al., 2015:745, 746). Metagenomics also allows for the comparison of the cultured to the uncultured based on their genetic information; for example, protein families and common or corresponding gene clusters can be identified that link one organism to the same family, genus, or species based on the amount of similarity found between the organisms once analyzed using bioinformatics. This technique has great potential in assisting the discovery of soil, marine, and possibly, in the future, air-dwelling isolates in the actinobacterial community (Seshadri et al., 2022). In combination with metagenomics, metataxonomics (metabarcoding) allows for a better understanding of the bacterial diversity associated with a specific site. Understanding the diversity further assists with approaches in the isolation of bacteria, specifically actinobacteria (Compson et al., 2020).

Previous studies on actinobacterial diversity of marine environments have shown that in terms of bioactive compound production, *Streptomyces, Nocardiopsis, Micromonospora, Salinispora,* and *Verruscosispora* were the genera most reported on with regards to being isolated from the marine environment (Malinga et al. 2022; Shi et al. 2022; Yan et al., 2022: 8736). However, these were not the only genera reported to be present in this niche. Additional genera reported are *Microbacterium*, *Brachybacterium*, and *Solwaraspora*, to name a few. This

opens the door for the discovery of new bioactive compounds for use in many industries, including the pharmaceutical and biotechnology industries (De la Hoz-Romo et al., 2022). This information allows for the selection and use of various isolation media and techniques (Dhakal et al., 2017) in order to tap into this niche environment to access the potential antibiotic-producing actinobacteria from marine sediments, sponges, and seawater.

2.5 References

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Chapter 3

Actinobacterial community analyses and the selective isolation of actinobacterial strains

3.1. Introduction

Metabarcoding is a molecular tool used in order to provide an overview of the biodiversity in an environmental sample from soil or water containing bound DNA or environmental DNA. This overview provides researchers with information on the taxa present in a particular sample through DNA sequencing or in this case also known as metagenomic DNA sequencing (Compson et al*.,* 2020; Mzava et al*.,* 2022). Metagenomic DNA sequencing is a form of next-generation sequencing to characterise the composition of the microbial community being studied (Mzava et al., 2022).

The diversity of actinobacteria in the marine environment differs vastly from that of other ecosystems. Marine actinobacteria potentially produce different bioactive compounds to that of their terrestrial relatives. Marine actinobacteria may have to adapt to extreme conditions namely anaerobic conditions, high pressures, and varying temperatures – lows of 0-8°C on the floor of the deep sea and highs of 100°C close to hydrothermal vents close to ridges in the mid-ocean (Anandan et al*.,* 2016:6). Due to the daily tidal cycles, gradients in moisture, UV radiation, salinity, temperature fluctuation and nutrient availability, marine tidal pools are harsh environments for microorganisms (Benedetti-Cecchi & Trussel, 2014). A study conducted by Jose and Jha (2017) showed the isolation of seven different actinobacterial genera from an intertidal pool on Dui Island, Gujarat. The genera isolated were *Nocardia, Streptomyces, Micromonospora, Nocardiopsis, Saccharomonospora*, *Glycomyces*, and *Actinomadura.* When further analyses were performed on three of the isolated strains, it was found that they produced some known compounds as well as several novel metabolites. This indicates that the intertidal environment should be explored as it may lead to the isolation of novel actinobacteria as well as novel metabolites. However, these microorganisms are more difficult to culture under standard laboratory conditions due to their special growth requirements or their culture conditions being unknown (Dhakal et al., 2017).

Morphological characterisation of actinobacteria is important. Actinobacteria can be identified based on mycelia formation, production of pigmentation, and the colour of aerial and substrate mycelium (Anandan et al*.,* 2016:8). Modern isolation methods seem to involve five steps for the isolation of actinobacteria. These steps are comprised of the selection of isolation medium composition, pre-treatment, incubation conditions, as well as colony selection and purification, with the most crucial of the five factors being the selection of isolation media because this dictates the type of organism that will grow on the isolation plates (Okudoh, 2001).

In this part of the study, we tried to get a glimpse of the marine environment sampled, focusing on the physicochemical properties of the target environment and the composition of the actinobacterial community. Various isolation techniques were employed to try to access the actinobacterial diversity.

3.2 Materials and Methods

3.2.1 Sampling of marine sediments

The sediment samples for this study were collected from Glencairn tidal pool (Table 3.1 and Figure 3.1), which forms part of the Table Mountain National Park (known as TMNP2 in this study) along the coast of the South Peninsula in Cape Town, Western Cape, South Africa (SANParks permit: CRC/2019-2020/013--2019/V1). Three subsites were selected: an area close to a rocky area, the ocean (tidal pool), and a dry subsite that were all within 10 m of one another. From each subsite, three points were chosen that were equidistant and 2 m apart (Figure 3.2). Sediment samples were collected from each point. Samples for metabarcoding (approximately 10-15 g) were placed in sterile 50 mL Greiner tubes and stored at 4°C (in a refrigerated unit) for transport to the laboratory, where they were processed within 24 hrs of sampling. Bulk sediment samples (>500 g) were collected in sterile plastic bags and submitted to Bemlab (Strand, South Africa) for the determination of the physicochemical properties and were also used in the isolation of marine actinobacteria. Physiochemical parameters tested were pH, resistance, stone volume, P Olsen, P Bray II, sodium, potassium, calcium, magnesium, copper, and zinc. Sediment samples were visualized under a light microscope (UB203i microscope with camera fitting, UOP, China) in order to determine the ease of bacterial isolations.

Table 3.1: Description and location of the Tidal pool and environment where the samples were obtained from.

Location Name	**Tidal Pool, Glencairn, Table Mountain National Park**
Province	Western Cape
Country	South Africa
Date	25.10.2019
GPS coordinates	-34.162481, 18.432022
Temperature: Ocean	16°C
pH: Tidal Pool water	pH 8.02

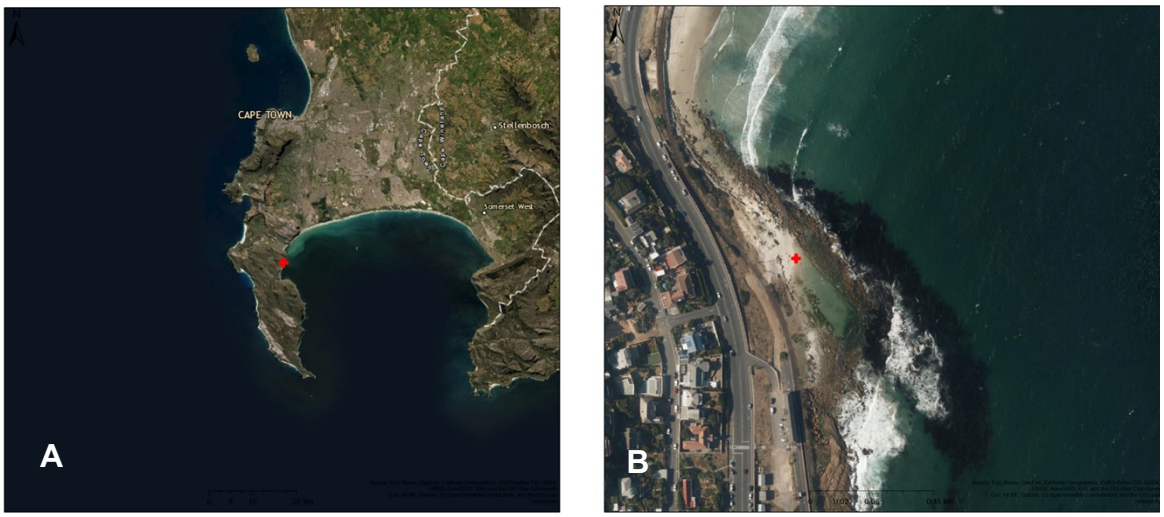

Figure 3.1: Images of the sampling site. A – Location of the Glencairn tidal pool on the Southern Peninsula; B – Zoomed-in image of the tidal pool sampled (images were obtained from Google Earth).

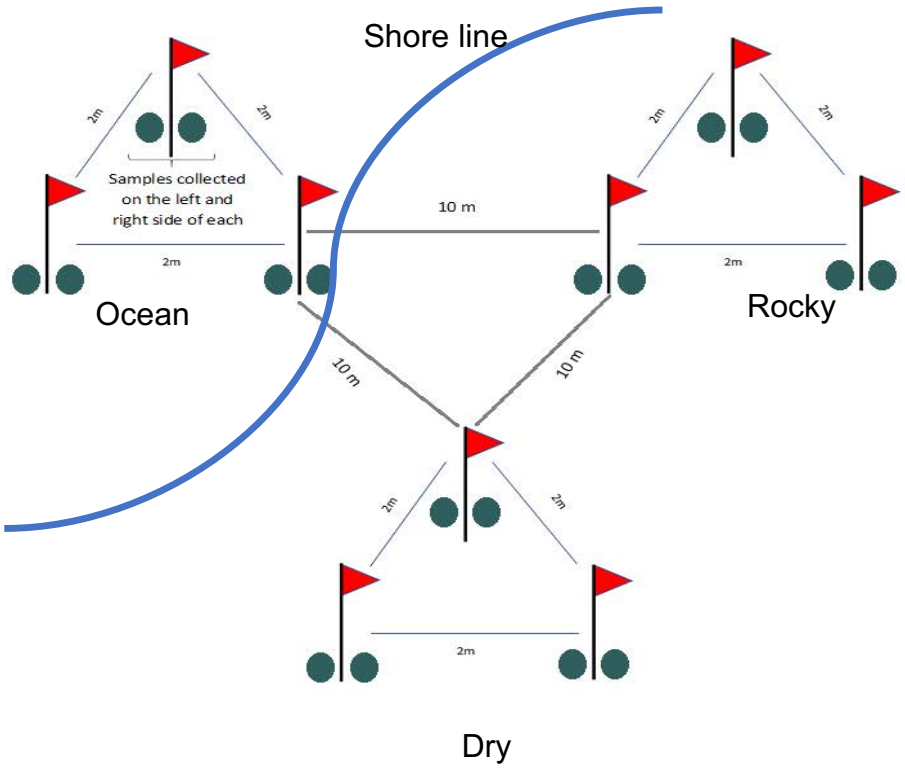

Figure 3.2: Layout on how samples were collected at each sampling site, with each set of flags representing a subsite of 'Rocky', 'Dry', and 'Ocean' (tidal pool) samples.

3.2.2 Amplification and sequencing of actinobacterial-specific 16S rRNA genes for metabarcoding

Metagenomic DNA (mgDNA) was isolated from 0.25 g of each sediment sample using the Qiagen DNeasy PowerSoil DNA Isolation Kit following the manufacturer's instructions.

Confirmation of a successful extraction was determined by gel electrophoresis by loading 5 µl of the mgDNA along with 1 µl loading dye onto a 0.8% (w/v) agarose gel prepared in 1x TAE (containing 10 µg/mL ethidium bromide) and visualised under UV light.

The method described by Schäfer et al. (2010:105) for the amplification of the 16S rRNA gene was performed. The actinobacterial-specific 16S rRNA gene primer pair (Com2xf: 5'-AAACTCAAAGGAATTGACGG-3'; Ac1186r: 5'-CTTCCTCCGAGTTGACCC-3') was used in the following PCR reaction: 1x KAPA Taq Readymix (1.5 mM $MgCl_2$, 0.2 mM of each dNTP), 0.2 µM of each primer, 1 µL of template mgDNA and water to bring the mixture up to a final volume of 25 µL. Amplification was performed using a BIO-RAD T100 thermal cycler. The amplification process occurred for 25 cycles using the following steps: Denaturation of DNA at 95°C for 30 seconds, annealing gradient between 51.6°C – 60.2°C, and lastly, an extension step at 72°C for 30 seconds. On the final cycle, the extension step was performed at 72°C for 15 minutes. The positive control was DNA from *Streptomyces polyantibioticus* SPR^T, while the negative control contained all the reagents, except mgDNA. Amplicons generated by the PCR step were analysed using electrophoresis as described before and visualised under UV light. The amplicons were cut from the agarose gel and purified using the Machery-Nagel gel purification kit (purchased from Separations). The purified amplicons were submitted to the DNA sequencing facility at the Central Analytical Facility (CAF) situated at Stellenbosch University, South Africa. Sequencing was performed using the Ion Torrent S5, using two 530 chips with an average of 250 000 total sequencing reads per sample. For the metabarcoding, 400bp chemistry was used. The library preparation kit used is the Ion AmpliSeq™ Library Kit 2.0 (ThermoFisher Scientific). Raw data in fastq file format was generated and subsequently used in the metabarcoding analysis.

3.2.3 Metabarcoding: Data processing and analyses

Mothur v1.44.0 (Schloss et al., 2009:7538-7540) was used through the Centre for High Performance Computing (CHPC) platform situated at the Council for Scientific and Industrial Research (CSIR), Rosebank Campus (Cape Town, South Africa) to process the raw data. All dependencies in the mothur installation are self-contained and did not require the download of additional components. The raw reads were quality-filtered, ambiguous bases were removed, and the reads combined into a single file. The VSEARCH algorithm was used to remove all chimeras. A Bayesian classifier was used to classify reads, and the SILVA 16S rRNA gene database (v138, Quast et al., 2013) was used as a reference database. After all non-prokaryotic sequences were removed, the remaining sequences were aligned with the reference database and curated to ensure that the region of interest was overlapping for all

sequences. Singletons were removed, and subsampling was performed to normalize the data. The sequences were clustered into Operational Taxonomic Units (OTUs) with the default distance-matrix cut-off of 0.03, and an OTU table and a taxonomy file were generated for downstream analysis. The MicrobiomeAnalyst 2.0 platform (Lu et al., 2023) was used to determine the alpha diversity and core microbiome using the default parameters. The uploaded OTU files were rarefied to the minimum library size to reduce the variability and the sparsity of the data between libraries.

3.2.4 Isolation of marine actinobacteria

Actinobacteria were isolated on the following media types: ISP2-C (g/L: 10 malt extract, 4 yeast extract, 4 glucose, 2 calcium carbonate), ISP2-C diluted 1:10, M1 (g/L: 10 starch, 4 yeast extract, 4 peptone), ISP5 (g/L: 1 L-asparagine monohydrate, 10 glycerol, 1 di-potassium hydrogen phosphate, 1 mL trace salts solution – 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $MnCl_2 \cdot 4H_2O$ and 0.1 g $ZnSO_4 \cdot 7H_2O$ was added to 100 mL of distilled water and filter sterilized through a 0.22 µm filter), Chitin agar (g/L: 2 chitin from shrimp), Seawater agar (g/L: 38.2 Red Sea salt). Each media type was prepared with 38.2 g/L Red Sea salts (https://g1.redseafish.com/red-sea-salts/red-sea-salt/) and without, except for the seawater agar, where the Red Sea salt was the only component in the media other than the bacteriological agar. For the media prepared with Red Sea salts, the salt concentration was approximately 3.5 % or 35 parts per thousand (ppt), which means that for every 1 L of water, there is approximately 35 g of salts. For the media that contained the Red Sea salt, the salt was allowed to completely dissolve before all other media components were added to 1 L of distilled water. Once dissolved, the pH was adjusted to 7.2 (7.0 for ISP5) with KOH (HCl for ISP2-C 1:10); thereafter, 18 g of bacteriological agar was added. The media was then autoclaved at 15 psi for 20 minutes (121°C) and allowed to cool to a temperature of 50°C ± 5°C. Five sets of M1, ISP2-C, ISP2-C:1:10, and ISP5 were prepared and were supplemented with the following antibiotics: 15 µg/mL rifampicin and 50 µg/mL potassium dichromate (R15/K), 25 µg/mL rifampicin and 50 µg/mL potassium dichromate (R25/K), 15 µg/mL rifampicin and 100 µg/mL cycloheximide (15R/C), 25 µg/mL rifampicin and 100 µg/mL cycloheximide (25R/C) or 100 µg/mL penicillin and 50 µg/mL potassium dichromate (P/K). Only one set of seawater and chitin agar was prepared and supplemented with 25 µg/mL nalidixic acid and 100 µg/mL cycloheximide. These antibiotics were included to limit the growth of unwanted microbial species such as fungi and Gram-negative bacteria.

3.2.5 Pre-treatment of sediment samples for actinobacteria isolation

In addition to using an untreated sediment sample, two different pre-treatment methods (physical and mechanical) were also employed for the isolation of actinobacteria from the

marine sediment samples. Furthermore, the stamp method was performed as an alternative dilution method for the untreated and physical treated sediment samples (see Figure 3.3 for the overview of the isolation approach).

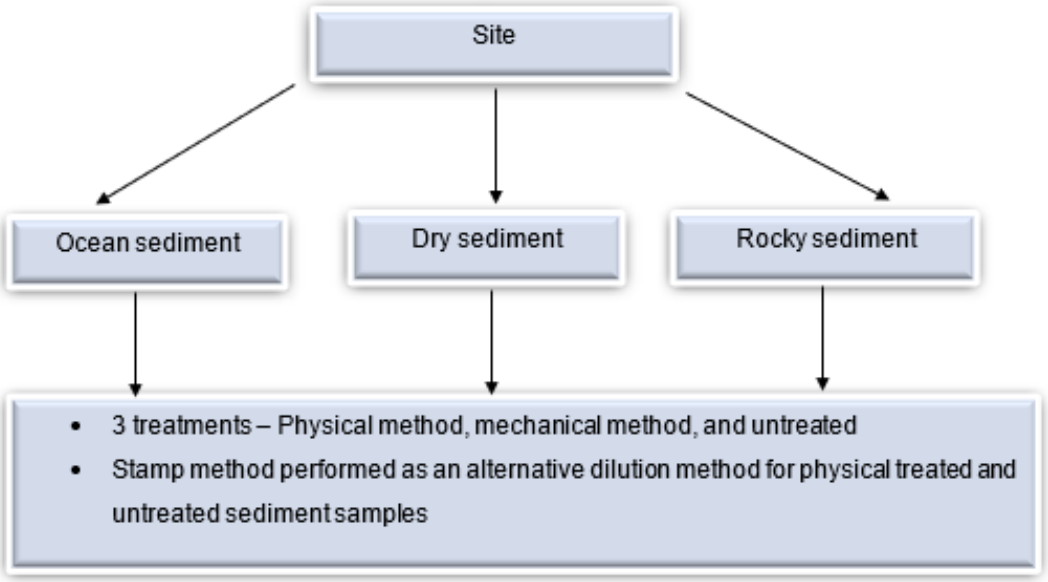

Figure 3.3: Diagram of how the plating of each subsite sample was performed for the isolation of marine actinobacteria from the TMNP2 sampling site.

I. Untreated method

Untreated sediment samples were processed by adding 1 g of each sample to 9 mL of Ringer's solution (g/L: 7.2 NaCl, 0.17 $CaCl_2$, and 0.37 KCl, pH adjusted to 7.3-7.4). The contents were mixed using a vortex mixer, and 1 mL of this mixture was added to 9 mL of Ringer's solution to bring it to a dilution of 10^{-1}. This ten-fold dilution was continued until a 10^{-3} dilution was obtained. 100 µL of each dilution was spread plated onto the isolation media in duplicate and incubated at 30 ± 3 °C.

II. Physical method

5 g of each sediment sample was added to a sterile petri dish and placed at room temperature (22 ± 3 °C) for 24 hours to air dry. 1 g of the dried-out sediment was added to 9 mL of Ringer's solution and diluted (as described in Section 3.4.1). 100 µL of each dilution was then spread plated onto the different isolation media in duplicate and incubated at 30 ± 3 °C.

III. Mechanical method

Eleven glass beads were added to sterile 15 mL tubes containing 9 mL of Ringer's solution and then autoclaved. 1 g of each sediment sample was added to a 15 mL tube. The sample

was then mixed on a rotor mixer for 15 minutes at 15 rpm at room temperature (22 ± 3 °C). 1 mL of the sample was added to 9 mL of Ringer's solution to bring it to a dilution of 10^{-1}. A ten-fold dilution was continued until a 10^{-3} dilution was obtained. 100 µL of each dilution was then spread plated onto the different isolation media in duplicate and incubated at 30 ± 3 °C.

IV. Stamping (alternative to serial dilution)

5 g of the untreated and physical treated sediment samples were added to sterile petri dishes. Sterile cotton wool (2 x 2 cm) was tapped gently onto the sediment sample. The agar plates were then stamped following a clockwise spiral pattern (Figure 3.4). No serial dilution was performed for this method, as the stamp method acted as a serial dilution in itself.

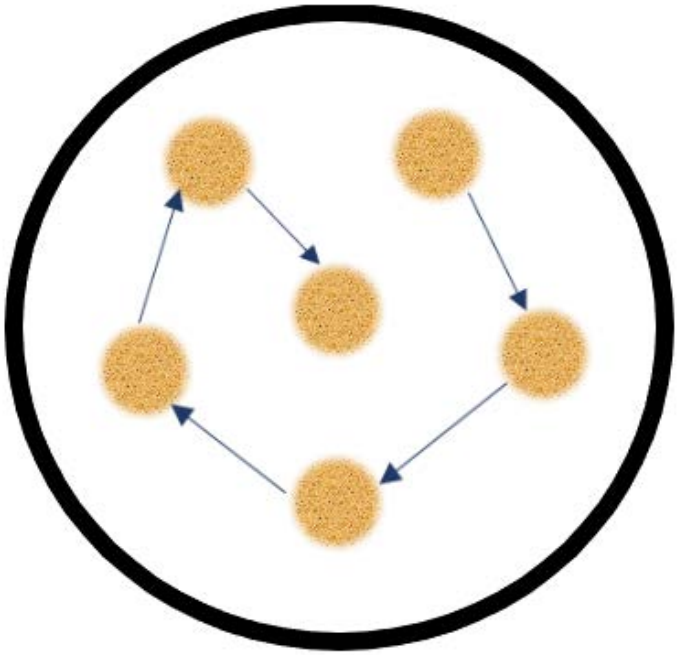

Figure 3.4: Layout of how the stamp method was performed. Stamping of the sample is performed in a consecutive stamping of the samples in a clockwise manner, resulting in a dilution of the amount of sample applied to the agar plate.

3.2.6 Colony picking and obtaining pure cultures

Actinobacteria can take between 15 to 30 days before growth is seen on isolation media. Isolation plates were checked after 1 month of incubation. If there were colonies present on the agar plates that had phenotypic properties of actinobacteria, individual colonies were selected and streaked onto fresh agar plates made of the same media, this time, omitting the antibiotics to obtain pure strains of the selected isolates. The same procedure was performed again after 2 months of incubation. Pure cultures were confirmed by performing the standard

Gram-staining technique. Once pure isolates were visually observed, liquid cultures were prepared by inoculating the strains into 10 mL of the isolation media in broth form. This was then incubated at 30°C for 7 days, shaking at 160 rpm on a rotary shaker. When sufficient growth of the bacterial strain was observed, purity was confirmed via standard Gram-staining, and 600 µL of the pure culture was added to 400 µL of a 50% (v/v) autoclaved glycerol solution and placed into a -80°C ultra-low freezer for preservation.

All reagents used in this study were sourced from Merck Millipore unless otherwise stated.

3.3 Results and Discussion

3.3.1 Physiochemical properties of sediment samples collected from TMNP2

The literature clearly shows that actinobacteria generate a wide range of bioactive compounds that are potentially significant lead compounds for therapeutic applications (Ngamcharungchit et al., 2023). Novel compounds are continuously searched for to develop new products and procedures for pharmaceutical, environmental, and industrial domains (Newman & Cragg, 2020:799-800).

Since the actinobacterial population will be driven by the physicochemical parameters of the source environment, it is important to know what trace metals and other elements are present, as well as the physical structure of the sediments. The fine sediments of TMNP2 (Figure 3.4) allow for a large attachment area for the actinobacteria (and other microorganisms), highlighting the importance of using different isolation approaches in order to ensure the release of the actinobacteria from the attachment surfaces. For example, in this study, the inclusion of a mechanical pre-treatment step would be essential to maximise the release of bacteria.

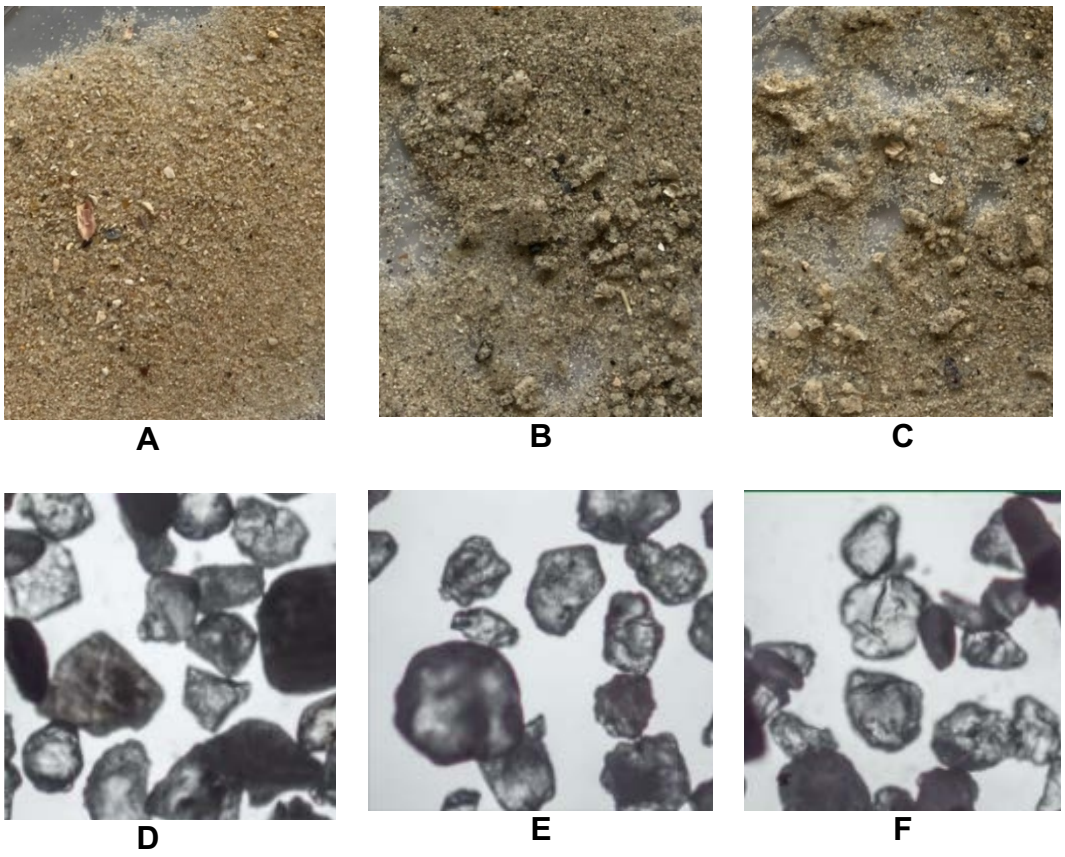

Figure 3.5: A – Sediment sample obtained from the TMNP2 Dry site. B – Sediment sample obtained from the TMNP2 Rocky site. C – Sediment sample obtained from the TMNP2 Ocean (tidal pool) site. D, E, and F are the microscopic views of the sediments in A, B, and C, respectively.

The six most prevalent ions in seawater are potassium (K^+), magnesium (Mg^{2+}), sodium (Na^+), sulphur in the form of sulphate (SO_4^{2-}), and chloride (Cl^-) in the form of potassium chloride (KCl). These ions comprise approximately 99% of all sea salts by weight (Devlin and Brodie, 2023:78). The salinity of the open ocean has been found to vary between 34 and 37 parts per thousand (ppt) (Byrne et al., 2023). This is seen in the physiochemical data obtained for the sediment samples collected from the TMNP2 sampling site across all three subsites, Dry, Ocean (tidal pool), and Rocky (Table 3.2). Among the numerous minor dissolved chemical elements, the presence of phosphorus (P Olsen and P Bray II) stands out the most because it is crucial to the development of marine organisms (Devlin & Brodie, 2023:78).

Calcium (Ca^{2+}) is an important element in seawater as it is the main component in calcium carbonate which is an important compound of the oceanic carbon cycle (He et al., 2020). There are two oxidation states of dissolved iron (Fe^{3+}) in seawater, i.e., Fe (II) and Fe (III), and it can be found as free iron or complexed with other organic and inorganic ligands. Fe^{3+} is a macronutrient that is required for the growth of phytoplankton in the marine environment (Liu & Millero, 2002:44). High levels of these elements in the TMNP2 samples, reflect a healthy and functional marine environment, which is also supported by the observation of various

marine macroalgae, fish and a cephalopod at the time of sampling as can be seen in the images provided in Appendix A.

Table 3.2: Physiochemical properties of the marine sediment samples collected from the tidal pool at Glencairn (TMNP2). Results were provided by Bemlab.

Sample	TMNP2 Dry	TMNP2 Ocean	TMNP2 Rocky
Soil type	Sand	Sand	Sand
pH (KCl)	9.1	8.8	8.8
Resistance (ohm)	500	70	80
Stone Vol (%)	2	1	3
P Olsen (mg/kg)	2.3	3.78	3.2
P Bray II (mg/kg)	11.2	9.69	10.36
Na (cmol/kg)	0.65	6.6	5.69
K (cmol/kg)	0.04	0.2	0.17
Ca (cmol/kg)	14.18	21.24	14.38
Mg (cmol/kg)	0.51	1.86	1.56
Cu (mg/kg)	0.16	0.05	0.14
Zn (mg/kg)	0.36	0.27	0.4
B (mg/kg)	0.79	1.53	1.6
Fe (mg/kg)	7.19	10.48	9.95
Soluble S (mg/kg)	30.77	182.3	149.43
C %	0.32	0.16	0.12

3.3.2 Metabarcoding of TMNP2 sediment samples

Metagenomic DNA (mgDNA) was isolated from each of the eighteen sediment samples collected, as represented in Figure 3.2. The Alpha-diversity of the microbial community of the eighteen samples is shown in Figure 3.5. This showed that even though samples were from the same subsite, the six samples obtained from the Rocky subsite had different microbial diversity. The six samples obtained from the Dry and Ocean (Tidal pool) subsite were closer related in terms of microbial diversity. Furthermore, samples obtained from the Dry subsite mostly showed a higher microbial diversity than the Rocky and Ocean (Tidal pool) subsite. Only one of the Rocky samples had high microbial diversity (a Shannon index >5). The lower microbial diversity for the samples obtained from the Ocean (Tidal pool) and Rocky subsite could be due to the salinity of the seawater, which has an effect on the microbial population (Shao et al*.,* 2020). This is also supported by the low resistance reading observed for the Rocky and Ocean samples; a low resistance is an indication of a high concentration of soluble salt, and sediments with a resistance of <300Ω are regarded as saline (Bemlab, 2020).

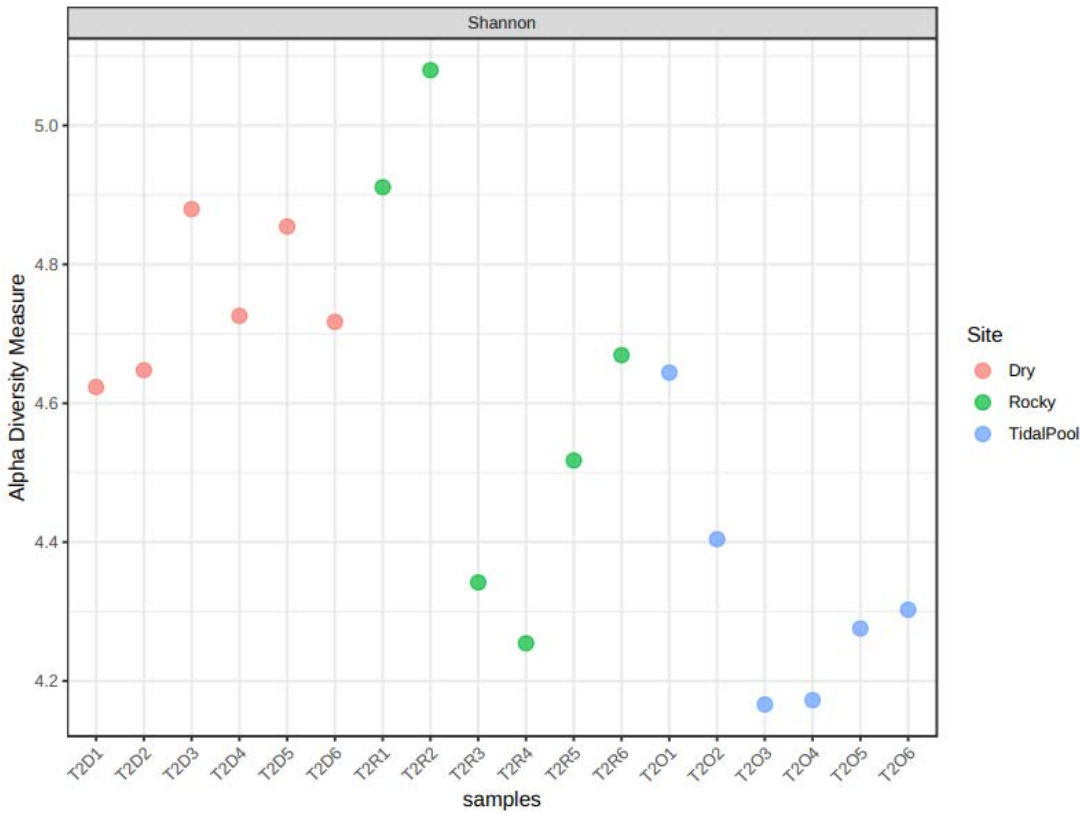

Figure 3.6: Scatter plot to show the Alpha-diversity (based on the Shannon index) of each of the eighteen samples that metagenomic DNA (mgDNA) was extracted from. T2: TMNP2; D1-6: Dry samples 1-6; R1-6: Rocky samples 1-6; O1-6: Ocean/tidal pool samples 1-6.

From the abundance of the actinobacterial community, it was seen that there is a significant amount of unclassified and uncultured actinobacteria present throughout the eighteen sediment samples (Figure 3.7). In a review, Ward and Bora (2006:281) stated that more novel actinobacteria are being isolated from marine sediment samples and that these newly discovered actinobacteria contribute significantly to the discovery of new drugs. The known actinobacterial genera present in the sediment samples are *Nocardioides, Streptomyces, Arthrobacter,* and *Micromonospora* (Figures 3.7 and 3.8). Previous studies have shown the potential to isolate novel actinobacteria from marine sediments belonging to genera that are known to produce bioactive compounds, such as *Nocardioides,* which has been found to have antimicrobial activity against *S. aureus*, and *Arthrobacter,* which has been known to produce bioactive compounds that assist in biodegradation (Gobbetti & Rizzello, 2014:69; Amaning Danquah et al., 2022). *Streptomyces* and *Micromonospora* are well known for their ability to produce antimicrobial compounds active against pathogenic bacteria (Manivasagan et al., 2014:173,186; Hassan et al., 2017:35). This prompted the use of different isolation approaches during this study to access as much of the actinobacterial diversity present in order to explore them for novel natural bioactive compound production.

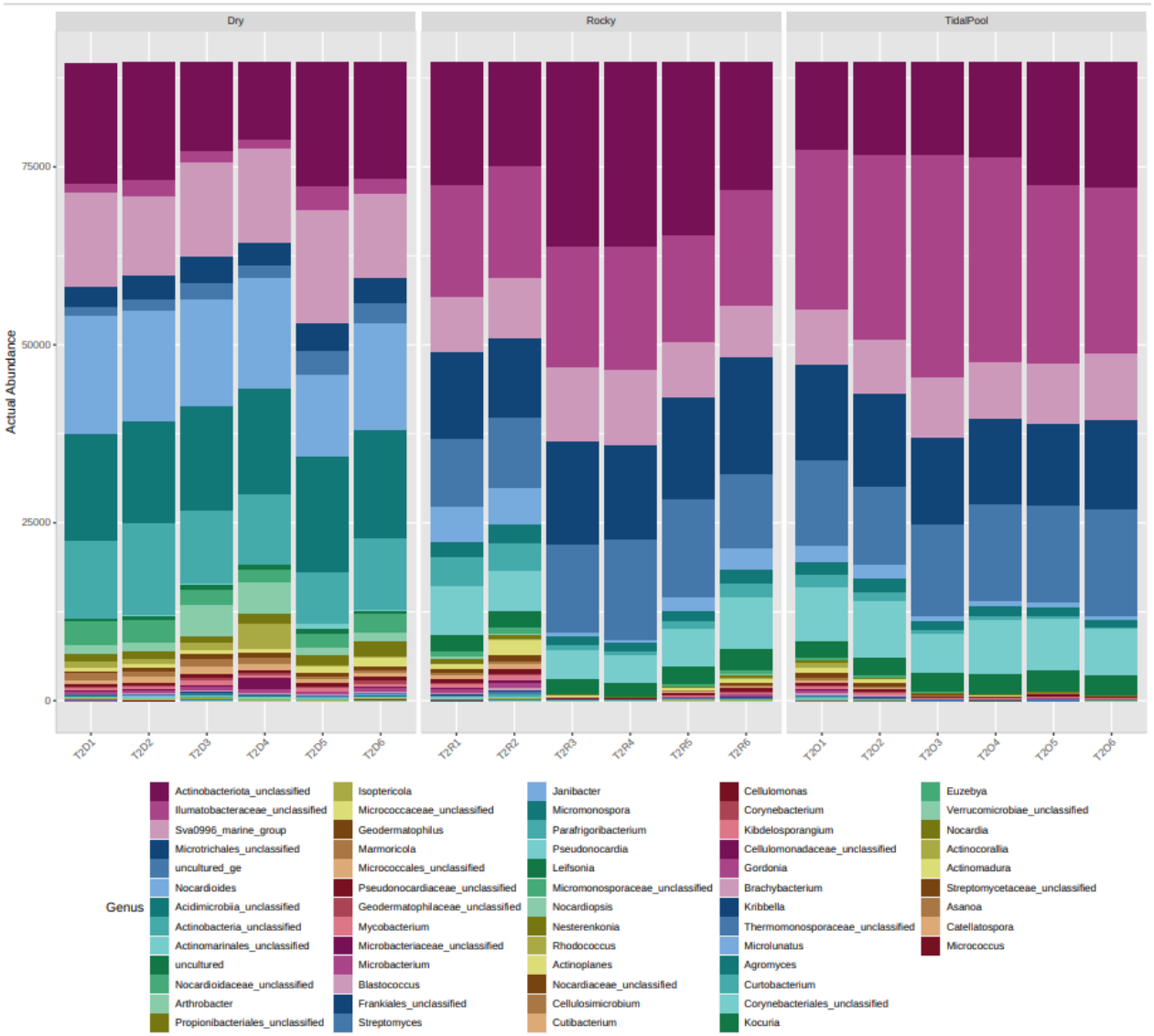

Figure 3.7: The actual abundance of the actinobacterial community (Genus level) based on the metagenomic DNA (mgDNA) isolated from TMNP2 Dry (T2D), Rocky (T2R), and Tidal pool (Ocean) (T2O) subsites.

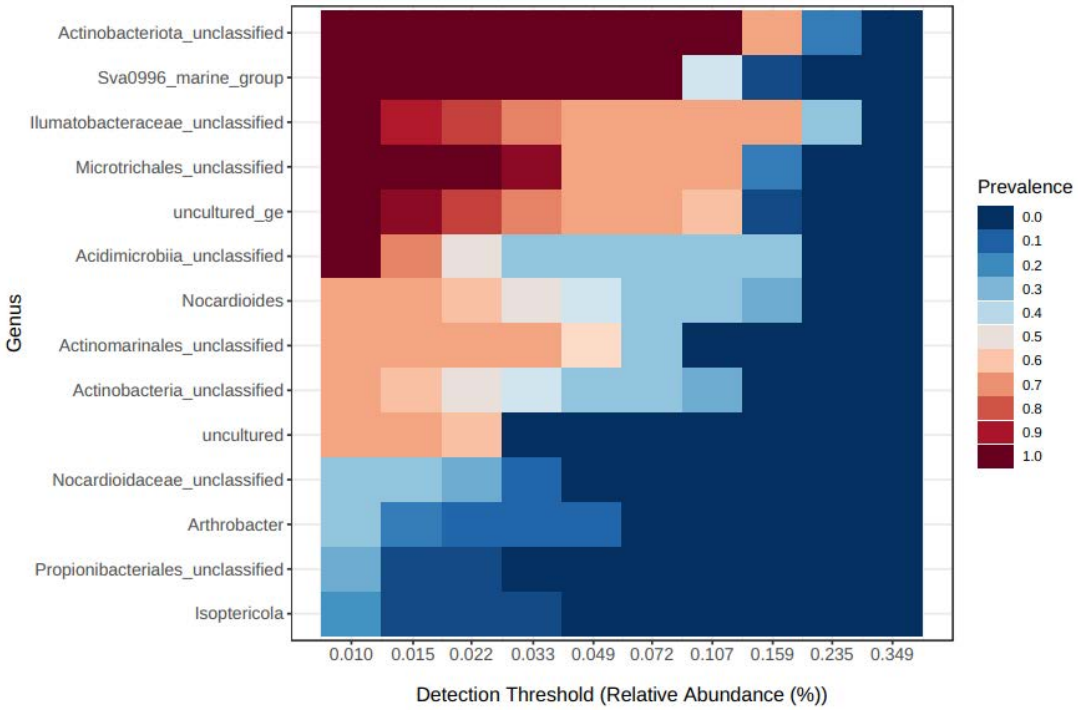

Figure 3.8: The combined relative abundance of actinobacteria in the collected sediment samples, indicating the core (dark red shading) actinobacterial microbiome consisting of unclassified and uncultured actinobacteria.

3.3.3 Isolation of marine actinobacteria

Actinobacteria are slow-growing microorganisms. The use of antibiotics in initial isolation media allows for the actinobacterial strains to grow while also minimising or eliminating the growth of fast-growing microorganisms (Faja et al., 2017:213). By using the isolation techniques outlined in this study, over 60 strains of actinobacteria were isolated. From these 60 strains isolated, 20 were selected for further analysis. Nine isolates were obtained from the 'Ocean' sample from the following pretreatment methods: two from the physical method, five from the untreated sample, one isolate obtained from the stamp method, and two from the mechanical method. Three isolates were obtained from the 'Dry' samples; two were isolated from the untreated samples, where one of the untreated isolates was obtained with the stamp method, and the last isolate was obtained from the mechanically treated sample. Lastly, eight isolates were obtained from the 'Rocky' sediment sample; two were isolated from the mechanically treated samples, three were isolated from the physically treated samples, and three were from the untreated sediment sample (Table 3.3). In Figure 3.7 it is also seen that there is an abundance of *Streptomyces* present in the 'Rocky' and 'Dry' subsites. This makes it more likely to isolate *Streptomyces* from these two samples.

Table 3.3: Isolates obtained from the sediment samples collected from the three subsites at the Glencairn tidal pool (TMNP2): Isolate designation, sediment sample isolated from, the pre-treatment method used, which serial dilution plate, isolation medium, and antibiotics in the medium, the isolates were obtained from.

Isolate designation	Extended strain name	Type of sediment sample obtained from	Pre-treatment method	Dilution plate	Isolation medium	Antibiotics in medium
R-1	TMNP2 OP STAMP EX ISP5 P/K #1	Ocean	Physical	Stamp	ISP5	100 µg/mL Penicillin and 50 µg/mL potassium dichromate
R-2	TMNP2 D STAMP EX ASW #2(2)	Dry	Untreated	Stamp	Seawater agar	25 µg/mL Nalidixic acid and 100 µg/mL Cycloheximide
R-6	TMNP2 RM 10^0 EX CHITIN #2(2)	Rocky	Mechanical	10^0	Chitin	25 µg/mL Nalidixic acid and 100 µg/mL Cycloheximide
R-21	TMNP2 OU 10^0 EX ISP2C 1:10 15R/C #7(2)	Ocean	Untreated	10^0	ISP2-C 1:10	15 µg/mL Rifampicin and 100 µg/mL Cycloheximide
R-23	TMNP2 RP 10^0 EX M1 R15/K #10	Rocky	Physical	10^0	M1	15 µg/mL Rifampicin and 50 µg/mL potassium dichromate
R-24	TMNP2 RP 10^0 EX M1 R15/K #7	Rocky	Physical	10^0	M1	15 µg/mL Rifampicin and 50 µg/mL potassium dichromate
R-30	TMNP2 RU 10^0 EX CHITIN #1(2)	Rocky	Untreated	10^0	Chitin	25 µg/mL Nalidixic acid and 100 µg/mL Cycloheximide
R-31	TMNP2 RM 10^-2 EX ISP2C R15/K #3	Rocky	Mechanical	10^{-2}	ISP2-C	15 µg/mL Rifampicin and 50 µg/mL potassium dichromate
R-32	TMNP2 RU 10^0 EX CHITIN #9(2)	Rocky	Untreated	10^0	Chitin	25 µg/mL Nalidixic acid and 100 µg/mL Cycloheximide
R-34	TMNP2 OM 10^0 EX CHITIN #5(2)	Ocean	Mechanical	10^0	Chitin	25 µg/mL Nalidixic acid and 100 µg/mL Cycloheximide
R-35	TMNP2 O STAMP EX ISP2C 25R/C #4(2)	Ocean	Untreated	Stamp	ISP2C	25 µg/mL Rifampicin and 100 µg/mL Cycloheximide
R-36	TMNP2 OM 10^0 EX ASW #5(2)	Ocean	Mechanical	10^0	Seawater agar	25 µg/mL Nalidixic acid and 100 µg/mL Cycloheximide
R-37	TMNP2 OP STAMP EX M1 R15/K #7	Ocean	Physical	10^0	M1	15 µg/mL Rifampicin and 50 µg/mL potassium dichromate
R-38	TMNP2 RP 10^0 EX M1 R15/K #3	Rocky	Physical	10^0	M1	15 µg/mL Rifampicin and 50 µg/mL potassium dichromate

Isolate designation	Extended strain name	Type of sediment sample obtained from	Pre-treatment method	Dilution plate	Isolation medium	Antibiotics in medium
R-41	TMNP2 DM 10^-2 EX M1 R15/K#1	Dry	Mechanical	10^{-2}	M1	15 µg/mL Rifampicin and 50 µg/mL potassium dichromate
R-43	TMNP2 OU 10^0 EX CHITIN #4(2)	Ocean	Untreated	10^{0}	Chitin	25 µg/mL Nalidixic acid and 100 µg/mL Cycloheximide
R-F	TMNP2 OU 10^0 EX ISP2C 1:10 R25/K #5	Ocean	Untreated	10^{0}	ISP2-C 1:10	25 µg/mL Rifampicin and 50 µg/mL potassium dichromate
R-J	TMNP2 OU 10^0 EX ISP2C 1:10 R15/K #7	Ocean	Untreated	10^{0}	ISP2-C 1:10	15 µg/mL Rifampicin and 50 µg/mL potassium dichromate
R-M	TMNP2 DU 10^-2 EX ISP2C 1:10 R25/K #2	Dry	Untreated	10^{-2}	ISP2-C 1:10	25 µg/mL Rifampicin and 50 µg/mL potassium dichromate
R-N	TMNP2 RU 10^-1 EX ISP2C 1:10 R15/K #1	Rocky	Untreated	10^{-1}	ISP2-C 1:10	15 µg/mL Rifampicin and 50 µg/mL potassium dichromate

The goal of the current study was to determine the antimicrobial potential of actinobacteria isolated from marine sediments, specifically from TMNP2. Actinobacteria isolated from this environment have a higher probability of producing interesting metabolites as the tidal pool environment is vastly underexplored. This strategy was employed to increase the possibility of finding novel bioactive compounds against the ESKAPE organisms, and the approach taken in this study is outlined and discussed in Chapters 4 and 5.

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Chapter 4

Screening marine actinobacteria for anti-ESKAPE bioactivity

4.1 Introduction

Marine organisms have given rise to many antibiotics, and currently, two-thirds of these natural products have been derived from actinobacteria. Antibiotics derived from these organisms are applied against microbial infections more efficiently as terrestrial organisms have not yet built-up immunity/resistance against them (Vimal et al*.,* 2009; Durand et al*.,* 2019). As indicated previously, the continuous isolation of the microorganisms that have already been discovered in the terrestrial environment has led to the marine environment being targeted since it shows great promise for novel natural compounds. This is due to their structural diversity and their unique bioactive capabilities (Gerwick & Moore, 2012). The initial focus on marine natural products was mainly related to organisms that were easily accessible, such as seaweed and sea sponges. With the rediscovery of secondary metabolites, attention began to shift to smaller organisms that have been previously overlooked. Since this shift in research, the culturing of marine microorganisms from deep and shallow sediments, as well as animate and inanimate surfaces, began taking place (Gerwick & Moore, 2012). Other than marine sediments that are being explored for actinobacteria, marine sponges are a popular source for isolating actinobacteria. For example, Kim et al. (2006:2121) reported that after chemical analysis was performed on *Salinispora* M403, isolated from a marine sponge, it was observed that this microorganism can produce rifamycin B and SV. However, the genes present in this *Salinispora* strain were closely related to *Amycolotopsis mediterranei* but were not identical. This allows for the idea that novel compounds could be isolated from *Salinispora* M403 and other marine actinobacteria isolated from unique environments (Kim et al., 2006:2121).

Secondary metabolites derived from actinobacteria are well known for their bioactivity against pathogenic microorganisms. The marine environment has lately become the best source for novel secondary metabolite discovery, where most of the compounds are derived from members of the genus *Streptomyces*. *Streptomyces* species thrive in the marine environment, and most of the secondary metabolites that are isolated from the marine environment exhibit bioactive properties, which include but are not limited to antibacterial and antifungal agents (Manivasagan et al., 2014:263; Bibi et al., 2020). In this part of the study, strains isolated from the TMNP2 sediments were screened for their potential to exhibit anti-ESKAPE activity, and strains exhibiting bioactivity were identified by 16S rRNA gene sequencing.

4.2 Materials and Methods

4.2.1 Bioactivity screening

The twenty strains listed in Table 3.3 were subjected to bioactivity screening. Two pre-screening methods were employed to determine antimicrobial activity against ten American Type Culture Collection (ATCC) ESKAPE pathogenic test strains (Table 4.1). First, the cross-streak method was employed for all 20 strains selected for screening. Once it was confirmed that an isolate displayed bioactivity against the test strains, the isolates that displayed no activity were excluded from further study. The second pre-screening technique was based on a filter disc assay for extracts prepared from mono-cultures of the strains that exhibited activity in the cross-streak experiment.

Table 4.1: The test trains used in this study, the media that allows for optimal growth, and the antibiotics they display resistance to.

Test Strain designation	Organism	Optimal growth conditions	Antibiotic resistance to
1	*Acinetobacter baumannii* ATCC BAA-1605	Tryptic soy broth/agar (TSB/TSA), 37°C	Ceftazidime, gentamicin, ticarcillin, piperacillin, aztreonam, cefepime, ciprofloxacin, imipenem and meropenem
2	*Enterobacter cloacae* ATCC BAA-1143	TSB/TSA, 37°C	Aminopenicillins and cefazolin
3	*Escherichia coli* ATTC 25922	TSB/TSA, 37°C	Ampicillin, amoxicillin, tetracycline, trimethoprim and sulfamethoxazole
4	*Pseudomonas aeruginosa* ATCC 27853	TSB/TSA, 37°C	Cefoxitin, flomoxef and kanamycin
5	*Staphylococcus aureus* ATCC 29213	TSB/TSA, 37°C	Penicillin and erythomycin
6	*Acinetobacter baumannii* ATCC 19606	Nutrient Broth/Agar (NB/NA), 37°C	Ampicillin, amoxicillin/clavulanic acid, cefazolin, cegoxitin, nitrofuratioin, trimethoprim and trimethoprim
7	*Klebsiella pneumoniae* ATCC 700603	NB/NA, 37°C	Resistant to most beta-lactam antibiotics
8	*Staphylococcus aureus* ATCC 33591	NB/NA, 37°C	Methicillin
9	*Enterococcus faecalis* ATCC 29212	Brain Heart Infusion Broth/Agar, 37°C (BHI-B/BHI-A)	Gentamicin and streptomycin
10	*Enterococcus faecalis* ATCC 51299	Brain Heart Infusion Broth/Agar with vancomycin (BHI-B+Van/BHI-A+Van), 37°C	Vancomycin

4.2.2 Cross-streak method

The marine actinobacterial isolates were streaked in a straight line down the center of ISP2 agar (g/L: 10 malt extract, 4 yeast extract, 4 glucose, 15 bacteriological agar), ISP2+ agar (g/L: 10 malt extract, 4 yeast extract, 4 glucose, 38.2 Red Sea salts, 15 bacteriological agar), M19 agar (g/L: 20 mannitol, 10 casamino acids, 20 peptone, 15 bacteriological agar), and M19+ agar (g/L: 20 mannitol, 10 casamino acids, 20 peptone, 38.2 Red Sea Salts, 15 bacteriological agar) in duplicate and incubated for 10 days at 30°C. Test strains (Table 4.1) were streaked out onto their respective media on day 9 and incubated overnight. After incubation, the test strain agar plates were removed from incubation and visually checked for contamination, thereafter, these plates were sealed with parafilm and placed in a fridge at 2 - 4 °C. On the 11^{th} day, the test strains (Table 4.1) were streaked from the outer end of the agar towards and touching the marine actinobacterial isolate (Figure 4.1). These plates were then incubated overnight at 37°C. The following day, the plates were observed for the inhibition of the growth of the test bacteria which determined if the marine actinobacterium produced bioactive compounds against the test strains. Before the cross-streak method was conducted, the test strains were streaked onto ISP2, ISP2+, M19, and M19+ agar plates to observe their growth on each media type in order to avoid false positive results (Figure 4.2).

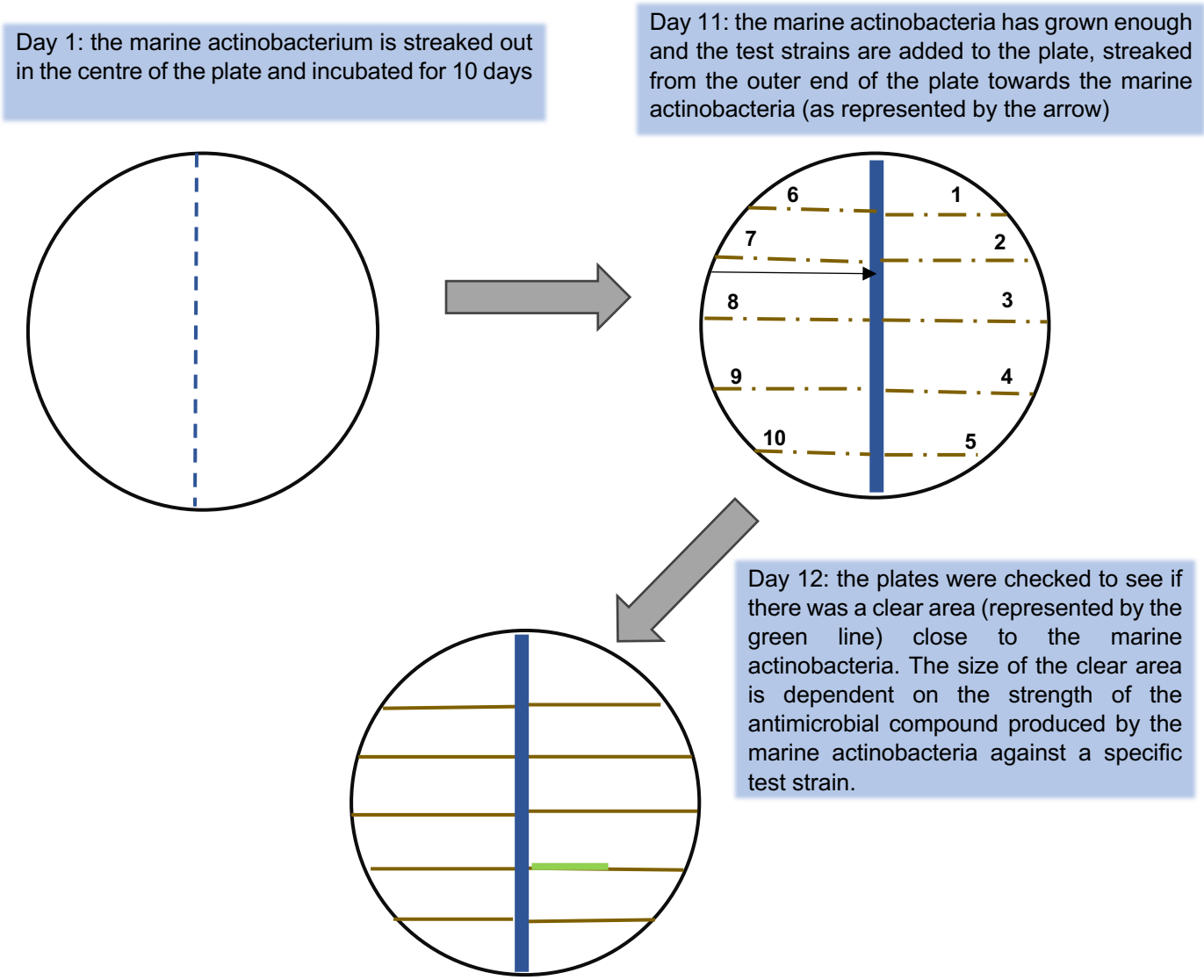


Figure 4.1: Demonstration of how the cross-streak experiment was performed to test bioactivity of the actinobacterial isolate against ESKAPE organisms. 1-10 represent the test strains used (Table 4.1).

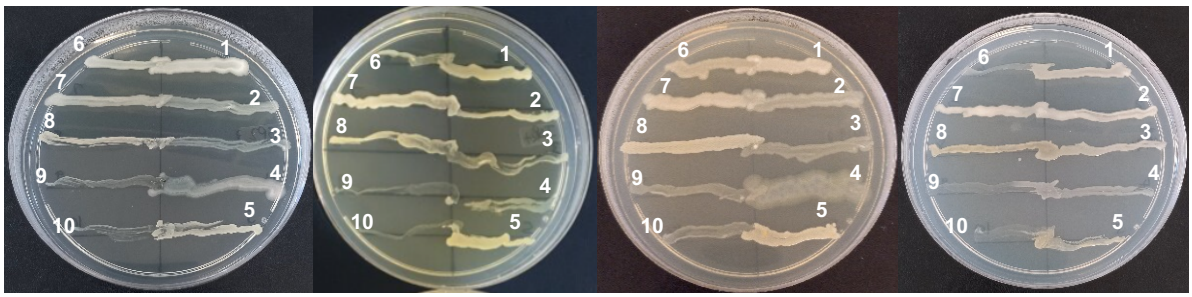


Figure 4.2: Control plates for A) ISP2, B) ISP2+, C) M19, and D) M19+ inoculated with test strains to confirm sufficient growth of test strains on the respective media types.

4.2.3 Preparation of crude extract for filter disc assays

I. Seed culture preparation

Liquid ISP2+, ISP2, M19+, and M19 were prepared, and 10 mL of each medium was pipetted into 50 mL Erlenmeyer flasks, covered with foil, and autoclaved at 121°C, 15 psi for 25 minutes. Once the flasks cooled down to room temperature (22 ± 3°C), the liquid media was inoculated with the marine actinobacterial strains selected based on the cross-streak results; each strain was inoculated in triplicate. These cultures were incubated at 30°C shaking at 160 rpm for 5 days on a rotary shaker. A triplicate set of media controls was also included.

II. Main culture preparation

M19+, M19, ISP2, and ISP2+ liquid media were prepared, and 10 mL of the media was pipetted into 50 mL Erlenmeyer flasks and autoclaved as described before. Once the flasks cooled down, 12 flasks of each media type were inoculated with 200 µl of the 5-day-old seed culture prepared for each actinobacterial strain. In addition, 12 flasks of each media type were left uninoculated (media controls). All the flasks were incubated at 30°C on a rotary shaker at 160 rpm. After 1 (T_1), 5 (T_2), 7 (T_3), and 10 (T_4) days of growth, triplicate flasks of each media type that was inoculated, as well as triplicate flasks of each media control were removed from the incubator and processed for crude extract preparation.

III. Crude extract preparation

On each sampling day, 0.5 g of DIAION HP-20 was added to each of the individual flasks and incubated overnight at 30°C, shaking on a rotary shaker at 160 rpm. The following day the cultures were filtered through coffee filters and washed with 5 volumes of sterile dH_2O. Once the dH_2O had passed through the filter, the washed cells and DIAION were collected off the coffee filter, transferred back into the same 50 mL Erlenmeyer flasks, and 10 mL of methanol was added. This solution was covered with foil and incubated at 30°C at 160 rpm overnight. The next day, the extracts were filtered through coffee filters into previously weighed McCartney bottles and placed in the fume hood until the methanol had completely evaporated, leaving only the dried extract in the bottle. Once completely dried, the McCartney bottles were weighed again. The weight of the crude extract was determined by subtracting the initial weight of the bottle from the final weight of the bottle:

$$(weight\ of\ McCartney\ bottle + dried\ extract) - Initial\ weight\ of\ McCartney\ bottle = weight\ of\ dried\ extract$$

After determining the weight of the dried extract, dichloromethane:methanol:water (64:36:8, v/v/v) was added to resuspend the extract at the desired concentration of 10 mg/mL. The extract was transferred into HPLC vials and stored at -20°C until further analysis was performed on it. Extracts were also prepared from pre-culture flasks (T_0).

4.2.4 Filter disc assay

Liquid media for the inoculation of the ESKAPE organisms (test strains) was prepared (Table 4.1). 10 mL of the media was pipetted into 50 mL Erlenmeyer flasks and autoclaved. Once cooled, the flasks were inoculated with the respective test strains and incubated at 37°C on a rotary shaker at 160 rpm overnight. The following day the OD_{600} was determined using a PerkinElmer Lambda 25 spectrophotometer. The culture was then diluted with their corresponding liquid media until the OD_{600} was at 0.5. 100 µL of the diluted test strains were then spread plated onto their corresponding optimal growth medium agar plates.

Filter discs (5 mm diameter) were prepared from Whatman No. 1 filters, autoclaved, and dried at 37°C before use. 5 µL of resuspended extract (at 10 mg/mL concentration) was spotted on a filter disc, giving a final concentration of 50 µg of extract per disc. The solvent mix was also spotted on filter discs to determine if it had an effect on the test strains and could potentially give a false positive result. In addition, 5 µL of ampicillin (100 mg/mL) and 5 µL of gentamycin (20 mg/mL) were also spotted on filter discs as positive controls against the test strains. Thereafter the filters containing the extracts and controls were placed onto labelled positions on the spread plates containing the different test strains and incubated overnight at 37 °C. The next day, zones of clearing were observed as an indicator of bioactivity.

4.2.5 16S rRNA gene sequence analysis

For the marine isolates that displayed promising results, colony PCR was performed. 100 µl of dH_2O (autoclaved, filter sterilised, and UV treated for 30 minutes) was added to a sterile 1.5 mL Eppendorf tube. An actinobacterial colony was then picked up using sterile toothpicks and placed into the sterile dH_2O. This mixture was vortexed for 10-15 seconds and heated in a heating block for 15 minutes at 95°C. Once removed from the heating block, the Eppendorf tubes were placed on ice for 10 minutes. These samples were used as DNA template. The 16S rRNA gene was amplified using universal bacterial 16S rRNA primers, F1 (5'-AGAGTTTGATCITGGCTCAG-3') and R5 (5'-ACGGITACCTTGTTACGACTT-3') (Cook & Meyers, 2003) in the following PCR reaction: 0.5 µM (1 µL) forward primer, 0.5 µM (1 µL) reverse primer, 12.5 µl of PCR master mix (ThermoStarr 2x PCR mastermix), 1 µl colony PCR sample and made up to a final volume of 25 µl using dH_2O. Amplification was performed using a BIO-RAD T100 thermal cycler. The amplification process occurred for 25 cycles using the following steps: denaturation of DNA at 95°C for 30 seconds, annealing at 56°C – 60.2°C, and lastly, an extension step at a temperature of 72°C for 30 seconds. On the final cycle, the extension step was performed at a temperature of 72°C for 15 minutes. The positive control used was DNA from *Streptomyces polyantibioticus* SPR^T. Amplicons generated by the PCR step were analysed using electrophoresis on a 0.8% (w/v) agarose gel containing 10 µg/mL ethidium bromide. A DNA ladder (NEB Fast DNA Ladder) was included to determine if the

amplicons were of correct size, approximately 1500 bp long. The electrophoresed gel was then visualized using a Gel Doc XR+ (BIO-RAD). The PCR amplicons were cleaned up using the MSB Spin PCRapace PCR purification kit (Invitek) following the manufacturer's instructions. The purified amplicons were submitted to the DNA sequencing facility at CAF, Stellenbosch University, for sequencing. The sequences were analyzed and edited using DNAman and submitted to EzBioCloud to determine the identity of the isolates.

4.2.6 Phylogenetic analysis

Phylogenetic analysis was performed to show the position of the top eight isolates in comparison to validly published species. The 16S rRNA gene sequences of the top validly published strain hits were downloaded from EzBioCloud and used in a multiple sequence alignment (ClustalX) with the 16S rRNA gene sequences of the actinobacterial isolates obtained in this study. The strain *Micromonospora tulbaghia* DSM 45142 was used as an outgroup. Default parameters on MEGA7 (Kumar et al., 2016) were used to prepare a neighbour-joining tree using 1000 bootstrap analyses. The accession numbers of the validly published strains were provided in the tree.

All reagents used in this study were sourced from Merck Millipore unless otherwise stated.

4.3 Results and Discussion

Screening for antibiotic activity is an important step in the process of identifying actinobacterial strains that could potentially produce useful antibiotics. This process aids in the isolation and characterisation of strains with the ability to inhibit the growth of pathogenic bacteria. There are many techniques that can be used to screen for antimicrobial compounds. The overlay technique, a common approach, involves pouring a layer of semi-solid agar containing the test strain over a solid medium containing the potential antibiotic-producing microorganism (Maricic & Dawid, 2014). Another agar-based screen is the agar well diffusion assay, which involves seeding an agar plate with a test organism, and the actinobacterial culture extracts are added to wells that have been made in the agar (Gonelimali et al., 2018). The presence of zones of inhibition around the well indicates the presence of antibiotics against the test strain. For pure or partially pure extracts, the microdilution assay can be used, which involves serially diluting extracts obtained from the actinobacterial strain in microplates to determine the minimum inhibitory concentration (MIC) against the test strain (Manandhar et al*.,* 2019). All of these techniques are typically used in dereplication in order to ensure that effort is placed on accessing potentially novel compounds. Dereplication may also involve the comparison of the spectra and characteristics of bioactive compounds with existing databases to identify novel compounds. Newer techniques used for screening are microfluidics and mass spectrometry.

These techniques, however, require highly specialised instrumentation, which puts smaller institutions at a disadvantage in the field of antibiotic discovery (Murry et al*.,* 2019:15415).

Since there were ten test strains used in this study, the cross-streak method and the filter disc assay were used for screening of antimicrobial activity. The cross-streak method was selected as this allows more than one test strain to be tested on the same agar plate at a time (Figure 4.2) which reduces the amount of agar plates needed in comparison to the overlay technique. The filter disc assay was used to determine if consistent results could be seen across liquid media and solid media assays with regard to the production of bioactive compounds against the ten test strains.

4.3.1 Cross-streak

The cross-streak method was used to primarily determine if the actinobacterial strains chosen for this study displayed bioactivity against the ten ESKAPE ATCC test strains. Eight actinobacterial isolates (Figure 4.3) displayed activity against various test strains on the four different media types used (Table 4.2, Figure 4.4). Six isolates displayed activity on M19 agar. R-2, R-21, and R-23 displayed activity against *P. aeruginosa*; R-21, R-30, and R-35 displayed activity against both *S. aureus* strains, and both *E. faecalis* strains; R-30 showed weak activity against both *A. baumannii* test strains, while R-35 showed activity against both *A. baumannii* test strains where *A. baumannii* ATCC BAA-1605 was inhibited less than *A. baumannii* ATCC 19606, and R-J displayed activity against *A. baumannii* ATCC 19606 and *S. aureus* ATCC 33591. Images of all isolates with activity can be seen in Appendix B

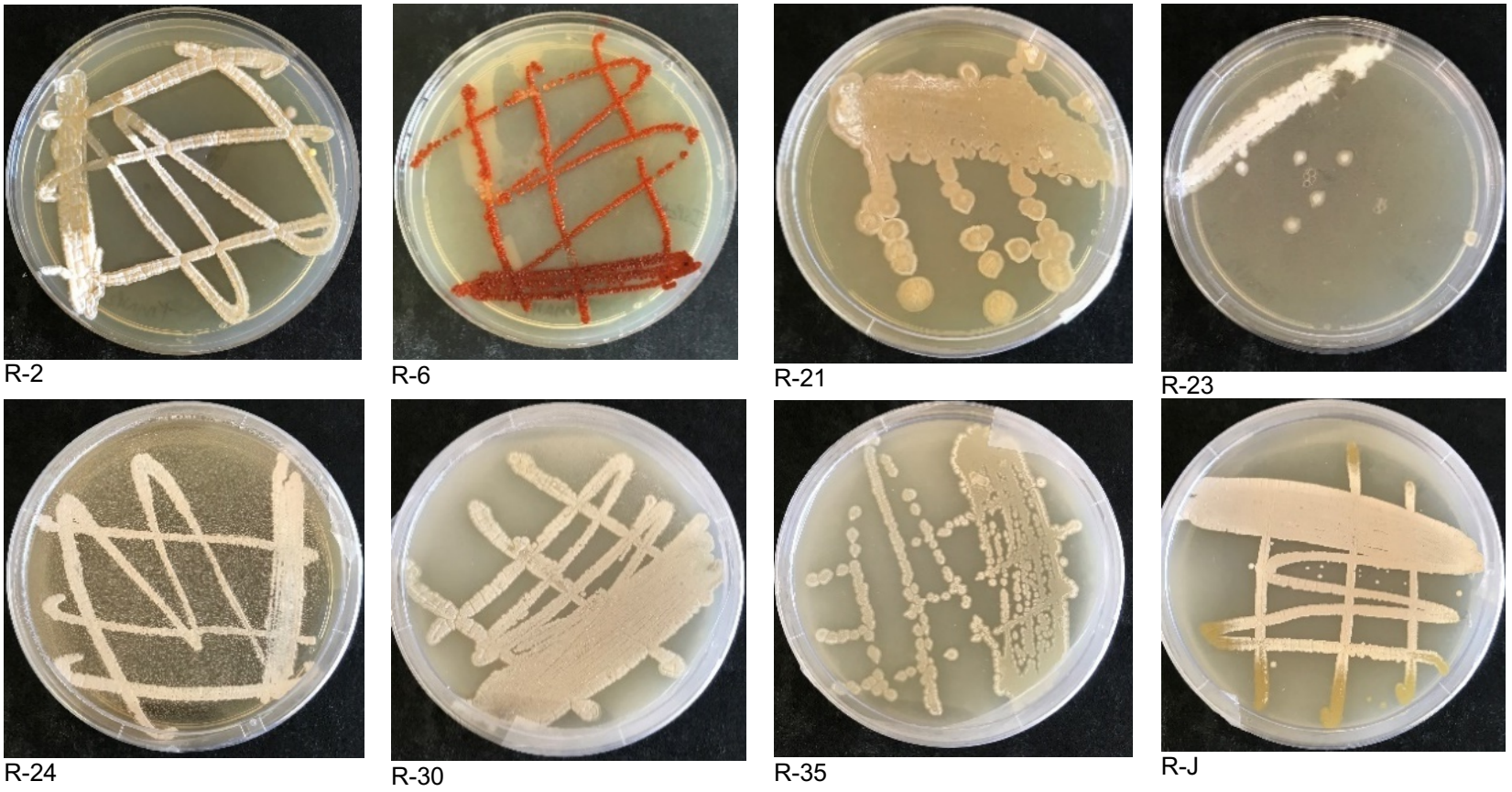
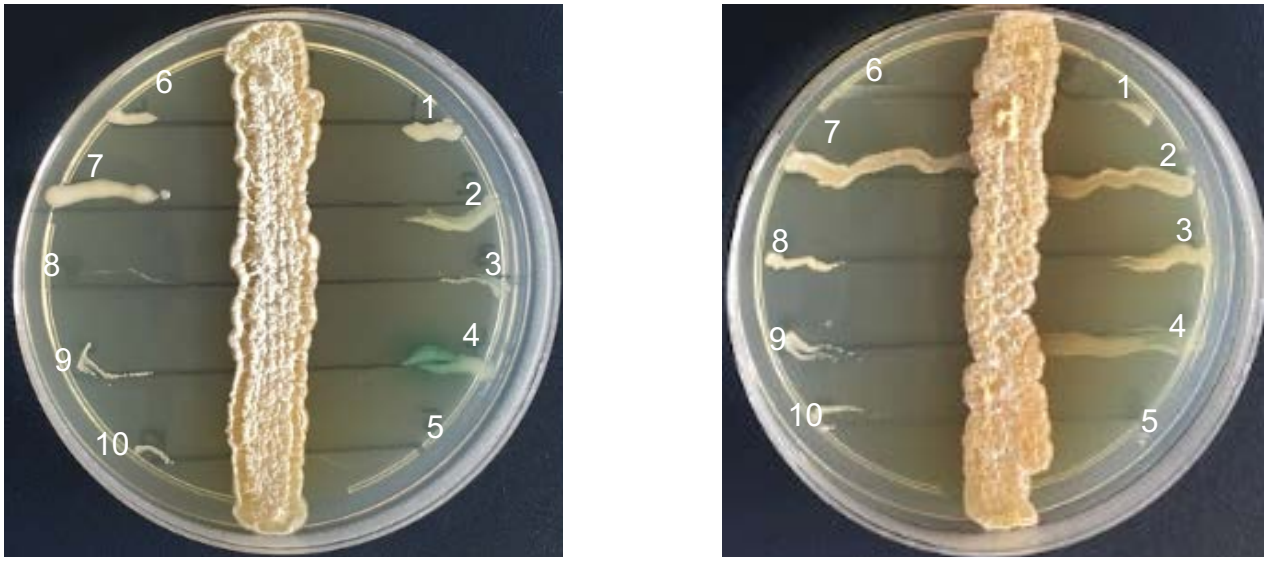


Figure 4.3: Top eight strains exhibiting bioactivity against the ten ATCC test strains selected for further analyses.

Table 4.1: Isolates displaying activity against the ESKAPE test strains on ISP2, ISP2+, M19, and M19+ cross-streak plates.

Isolate designation	Screening media	1. *A. baumannii* ATCC BAA – 1605	2. *E. cloacae* ATCC BAA-1143	3. *E. coli* ATCC 25922	4. *P. aeruginosa* ATCC 27853	5. *S. aureus* ATCC 29213	6. *A. baumannii* ATCC 19606	7. *K. pneumoniae* ATCC 700603	8. *S. aureus* ATCC 33591	9. *E. faecalis* ATCC 29212	10. *E. faecalis* ATCC 51299
R-2	M19	N	N	N	Y	N	N	N	N	N	N
R-2	M19+	N	N	N	N	N	Y	N	N	N	N
R-2	ISP2	N	N	N	Y	N	N	N	N	N	N
R-2	ISP2+	N	N	N	N	N	Y	N	N	N	N
R-6	ISP2+	N	N	N	N	N	N	N	Y	N	N
R-21	M19	N	N	N	Y	Y	N	N	Y	Y	Y
R-21	M19+	N	N	N	N	Y	Y (re-growth)	N	Y	Y	Y
R-21	ISP2	Y	Y	N	Y	Y	Y	N	Y	Y	Y
R-21	ISP2+	N	N	Y (re-growth)	N	Y	N	N	Y	Y	Y
R-23	M19	N	N	N	Y	N	N	N	N	N	N
R-23	ISP2	N	N	N	Y	N	N	N	N	N	N
R-23	ISP2+	Y	N	N	N	N	Y	N	N	N	N
R-24	M19+	N	N	N	N	Y (weak)	N	N	Y (weak)	N	N
R-24	ISP2	N	N	N	Y (weak)	Y (weak)	N	N	Y (weak)	Y (weak)	Y (weak)
R-24	ISP2+	N	N	N	N	Y	N	N	Y	Y	Y
R-30	M19	Y (weak)	N	N	N	Y	Y (weak)	N	Y	Y	Y
R-30	M19+	Y	N	Y	Y (weak)	Y	Y	N	Y	Y	Y
R-30	ISP2	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
R-30	ISP2+	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
R-35	M19	Y (weak)	N	N	N	Y	Y	N	Y	Y	Y
R-35	M19+	Y	N	Y	N	Y	Y	N	Y	Y	Y
R-35	ISP2	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
R-35	ISP2+	Y	Y	Y	N	Y	Y (weak)	N	Y	Y	Y
R-J	M19	N	N	N	N	N	Y	N	Y	N	N
R-J	ISP2	N	N	N	N	N	N	N	Y (weak)	N	N
R-J	ISP2+	N	N	N	N	N	Y	N	Y (weak)	Y	Y



A B

Figure 4.4: A) Image of the cross-streak of R-30 on ISP2 media against the ten test strains where *S. aureus* ATCC 29213 (5) is completely inhibited, and R-30 has strong activity against the remaining nine test strains. B) Cross-streak of R-35 on M19+ displaying complete inhibition of *S. aureus* ATCC 29213 (5) with strong activity against six other test strains and no activity against the three remaining strains.

Five isolates had activity on M19+. R-2 had activity against *A. baumannii* ATCC 19606. R-24 showed weak activity against both *S. aureus* test strains. R-21 had activity against both *S. aureus* strains, *A. baumannii* ATCC 19606, and *E. faecalis* strains. R-30 and R-35 had activity against seven of the test strains: both *A. baumannii* strains, *E. coli*, both *S. aureus* strains, and both *E. faecalis* strains. R-30 also showed weak activity against *P. aeruginosa*.

Seven isolates had activity when cross-streaked on ISP2. R-2 and R-23 had activity against *P. aeruginosa* only. R-21 had activity against eight of the test strains with no activity observed against *E. coli* and *K. pneumonia.* R-24 showed weak activity against *P. aeruginosa*, both *S. aureus* strains, and both *E. faecalis* strains, while R-J showed weak activity against *S. aureus* ATCC 33591. The best activity was seen from isolates R-30 and R-35, which showed nearly complete inhibition of all ten test strains on ISP2.

Lastly, all eight isolates showed activity when cross-streaked on ISP2+. R-2 only showed activity against *A. baumannii* ATCC 19606, and R6 only showed activity against *S. aureus* ATCC 33591. R-21 and R-24 had activity against both *S. aureus* strains and both *E. faecalis*

strains, while R-21 also showed weak activity against *E. coli.* The bacteriostatic effect of the compound produced by R-21 against *E. coli* allowed for regrowth of the test bacteria. R-23 showed activity against both *A. baumannii* strains. R-J had activity against *A. baumannii* ATCC 19606, both *E. faecalis* strains and weak activity was observed against *S. aureus* ATCC 33591. R-35 showed strong activity against seven test strains, weak activity against *A. baumannii* ATCC 19606, and no activity observed against *P. aeruginosa* and *K. pneumoniae.* R-30 once again displayed activity against all ten test strains.

From the cross-streak method, the isolates that displayed activity, displayed activity in more than one media type and in most cases, activity was seen across all media types and often against different test strains. This could explain the 'One Strain-Many Compounds (OSMAC)' theory by using the 'one factor at a time (OFAT)' approach. This indicates that different, usually silent, biosynthetic pathways may become active when the medium used to observe secondary metabolite production against pathogenic bacteria is changed (Djinni et al*.,* 2019:3; Ngamcharungchit et al*.,* 2023:17). Although the medium used was the factor that changed, it showed that different bioactive compounds could be activated against different test strains when different nutrients are present at different concentrations.

The best activity seen was for strains R-30 and R-35, which had activity against all of the test strains across all four media types used (Figure 4.4). For this reason, no media types could be excluded for the preliminary screening in liquid media for the filter disc assay test of the single cultures. The isolates selected for the cross-streak method that showed no activity against the test strains were eliminated for further study.

4.3.2 Filter disc assays

As desired, no activity was seen by the media controls and the solvent DCM:Methanol:water (64:36:8), which shows that the media and the solvent that the extracts were resuspended in had no effect on the results obtained from this experiment. Ampicillin had activity against *E. coli, P. aeruginosa,* both *S. aureus* strains, *A. baumannii* ATCC 19606, and both *E. faecalis* strains. Gentamycin had activity against all ten test strains, however, weak activity was seen against *A. baumannii* ATCC BAA-1605.

Isolate R-6 displayed weak activity against both *E. faecalis* strains at T_2, T_3, and T_4 in ISP2+ media and very weak activity against *S. aureus* ATCC 29213 and *A. baumannii* ATCC 19606 at T_4 in ISP2+ (Table 4.4). Isolate R-21 showed strong activity against *S. aureus* ATCC 29213 at T_2 in ISP2 and weak activity against this test strain at T_3 in ISP2. Isolate R-21 also showed strong activity against *A. baumannii* ATCC 19606 at both T_2 and T_3 in ISP2 and weak activity against *A. baumannii* ATCC 19606 at T_3 in ISP2+ media. Isolate R-30 showed strong activity against this test strain at T_0 and T_3 in both ISP2 and ISP2+. At T_2 R-30 had strong activity against *A. baumannii* ATCC 19606 in ISP2 but weak activity in ISP2+. Weak activity was also seen for R-30 in M19+ at T_3 against *A. baumannii* ATCC 19606. R-30 also had strong activity against *S. aureus* ATCC 29213 at T_0 in both ISP2 and ISP2+ and only had activity in ISP2 at T_2. Furthermore, R-30 had weak activity against *S. aureus* ATCC 29213 at T_3 in ISP2+. Isolate R-35 had strong activity against both *A. baumannii* ATCC 19606 and *S. aureus* ATCC 29213 at T_0 in ISP2 and ISP2+, as well as at T_2 and T_3 in ISP2. Strong activity was seen at T_3 in ISP2+ against *A. baumannii* ATCC 19606 and weak activity against *S. aureus* ATCC 29213. Strong activity was also seen against *A. baumannii* ATCC 19606 at T_2 in both ISP2+ and M19+. Furthermore, weak activity was seen against *A. baumannii* ATCC 19606 at T_3 in M19+ and at T_4 in ISP2.

Throughout the filter disc assay, very weak activity was seen against *P. aeruginosa* ATCC 27853 (Figure 4.5). The weak activity seen for all the isolates against *P. aeruginosa* could indicate that the pathogen was utilizing its efflux ability, as described by Lorusso et al. (2022) that allows for *P. aeruginosa* to expel toxic compounds when a threat is detected in time before growth inhibition is accomplished by bioactive compounds. Images of strong and weak (excluding very weak activity against *P. aeruginosa*) activity observed can be seen in Appendix C.

Table 4.2: Activity of monoculture extracts against the ten test strains used in this study.

Isolate	Screening media	Sampling time	Plate number	*P. aeruginosa* ATCC 27853 (TSA)	*S. aureus* ATCC 29213 (TSA)	*A. baumannii* ATCC 19606 (NA)	*E. faecalis* ATCC 29212 (BHI)	*E. faecalis* ATCC 51299 (BHI + Van)
R-2	ISP2	T0	P1	Y (very weak)	N	N	N	N
R-2	ISP2+	T1	P6	Y (very weak)	N	N	N	N
R-2	M19+	T2	P11	Y (very weak)	N	N	N	N
R-2	ISP2	T3	P15	Y (weak)	N	N	N	N
R-2	ISP2+	T3	P15	Y (weak)	N	N	N	N
R-6	ISP2	T1	P7	Y (very weak)	N	N	N	N
R-6	ISP2+	T1	P7	Y (very weak)	N	N	N	N
R-6	M19	T1	P7	Y (very weak)	N	N	N	N
R-6	ISP2+	T2	P12	N	N	N	Y (weak)	Y (weak)
R-6	M19	T2	P13	Y (very weak)	N	N	N	N
R-6	M19+	T2	P13	Y (weak)	N	N	N	N
R-6	ISP2+	T3	P17	N	N	N	Y (weak)	Y (weak)
R-6	ISP2+	T4	P21	N	Y (very weak)	Y (very weak)	Y (weak)	Y (weak)
R-6	M19	T4	P22	Y (very weak)	N	N	N	N
R-6	M19+	T4	P22	Y (very weak)	N	N	N	N
R-23	ISP2	T3	P14	Y (very weak)	N	N	N	N
R-21	ISP2	T2	P12	N	Y	Y	N	N
R-21	ISP2	T3	P17	N	Y (weak)	Y	N	N
R-21	ISP2+	T3	P17	N	N	Y (weak)	N	N
R-M	ISP2+	T0	P2	Y (very weak)	N	N	N	N
R-M	ISP2+	T1	P7	Y (very weak)	N	N	N	N
R-M	ISP2+	T2	P12	Y (very weak)	N	N	N	N
R-M	M19	T2	P12	Y (very weak)	N	N	N	N
R-M	M19+	T2	P12	Y (very weak)	N	N	N	N
R-M	M19+	T3	P17	Y (very weak)	N	N	N	N
R-M	ISP2	T4	P20	Y (very weak)	N	N	N	N
R-M	M19+	T4	P21	Y (very weak)	N	N	N	N
R-30	ISP2	T0	P1	Y (very weak)	Y	Y	N	N
R-30	ISP2+	T0	P1	N	Y	Y	N	N
R-30	M19	T0	P2	Y (very weak)	N	N	N	N
R-30	M19+	T0	P2	Y (very weak)	N	N	N	N
R-30	ISP2	T1	P5	Y (very weak)	N	N	N	N
R-30	ISP2+	T1	P6	Y (very weak)	N	N	N	N
R-30	ISP2	T2	P10	Y (very weak)	Y	Y	N	N
R-30	ISP2+	T2	P10	Y (very weak)	N	Y (weak)	N	N
R-30	M19+	T2	P11	Y (very weak)	N	N	N	N
R-30	M19+	T2	P11	Y (very weak)	N	N	N	N
R-30	ISP2	T3	P15	Y (very weak)	N	Y	N	N
R-30	ISP2+	T3	P15	Y (very weak)	Y (weak)	Y	N	N
R-30	M19	T3	P16	Y (very weak)	N	N	N	N
R-30	M19+	T3	P16	Y (very weak)	N	Y (weak)	N	N
R-30	ISP2	T4	P19	Y (very weak)	N	N	N	N
R-30	M19	T4	P20	Y (very weak)	N	N	N	N

Isolate	Screening media	Sampling time	Plate number	*P. aeruginosa* ATCC 27853 (TSA)	*S. aureus* ATCC 29213 (TSA)	*A. baumannii* ATCC 19606 (NA)	*E. faecalis* ATCC 29212 (BHI)	*E. faecalis* ATCC 51299 (BHI + Van)
R-35	ISP2	T0	P2	N	Y	Y	N	N
R-35	ISP2+	T0	P3	Y (very weak)	Y	Y	N	N
R-35	M19	T0	P3	Y (very weak)	N	N	N	N
R-35	ISP2	T1	P7	Y (very weak)	N	N	N	N
R-35	ISP2	T2	P11	Y (very weak)	Y	Y	N	N
R-35	ISP2+	T2	P13	N	N	Y	N	N
R-35	M19+	T2	P13	Y (very weak)	N	Y	N	N
R-35	ISP2	T3	P16	Y (very weak)	Y	Y	N	N
R-35	ISP2+	T3	P17	N	Y (weak)	Y	N	N
R-35	M19+	T3	P17	Y (very weak)	N	Y (weak)	N	N
R-35	ISP2	T4	P21	Y (very weak)	N	Y (weak)	N	N
R-J	ISP2	T0	P2	Y (very weak)	N	N	N	N
R-J	ISP2+	T1	P7	Y (very weak)	N	N	N	N

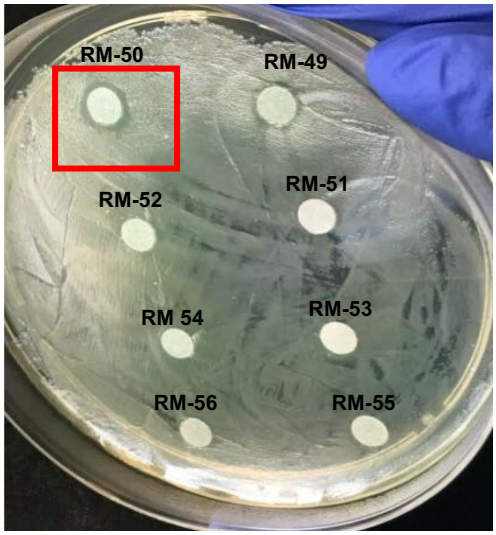


Figure 4.5: Weak activity observed against the test strain *P. aeruginosa*. This type of activity was seen across all media types, where 'very weak' activity was observed against *P. aeruginosa* (Table 4.4).

From the strains that displayed activity in the cross-streak method, only isolates R-30 and R-35 displayed very good activity in liquid media. Although the activity displayed from these two strains was not as prominent in the liquid media extracts as it was in the solid media cross-streak test, the extract obtained from strain R-30 consistently displayed good activity against *S. aureus* ATCC 29213 and *A. baumannii* ATCC 19606 at the various sampling times (T_0 – pre-culture, T_1 – 1-day old culture, T_2 – 3-day old culture, T_3 – 5-day old culture). The best activity was observed when the R-30 and R-35 strains were cultured in ISP2 media. For this reason, all other media types were eliminated, and only R-30 and R-35 were selected for further investigation in ISP2 media.

Many antibiotic-producing actinobacterial strains often exhibit enhanced secondary metabolite production on solid media (Davidson et al., 2008:319). The stress associated with nutrient limitation and competition for resources can cause the production of bioactive compounds. A significant fraction of the putative transporters are the ATP-binding cassette (ABC) transporters which are responsible for importing nutrients into the cell and exporting toxins and secondary metabolites out of the microorganism (Davidson et al*.,* 2008:319). In some cases, microorganisms, when cultivated under the right conditions in liquid media, can produce a high yield of bioactive compounds (Vijayakumari et al*.,* 2013:137). Unfortunately, this was not seen in this study when comparing the cross-streak method to the filter disc assay of the single cultures. This, however, aligns with what Gebreyohannes et al. (2013:433) observed, stating that the fluctuation in results of liquid media extracts when comparing it to that of the solid media results, could be due to the degradation of crude extract compounds after the use of organic solvents. Culture parameters play a major role in what bioactive compounds a microorganism produces. For this reason, the correct pH, temperature, and nutrients available determine if cryptic pathways will be activated or not (Djinni et al*.,* 2019), whereas the extracts obtained from the liquid culture had undergone multiple steps before the crude extract was ready for screening against the test strains.

4.3.3 16S rRNA gene sequences and phylogenetic analysis

Table 4.3 contains information on the 16S rRNA gene-based identification of the top eight bioactive strains, which showed that all of the top eight strains belonged to the genus *Streptomyces*, as confirmed by phylogenetic analysis (Figure 4.6). 16S rRNA analysis reported the genus correctly; however, the species was not accurately detected as the 16S rRNA amplification does not provide enough genetic material to correctly predict the species of the actinobacteria.

Table 4.3: Identification of the top eight bioactive strains Identification was performed using Ezbiocloud from the 16S rRNA gene sequence analyses.

Isolate code	Forward sequence identity	Reverse sequence identity	Consensus sequence identity	Consensus sequence length (bp)
R-2	*Streptomyces pratensis* (98.51% Similarity)	*Streptomyces pratensis* (97.77% Similarity)	*Streptomyces pratensis* (99.49% Similarity)	1432
R-6	*Streptomyces longisporoflavus* (98.56% Similarity)	*Streptomyces rosealbus* (96.94% Similarity)	*Streptomyces longhuiensis* (99.00% Similarity)	1434
R-21	*Streptomyces tunisiensis* (99.61% Similarity)	*Streptomyces tunisiensis* (97.17% Similarity)	*Streptomyces malachitospinus* (99.02% similarity)	1428
R-23	*Streptomyces lienomycini* (96.78% Similarity)	*Streptomyces ambofaciens* (97.47% Similarity)	*Streptomyces tibetensis* (98.72% similarity)	1421
R-24	*Streptomyces sedi* (95.44% similarity)	*Streptomyces zhaozhouensis* (96.23% similarity)	*Streptomyces qinglanensis* (98.95% Similarity)	1434
R-30	*Streptomyces labedae* (96.53% Similarity)	*Streptomyces althioticus* (95.98% Similarity)	*Streptomyces tunisiensis* (99.42% Similarity)	1437
R-35	*Streptomyces griseoincarnatus* (83.49% Similarity)	*Streptomyces griseoincarnatus* (81.73% Similarity)	*Streptomyces tunisiensis* (99.42% Similarity)	1433
RJ	*Streptomyces parvulus* (95.77% Similarity)	*Streptomyces parvulus* (97.13% Similarity)	*Streptomyces parvulus* (98.67% Similarity)	1433

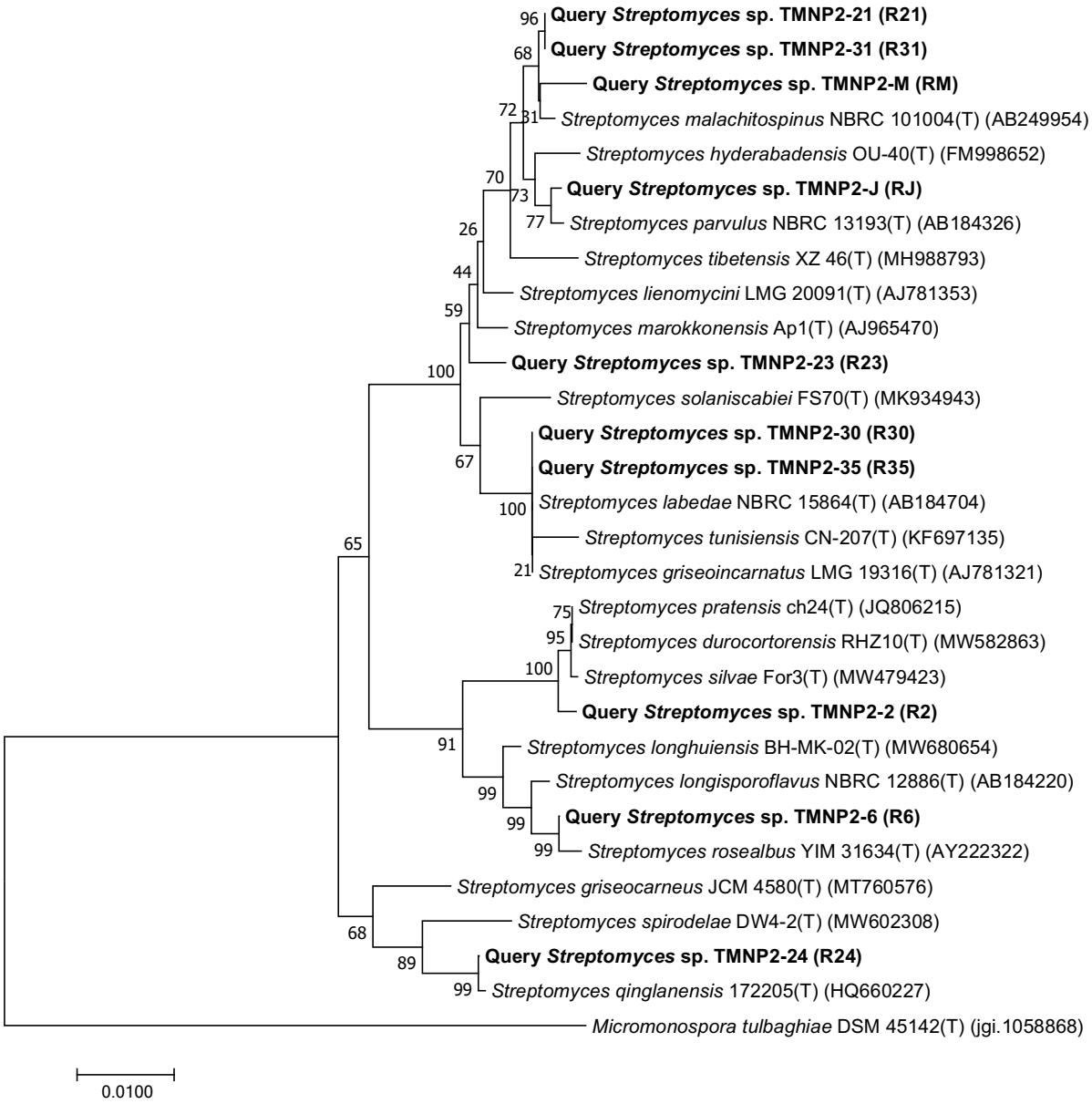


Figure 4.6: Neighbour-joining phylogenetic tree representing the relationship of the closest relatives of the TMNP2 strains with activity based on the 16S rRNA sequences.

Although there were various isolation techniques employed for actinobacteria isolation, the top eight strains that showed bioactivity were all streptomycetes. Indicating that the colony selection may have been biased towards 'typical' filamentous growth types which resulted in the picking of these streptomycetes. However, this is not seen as a bad outcome. *Streptomyces* species still prove to be a valuable source of natural antimicrobial compounds as they are solely responsible for approximately 75% of identified bioactive compounds (Pacios-Michelena et al., 2021). As the resistance to pathogenic bacteria increases, researchers have been implementing new ways to change the existing antibiotics or finding

novel antibiotics from this group of bacteria that are more effective against antimicrobial-resistant bacteria (Hasani et al., 2014:67). Methods typically used in the search for new antimicrobial compounds include bioprospecting – exploring the biodiversity of a selected environment for novel resources (Beattie et al., 2011), metagenomics, isolation and cultivation bio-assay fractionation – a technique used to isolate and purify active compounds which involves sequentially partitioning the crude extract and testing each fraction for bioactivity until the active compound is identified (Aliahmadi et al., 2014), and chemical synthesis and modification – chemically synthesizing analogs of naturally occurring marine compound or modifying compounds that are already in existence to increase their potency (Rowe & Spring, 2021). The techniques focused on in this study are mainly isolation and cultivation and the metagenomics and bio-assay approaches.

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Chapter 5

Co-culture, molecular networking, and genome mining

5.1 Introduction

Actinobacteria produces a wide range of bioactive metabolites, many of which are significant lead compounds for therapeutic applications. Consequently, their investigation may yield a vast reservoir of potentially active substances. New genera and species offer promise as sources of unique bioactive compounds. It is essential to understand the biodiversity of the strains, look into and identify new genera or species, and improve the compound production process (Ngamcharungchit et al., 2023:18-19). The genus *Streptomyces* is well-known for its intricate development cycle that has been thoroughly studied. Studies have shown that the production of secondary metabolites is induced as a defense mechanism against antagonistic microorganisms (Djinni et al*.,* 2019:2).

Naturally, microorganisms co-exist in a community which prompts the production of bioactive compounds to inhibit the growth of competitive microbial species within the same environment. In addition to secondary metabolite production, the development cycle also plays a significant role in the organic matter cycling within the soil and sediment ecosystem (Yu et al*.,* 2019). Co-cultivation of two or more bacterial strains enhances the possibility of activating silent biosynthetic pathways. In a study conducted by Yu et al. (2019), a marine-derived *Streptomyces rochei* MB037 was co-cultured with a *Rhinocladielle* sp. and this successfully activated the production of new secondary metabolites, previously reported as borrelidin, borrelidin F, borrelidin J, borrelidin K, and 7-methoxy-2,3-dimethylchromone-4-one.

In this study, indirect co-culture of actinobacteria:actinobacteria (R-30:R-35) and direct co-culture of actinobacteria:*Mycobacterium aurum* A+ (also an actinobacterium, but containing mycolic acids in its cell wall; R-30:*M. aurum* and R-35:*M. aurum*) was performed to determine the effects of co-culture on antimicrobial compound production against the ten test strains using the filter disc method for visualisation of bioactive compound production. The use of molecular networking (based on MS/MS fingerprinting) was also explored alongside the genome sequence of strain R-35.

5.2 Materials and Methods

5.2.1 Co-culture: Actinobacteria vs Actinobacteria (non-contact)

I. Pre-culture preparation

Two strains, R-30 and R-35 were selected for the co-culture experiment. Eight 50 mL shake flasks, each 10 mL ISP2 (pH 7 to 7.2), were prepared. Flasks were inoculated with half a loop of the outer edge of a colony of the actinobacterial strains; each strain was inoculated in triplicate. The last two flasks were not inoculated and served as media control flasks. All flasks were incubated at 30 °C, shaking on a rotary shaker at 160 rpm for 5 days. On day 5, three flasks that were inoculated with the same actinobacterial strains were combined into one 50 mL flask and used as the inoculum for the main culture.

II. Main culture preparation

The 250 mL co-culture flasks were set up with a 0.22 µm membrane filter between them, as shown in Figure 5.1. A clamp was used to secure the two flasks together to ensure that no media was leaking from the position where the filter was connecting the two flasks. Six sets of the co-culture flasks were set up for the experiment. ISP2 was prepared and autoclaved separately from the flasks, in order to prevent the media from leaking through the connection site due to the expansion of the glass and metal during sterilisation. After being autoclaved, 50 mL of ISP2 was dispensed into each of the flasks, and this was done under sterile conditions. Six single 250 mL flasks were also prepared for each strain (mono-cultures), as well as nine 50 mL flasks with 10 mL of ISP2 that served as media controls.

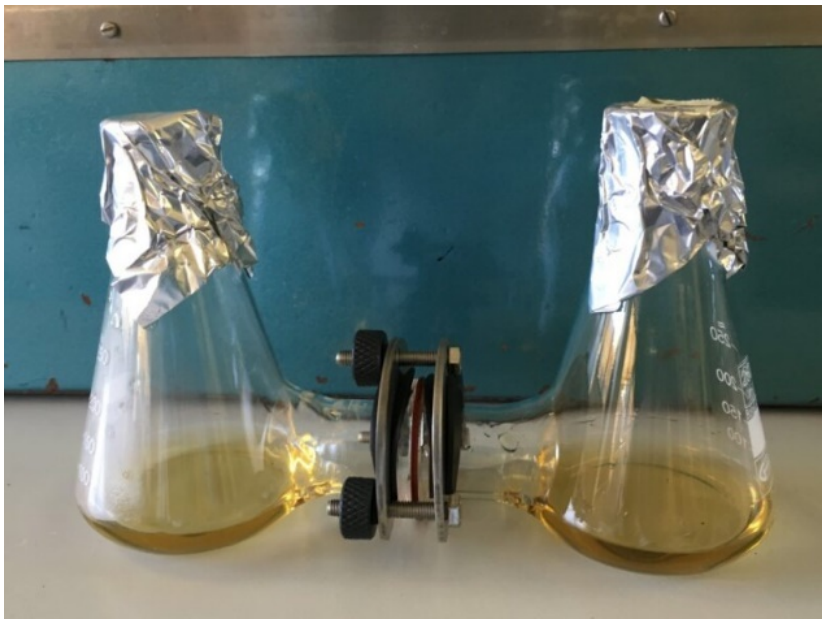


Figure 5.1: Non-contact co-culture setup of two 250 mL flasks containing 50 mL of ISP2 liquid media.

Each flask of the co-culture setup was then inoculated with 1 mL of pre-culture (strain R-30 into one and strain R-35 into the other). Mono-culture flasks for each strain were also inoculated with 1 mL of pre-culture. The co-culture, mono-culture, and media control flasks were incubated at 30 °C, shaking at 160 rpm. After 1 (T_1), 3 (T_2), and 5 (T_3) days of incubation, extracts were prepared from two co-culture setups, two mono-culture flasks of each strain, and three media control flasks.

III. Extract preparation from main cultures

On each sampling day, 2.5 g of DIAION HP-20 was added to the co-culture and mono-culture flasks, while 0.5g of DIAION HP-20 was added to the media control flasks. The flasks were incubated overnight at 30 °C, shaking at 160 rpm. The following day the cultures were filtered through coffee filters and washed with 5 volumes of sterile dH_2O. Once the dH_2O had passed through the filter, the washed cells and DIAION were collected off the coffee filter and transferred to clean 250 mL flasks (the co-culture set-up was then separated at this point). 50 mL methanol was added to each flask and incubated at 30 °C, shaking at 160 rpm overnight. Methanol extracts were filtered into pre-weighed McCartney bottles and the extracts were dried in a MiVac Quattro (method: -OH, 30 °C). The dried extract and McCartney bottles with dried extract were weighed, and the weight of the dried extract was calculated as before (Section 4.2.3). Extracts were resuspended in DCM:Methanol:water (64:36:8) to a concentration of 10 mg/mL.

5.2.2 Co-culture: Marine Isolates (R-30 and R-35) vs alive *Mycobacterium aurum* A+ and 'dead' *M. aurum* A+ (direct contact)

I. Pre-culture preparation

Pre-cultures were prepared by inoculating strains R-30, R-35, and *M. aurum* A+ in triplicate into 10 mL ISP2 and incubating at 30 °C for 5 days, shaking at 160 rpm. The pre-cultures were combined into one flask for R-30, R-35, and *M. aurum* A+ and used as the inoculum for the main culture.

II. Main culture preparation

For the co-culture, 300 µL of marine actinobacterium and 100 µL of the alive *M. aurum* A+ pre-cultures were inoculated into 10 mL ISP2 in 50 mL flasks in triplicate and incubated at 30 °C at 160 rpm. After 1 (T_1), 3 (T_2), and 5 (T_3) days of incubation, flasks were removed for extract preparation following the crude extract preparation method described in section 5.2.1 (III). On each sampling day, extracts were prepared from three R-30 mono-culture flasks, three R-35 mono-culture flasks, three *M. aurum* A+ mono-culture flasks, three media control flasks, three

R-30 plus *M. aurum* A+ co-culture flasks and three R-35 plus *M. aurum* A+ co-culture flasks. The same setup was used for the 'dead' *M. aurum* A+ test; however, the *M. aurum* pre-culture was autoclaved at 121 °C for 25 minutes before addition to the main culture flasks.

5.2.3 Co-culture: Marine isolates (R-30 and R-35) vs different concentrations of alive or 'dead' *Mycobacterium aurum* A+ (direct contact)

Pre-cultures were prepared by inoculating R-30 and R-35 in triplicate into 10 mL ISP2 and incubated at 30 °C for 5 days, shaking at 160 rpm. The three pre-culture flasks were combined into one inoculum for R-30 and R-35. For the main culture, 300 µL of the actinobacterial pre-culture was inoculated into 10 mL ISP2 in 50 mL flasks and incubated at 30 °C for four days at 160 rpm. On the fourth day, different concentrations of alive or 'dead' *M. aurum* A+ cells were added to the main cultures and allowed to grow overnight. The concentrations of *M. aurum* A+ used were: 0.1% (v/v) alive or 'dead', 0.5% (v/v) alive or 'dead', and 1% (v/v) alive or 'dead'. Samples that were extracted on day 5 are as follow, all in triplicate:

a. R-30 in 10 mL ISP2 (mono-culture)
b. R-35 in 10 mL ISP2 (mono-culture)
c. Alive *M. aurum* A+ in 10 mL ISP2 (mono-culture)
d. 'Dead' *M. aurum* A+ in 10 mL ISP2 (mono-culture)
e. 10 mL ISP2 (media control)
f. R-30 in 10 mL ISP2 + 0.1% alive *M. aurum* A+
g. R-30 in 10 mL ISP2 + 0.1% 'dead' *M. aurum* A+
h. R-35 in 10 mL ISP2 + 0.1% alive *M. aurum* A+
i. R-35 in 10 mL ISP2 + 0.1% 'dead' *M. aurum* A+
j. R-30 in 10 mL ISP2 + 0.5% alive *M. aurum* A+
k. R-30 in 10 mL ISP2 + 0.5% 'dead' *M. aurum* A+
l. R-35 in 10 mL ISP2 + 0.5% alive *M. aurum* A+
m. R-35 in 10 mL ISP2 + 0.5% 'dead' *M. aurum* A+
n. R-30 in 10 mL ISP2 + 1% alive *M. aurum* A+
o. R-30 in 10 mL ISP2 + 1% 'dead' *M. aurum* A+
p. R-35 in 10 mL ISP2 + 1% alive *M. aurum* A+
q. R-35 in 10 mL ISP2 + 1% 'dead' *M. aurum* A+

Crude extracts were prepared from each flask using the preparation method described in section 5.2.1 (III).

5.2.4 Filter disc assay

All extracts prepared from the mono-cultures, co-cultures (both non-contact and direct contact), and media controls were tested for bio-activity against the ESKAPE test strains with the filter disc assay as described in section 4.2.4.

5.2.5 LC-ESI-MS/MS acquisition

All the extracts prepared from the different co-culture setups, mono-cultures, and media controls were submitted to the LC-MS facility at Rhodes University for MS/MS fingerprinting and molecular networking. Dried extracts were resuspended in methanol at 1 mg/mL and analysed on a Bruker Compact EST-MS/MS, coupled to a Dionex Ultimate 3000 UHLPC equipped with a Waters Xselect CSH C18 column (2.1 × 150 mm, 2.5 µm). The injection volume was 5 µL, and the mobile phase consisted of water (A) and acetonitrile (B). Both were acidified with 0.1% (v/v) formic acid. The mobile phase was run on a gradient program with a flowrate of 0.4 mL/min: min 0-2 = 85/15 (A/B), min 2-3 = ramp to 60/40 (A/B), min 3-5 = hold, min 5-6 = ramp to 30/70 (A/B), min 6-8 = hold, min 8-10 = ramp to 0.1/99.9 (A/B), min 10-13 = hold, min 13-16 = re-equilibration. The electrospray source was operated in positive ionisation mode with an End Plate Offset voltage of 500 V, a capillary voltage of 4500 V, a nebulizer pressure of 3 bar, a dry gas flow of 9 L/min, and a dry temperature of 200 °C. A mass range from 50 to 2000 m/z was set for acquisition and collision energies for MS2 collision-induced dissociation were set to 330 and 60 eV (Multi-CE) and an isolation window width of 1.5 m/z. The number of precursors was set to 10 with active exclusion after 6 spectra and release after 0.5 min or when current intensity or previous intensity was less than or equal to 2. The trigger threshold was set to 1350 cts.

5.2.6 Data processing

The data was converted to mzXML format with Bruker Compass software and then processed in MZmine3 (Schmid et al., 2023). Mass detection was carried out with noise levels of 1000 for MS1 and 50 for MS2, followed by chromatogram assembly with the ADAP Chromatogram builder with a minimum of 3 consecutive scans, a minimum intensity for consecutive scans of 1200 and a minimum height of 1500, m/z tolerance was set to 0.001 m/z or 10 ppm. The chromatograms were deconvoluted using the local minimum feature resolver with a chromatographic threshold of 1%, a minimum search range of 0.1 min, a minimum height of 1500, a minimum edge-to-top peak ratio of 1.2, and a peak duration of 0.3 min. MS2 spectra were linked to MS1 features with a tolerance of 0.05 m/z and 0.2 min. Thereafter, the isotopic peaks finder was used with the elements C, H, N, O, P, S, Cl, and Br, with a maximum charge of 1 and a tolerance of 0.001 m/z.

The weight was set to 80 for m/z and to 20 for retention time. The aligned feature list was then filtered to only keep features between 1.2-4 min and with an associated MS2 spectrum. Gap-filling was carried out with the Peak finder module with tolerances of 10% for intensity, 0.2 min and 0.001 m/z. The Feature list blank subtraction module was used to remove all features present in the media controls, unless their peak area in one of the samples was more than 300% compared to the controls. The Duplicate feature filter was then used with tolerances of 0.2 min and 0.001 m/z, followed by reordering of the feature IDs. The final feature list defined for the global natural product server (GNPS) and SIRIUS5 was exported. In both cases, MS2 spectra were merged across samples with the weighted average mode, a mass tolerance of 0.001 m/z or 10 ppm, a minimum cosine of 0, a signal count threshold of 20%, and an isolation window width of 1.5.

5.2.7 Spectral matching and molecular networking with GNPS

Spectral matching and molecular networking with GNPS were carried out when a molecular network was created with the Feature-Based Molecular Networking (FBMN) workflow (Nothias et al., 2020) on GNPS (Wang et al., 2016). The mass spectrometry data was first processed with MZMINE2 (Dührkop et al., 2019), and the results were exported to GNPS for FBMN analysis. The precursor ion mass tolerance, as well as the MS/MS fragment ion tolerance, was set to 0.02 Da. A molecular network was created where edges were filtered to have more than 4 matched peaks and a cosine score above 0.7. When each of the nodes appeared in each other's respective top 10 most similar nodes, edges between two nodes were kept in the network. The lowest-scoring edges were removed from molecular families until the molecular family size was below 100. The analogue search mode was used by searching against MS/MS spectra, and library spectra were required to have a score above 0.7 and at least 4 matched peaks. MS/MS spectra were annotated by using the DEREPLICATOR (Mohimani et al., 2018), while Cytoscape software (Shannon et al., 2003) was used to visualise the molecular networks.

The results were further analysed through the SIRIUS5 suite (Dührkop et al., 2019) to achieve compound class and molecular structure predictions for the annotation of ion features that did not provide spectral matches through GNPS. The default parametrization was utilised except for the selected elements: C, H, N, O, P, S, Cl, and Br, with possible ionisations of [M+H]+ and [M+Na]+. For in silico structure prediction, all databases were selected.

5.2.8 Whole genome sequencing

In order to determine the biosynthetic capability of the actinobacterial strain, R-35, whole genome sequencing was performed. Three 50 mL flasks with 10 mL ISP2+ were prepared and inoculated with strain R-35. The flasks were incubated at 30°C for 5 days, shaking at 160 rpm.

The Zymo Research Quick-DNA Fecal/Soil Microbe Miniprep Kit was used as per the manufacturer's instructions for DNA extraction. The integrity of the extracted DNA was examined using 1% (w/v) agarose gel electrophoresis, and the concentration and purity were determined using a Genova Nano Micro-Volume Spectrophotometer (Jenway, Staffordshire, United Kingdom). In order to confirm that the DNA extracted was from the strain of interest, 16S rRNA gene amplification was performed as described in section 4.2.5. The amplicons were then analysed as previously described.

The extracted DNA was submitted for genome sequencing at CAF, Stellenbosch University, South Africa. The raw sequence data was generated on an Ion Torrent S5 platform using 200 base chemistry, and fastq files were generated. Sequenced data was assembled using SPAdes v3.31.12 on the viral and bacterial bioinformatics resource center website (https://www.bv-brc.org/). The assembled genome was submitted to antiSMASH 6.0 (Blin et al., 2021; https://antismash.secondarymetabolites.org/#!/start) to predict the different types of biosynthetic gene clusters that may be present in the genome and the potential antibiotics that the organism could produce. The assembled genome sequence was also submitted to the Type Strain Genome Server (TYGS; https://tygs.dsmz.de/; Meier-Kolthoff & Göker, 2019) at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) to determine if the actinobacterial strain may represent a novel or known species. The genome was visualised using Proksee (Grant et al., 2023), and specific features identified in the genome through the use of the Comprehensive Antibiotic Resistance Database (CARD) Resistance Gene Identifier (RGI) (version 1.1.1) tool (Alcock et al., 2020). The raw genome sequencing data has been submitted to the National Center for Biotechnology (NCBI) under the BioProject ID PRJNA1129156 (BioSample ID Accession Number: SAMN42145163; Short Read Archive (SRA) Accession: SRR29633055; https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1129156).

All reagents used in this study were sourced from Merck Millipore unless otherwise stated.

5.3 Results and Discussion

Although marine actinobacteria may produce some of the same compounds as their terrestrial relatives, in the case of rare marine actinobacteria, the potential to find novel compounds greatly increases. By manipulating the growth environment of the actinobacteria in a laboratory setting by introducing a competitor microorganism, co-culture can create a competitive environment that can both stimulate and improve the production of new secondary metabolites by activating silent gene clusters (Djinni et al*.,* 2019; Ngamcharungchit et al., 2023).

Only two of the eight actinobacterial strains that showed activity in the cross-streak test showed strong activity when cultured in liquid media. For this reason, the co-culture experiment was set up only with isolates R-30 and R-35 as the marine actinobacteria of interest to attempt to induce novel antimicrobial compounds under competitive culturing conditions.

5.3.1 Mixed culture fermentations of R-30 and R-35 (non-contact)

As with the initial screening assay, no activity was seen for the media control extracts and the solvent DCM:Methanol:water (64:36:8), which shows that the media and the solvent that the extracts were resuspended in had no effect on the results obtained from this experiment. Ampicillin had activity against *E. coli, P. aeruginosa,* both *S. aureus* strains, *A. baumannii* ATCC 19606, and both *E. faecalis* strains. Gentamycin had activity against all ten test strains, however, weak activity was seen against *A. baumannii* ATCC BAA 1605.

At T_1, only the mono-culture of R-35 had weak activity against *E. faecalis* ATCC 29212 (Table 5.1). When R-30 and R-35 were co-cultured together, R-30 showed activity against *P. aeruginosa, S. aureus* ATCC 29213, *A. baumanni* ATCC 19606, and both *E. faecalis* test strains at T_1, while R-35 had activity against *P. aeruginosa,* and *E. faecalis* ATCC 52199, as well as weak activity against *S. aureus* ATCC 29213 (Table 5.1). This indicated that co-culture of these two strains had a positive effect on the production of bioactive compounds at T_1. At T_2, R-35 co-culture extract showed activity against *A. baumannii* ATCC 19606. At T_3 in both the co-culture and mono-culture of R-30, only one of the duplicate extracts had weak activity against *S. aureus* ATCC 29213. At this time point, the mono-culture of R-35 had activity against *S aureus* ATCC 29213, *A. baumannii* ATCC 19606, and both *E. faecalis* test strains, while one of the replicates for R-35 co-culture only had activity against *A. baumannii* ATCC 19606. This indicated that co-culture had a negative impact on the bioactive compound production of R-35 at T_3. Examples of the bioactivities observed can be seen in Figure 5.2.

Non-contact co-culture with separation of the actinobacterial strains with semi-permeable membrane allows for only the exchange of nutrients and metabolic signals through the barrier and aids in mimicking the isolates' natural habitat. A favourable outcome would be to see more activity present from the isolates in co-culture. This can be seen in the results of this experiment performed at T_1 since co-culture of the two actinobacterial strains had a positive effect on the production of antimicrobial compounds. An experiment conducted by Hifnawy et al. (2020) showed that the co-culture of two marine-derived actinobacterial strains, *Micromonospora* sp. UR56 and *Actinokineospora* sp. EG49 yielded twelve metabolites from the extracts. Extracts obtained from the *Micromonospora* sp. UR56 indicated diverse metabolite production from different chemical classes. This diverse metabolite production was assumed to be due to a competitive environment or a chemical defence mechanism of *Micromonospora* sp. UR56. Another study conducted by Li et al. (2022) showed that co-culture of *Streptomyces albireticuli*

and *Streptomyces albofavus* produced more antimicrobial compounds than in mono-cultures. This confirms what was observed in the co-culture experiment conducted between R-30 and R-35.

Table 5.1: Activity of extracts when the two actinobacterial strains, R-30 and R-35, were co-cultured with one another.

Treatment	Replicate	Sampling time	*P. aeruginosa* ATCC 27853 (TSA)	*S. aureus* ATCC 29213 (TSA)	*A. baumannii* ATCC 19606 (NA)	*E. faecalis* ATCC 29212 (BHI)	*E. faecalis* ATCC 51299 (BHI + Van)
R35	1	T1	N	N	N	Y (very weak)	N
R35	2	T1	N	N	N	Y (very weak)	N
R30 (co-culture)	1	T1	Y	N	N	Y	N
R30 (co-culture)	2	T1	Y	Y	Y	Y	Y
R35 (co-culture)	1	T1	Y	Y (weak)	N	N	Y
R35 (co-culture)	1	T2	N	N	Y	N	N
R30 (co-culture)	1	T3	N	Y (very weak)	N	N	N
R30	1	T3	N	Y (very weak)	N	N	N
R35 (co-culture)	2	T3	N	N	Y	N	N
R35	1	T3	N	Y (weak)	Y	N	Y
R35	2	T3	N	Y	Y	Y	Y

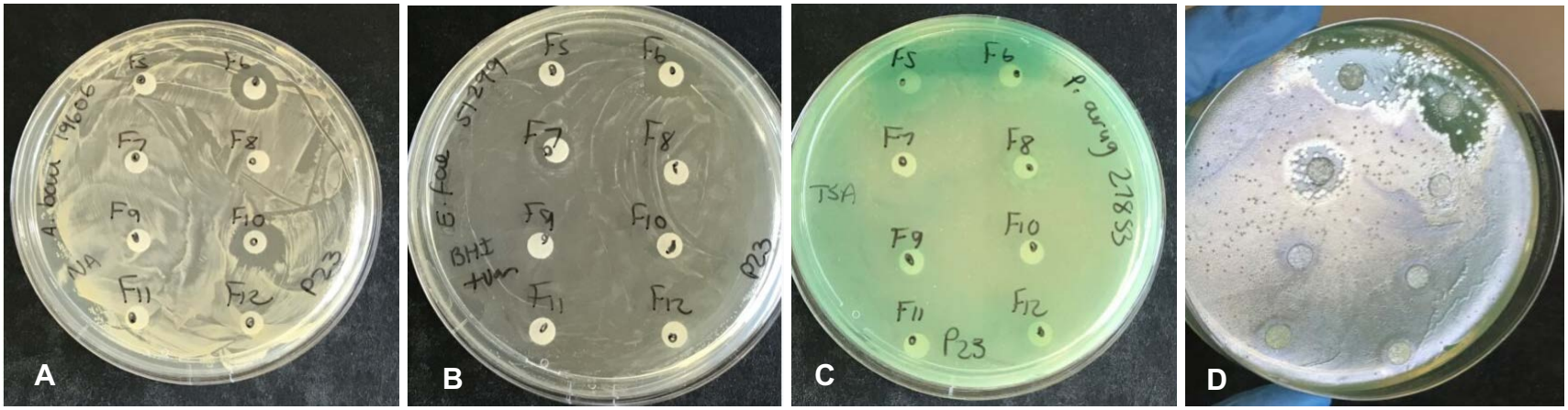


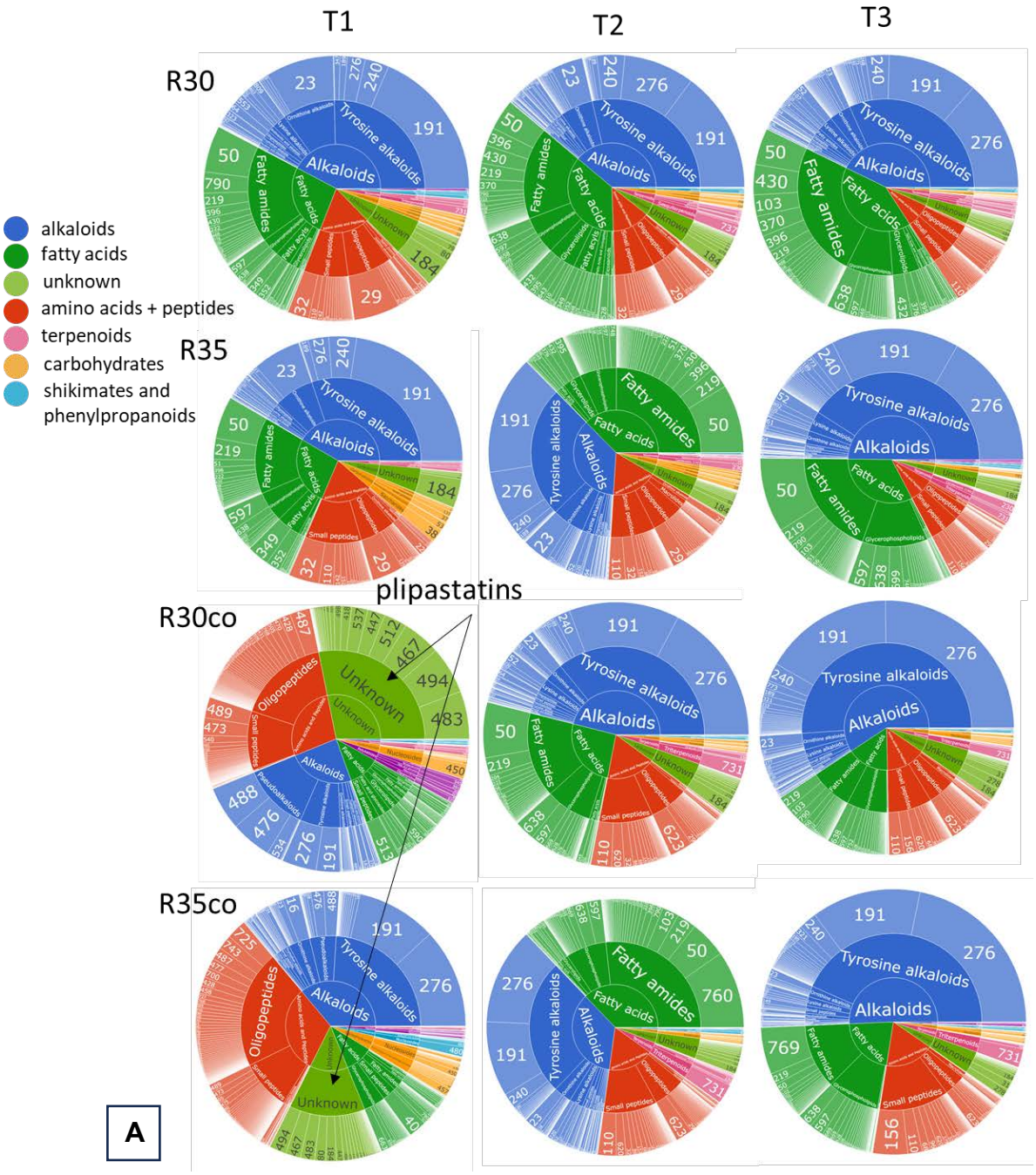
Figure 5.2: A) Extracts showing visible activity against *A. baumannii* ATCC 19606. B) Extracts displaying activity against *E. faecalis* ATCC 51299 and C) back of plate showing extracts with activity against *P. aeruginosa* ATCC 27853, and D) the front of plate showing the activity observed against *P. aeruginosa.*

5.3.2 Molecular networking of R-30 and R-35 in non-contact co-culture

As previously described, isolates R-30 and R-35 were cultured on their own and co-cultured with one another in the non-contact co-culture method. Each sample was produced in duplicate and sampled at three time points (T_1, T_2 and T_3). Organic solvent extracts were analysed using RP-LC-ESI-MS/MS in positive ionisation mode.

At T_1 both R-35 co-culture and R-30 co-culture produced features annotated as plipastatins surfactins, bacillaenes, mycosubtilins, threonine and serine containing aminolipids, and various unannotated distinct clusters (Figure 5.3). Plipastatins have been found to inhibit the growth of *S. aureus* by blocking quorum sensing (Gao et al., 2022:1177), surfactins have been found to inhibit the growth of multi-drug-resistant *S. aureus,* and *P. aeruginosa* (Meena & Kanwar, 2015), while mycosubtilins have been found to inhibit the growth of *P. aeruginosa* (Leclère et al*.,* 2005). Threonine and serine do not themselves display antimicrobial activity, however, they compromise the integrity of the pathogen's cell wall by making the pathogen susceptible to antibiotics (Pereira et al*.,* 2011), which could explain the activity seen against both *E. faecalis* strains and *A. baumannii* ATCC 19606 in this study. At later time points, these features were not detected.

For all cultures, towards later time points, the relative abundance of features annotated as desferrioxamines appears to increase, excluding R-35 co-culture. R-30 produced several distinct antimycins, also detected in R35 at T_1, while R-30 at later time points appears to shift towards containing a greater relative abundance of desferrioxamines and specific features annotated as aminolipids at T_2 and T_3, but not at T_1. R-35 produced no dominant features at T_1, while at T_2 and even less at T_3 various features annotated as antimycins were detectable. Towards T_3, many unannotated features increased in relative abundance in addition to features annotated as aminobacteriohopanetriols, which were also enriched in R-35 at T_3, as well as in R-30 and R-35 co-cultures at T_2 and T_3. Overall, metabolome shifts were observed, particularly between T_1 and T_2, in the co-cultures. Metabolomes further shifted from T_2 to T_3, which could relate to a maturing process of the cultures. The mono-cultures did not produce high abundances for most of the measured metabolites in T_1, whereas the co-cultures did. This could be due to a response to initial exposure of the co-cultured bacterium. The cause of the observed biological activities could, unfortunately, not be unraveled using molecular networking analyses. Singletons shown in Figure 5.3B could possibly represent new bioactive compounds that are shown by the unknown metabolites in Figure 5.3A. Molecular networking on mono-cultures and co-cultures for R-30 and R-35, can be found in Appendix E.


T1
T2
T3
R30
R35
R30co
R35co
plipastatins
alkaloids
fatty acids
unknown
amino acids + peptides
terpenoids
carbohydrates
shikimates and phenylpropanoids
Alkaloids
Tyrosine alkaloids
Fatty amides
Fatty acids
Unknown
Oligopeptides
Small peptides
Pseudoalkaloids
A

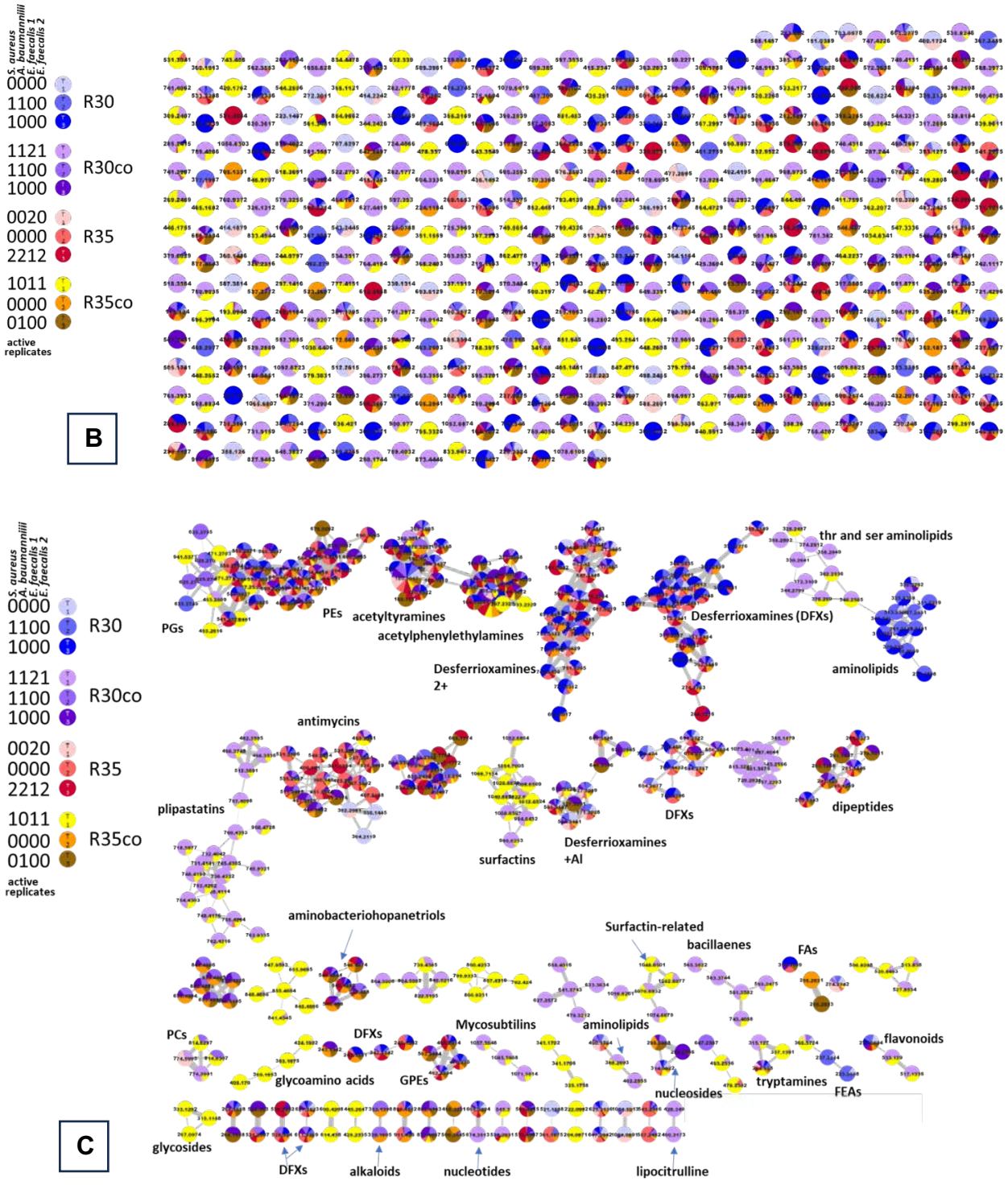


Figure 5.3: A) Sunburst charts created from CANOPUS NPClassifier output showing relative abundances of features per compound class (replicates were averaged), B) Singletons observed, and C) annotated molecular network clusters of the extracts obtained during non-contact co-culture.

5.3.3 Mixed culture fermentations

5.3.3.1 Mixed culture fermentaion of R-30 and R-35 vs *Mycobacterium aurum*

When direct contact co-culture was performed, R-35 produced activity against three of the test strains at different sampling times (Table 5.2). The extracts prepared from the T_3 mono-culture of R-35 showed activity against *S. aureus* ATCC 29213, *A. baumannii* ATCC 19606, and *E. faecalis* ATCC 51299. These results confirm the initial activity seen from the pre-screening for antimicrobial compounds against *S. aureus* ATCC 29213 and *A. baumannii* ATCC 19606 (Table 4.4).

At T_3 isolate R-35 showed activity against *S. aureus* ATCC 29213, *A. baumannii* ATCC 19606, and both *E. faecalis* test strains when co-cultured with alive *M. aurum* A+ in a 3:1 ratio. However, when co-cultured with the 'dead' *M. aurum,* it only showed activity against *S. aureus* ATCC 29213 and *A. baumannii* ATCC 19606. This indicated that the 'dead' *M. aurum* had no effect on the metabolite production of R-35, but the alive *M. aurum* induced metabolite production with activity against both *E. faecalis* test strains. Strain R-30 showed no activity in this co-culture experiment or in the mono-culture.

Table 5.2: Activity profiles of the extracts prepared from the co-culture of strain R-35 with *Mycobacterium aurum* A+ displaying a great increase in the activity profiles at different sampling points.

Treatment	Sampling time	*S. aureus* ATCC 29213 (TSA)	*A. baumannii* ATCC 19606 (NA)	*E. faecalis* ATCC 29212 (BHI)	*E. faecalis* ATCC 51299 (BHI + Van)
R35 + M. a live	T2	N	Y (weak)	N	N
R35	T3	Y	Y	N	Y
R35 + M. a live	T3	Y	Y	Y	Y
R35 + M. a dead	T3	Y	Y	N	N

5.3.3.2 Mixed culture fermentations of R-30 and R-35 vs *Mycobacterium aurum* at different concentrations

The most promising activity was seen in the third experiment conducted, where strain R-35 was co-cultured with different concentrations of *M. aurum* A+. For R-35 + 0.1% *M. aurum* alive, R-35 + 0.1% *M. aurum* 'dead', R-35 + 0.5% *M. aurum* alive, R-35 + 0.5% *M. aurum* 'dead', R35 + 1% *M. aurum* alive, and R-35 + 1% *M. aurum* A+'dead', the extracts obtained showed activity against eight of the ten test strains (Table 5.3).

Table 5.3: Activity profiles of the extracts prepared from the co-culture of strain R-35 with different concentrations of *Mycobacterium aurum* A+.

Treatment	*A. baumannii* ATCC BAA – 1605 (TSA)	*E. cloacae* ATCC BAA-1143 (TSA)	*E. coli* ATCC 25922 (TSA)	*S. aureus* ATCC 29213 (TSA)	*A. baumannii* ATCC 19606 (NA)	*S. aureus* ATCC 33591 (NA)	*E. faecalis* ATCC 29212 (BHI)	*E. faecalis* ATCC 51299 (BHI + Van)
R35	Y	Y	Y	Y	Y	N	Y	Y
R35 + 0.1% M. a live	Y	Y	Y	Y	Y	Y	Y	Y
R35 + 0.5% M. a live	Y	Y	Y	Y	Y	Y	Y	Y
R35 + 1% M. a live	Y	Y	Y	Y	Y	Y	Y	Y
R35 + 0.1% M. a 'dead'	Y	Y	Y	Y	Y	Y	Y	Y
R35 + 0.5% M. a 'dead'	Y	Y	Y (weak)	Y	Y	Y	Y	Y
R35 + 1% M. a 'dead'	Y	Y	Y	Y	Y	Y	Y	Y

As previously mentioned, mycolic acid-producing bacteria was found to enhance the production of antimicrobial compounds by actinobacteria (Bertrand et al*.,* 2014). In addition to the experiment conducted by Wang et al. (2021), who found that co-culturing produces a higher yield of secondary metabolites, Onaka et al. (2015) showed that *Tsukamurella pulmonic* TP-B0596 (mycolic acid-producing strain) induced secondary metabolite production by different *Streptomyces* species when co-cultured together.

A previous study showed that secondary metabolites are produced by one organism when it senses a secondary metabolite being produced by another organism (Bertrand, 2014:1186, 1189). During this study, the extracts prepared from the co-culture of R-35 and different concentrations of *M. aurum* A+ had strong activity against the test strains (Figure 5.2). This may provide the actinobacteria with a competitive advantage when in an unfavourable environment (Demain & Fang, 2000). The main difference between the two experiments is that for one experiment, *M. aurum* was cultured with a streptomycete on day one, and in the second experiment, it was added one day before optimal antibiotic production, which therefore acted as an inducer instead of a competitor. Even though *M. aurum* is a slow grower, it can still outcompete a streptomycete if placed in the same medium at the same time, making it a competitor. It is therefore not surprising that the presence of this mycolic-acid producer had a limited effect on strain R35's bioactivity (Table 5.2), but when added as an inducer, it had a much greater effect (Table 5.3) (Hoshino et al., 2019:367-369). Images for co-culture (non-contact and direct contact) can be seen in Appendix D.

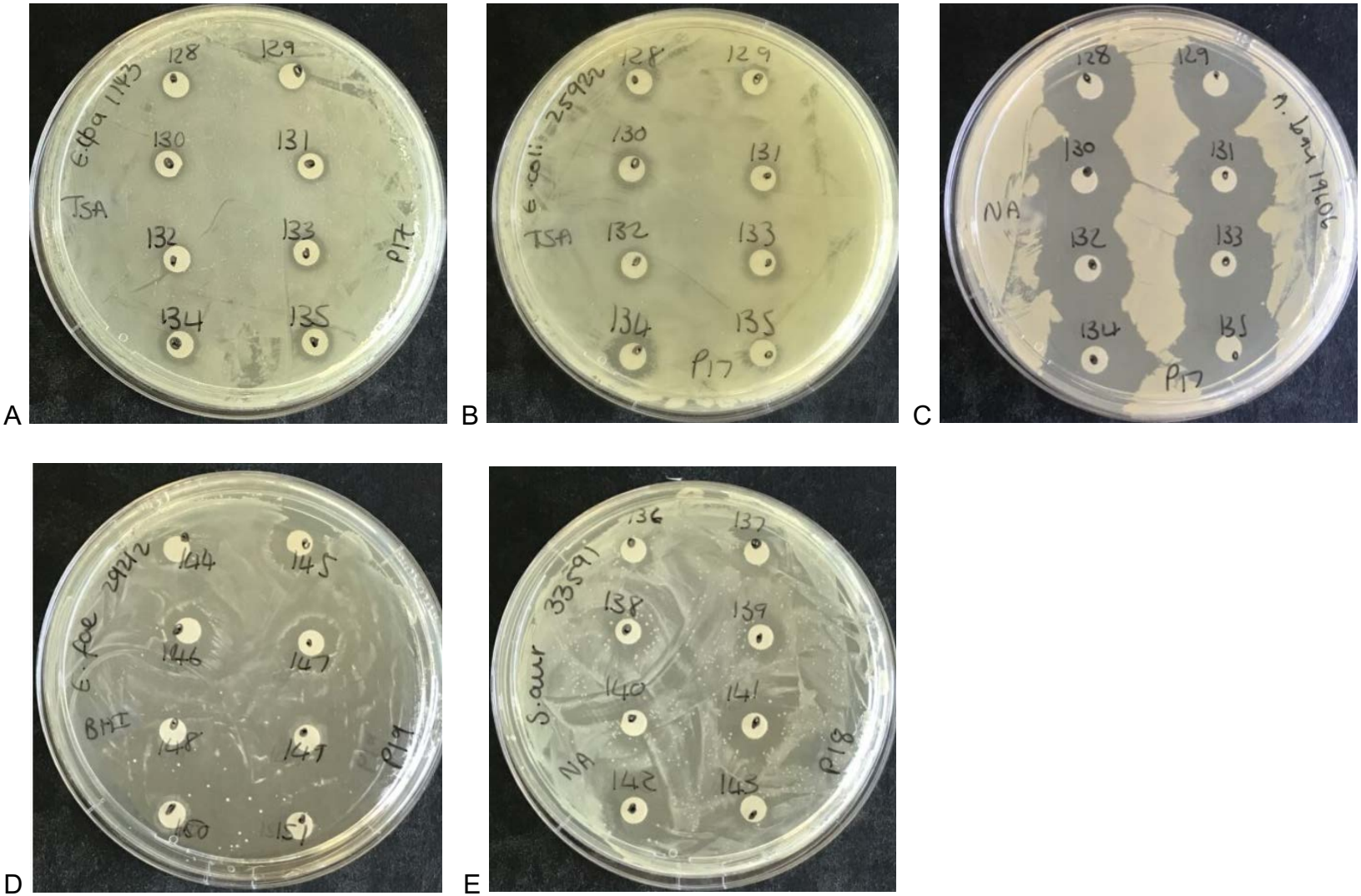


Figure 5.4: Pictures of disc diffusion assay plates of extracts prepared from the co-culture of R-35 and *M. aurum* A+ at different concentrations with activity against A) *E. cloacae,* B) *E. coli, C) A. baumannii* ATCC 19606, D) *E. faecalis* ATCC 29212, and E) *S. aureus* ATCC 33591.

5.3.4 Molecular networking R-35 and *M. aurum* A+ in direct contact co-culture

N-acetyltyramine and hydroxamate siderophores (desferrioxamines) were present in the R-35 and *M. aurum* alive samples (Figure 5.5). However, the hydroxamate siderophores seem to be enriched in the active samples. This could explain the activity seen against the eight test strains, as hydroxamate siderophores are known to exhibit synergy with other active compounds (He & Xie, 2011:10). The reason for observing activity may, therefore, be more complex than just one class of compounds being present at higher levels.

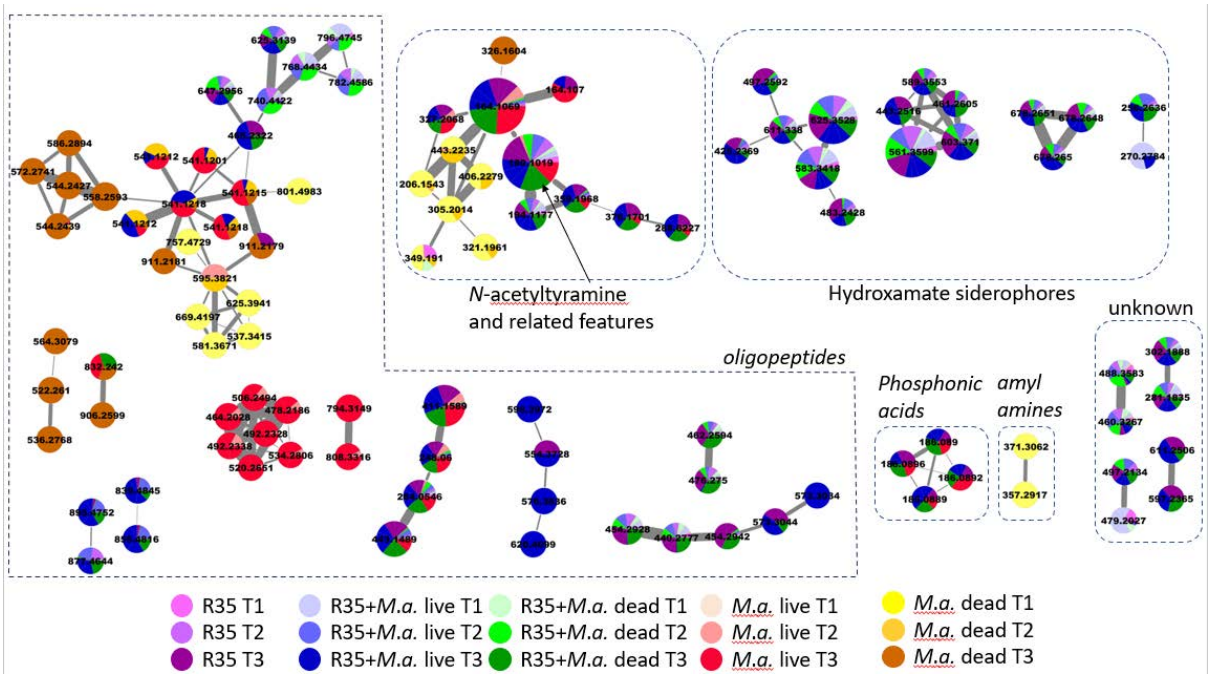


Figure 5.5: Annotated molecular network clusters for extracts from the direct co-culture of R-35 and *M. aurum* live and 'dead' at different sampling times.

In the extracts of R35 co-cultured with *M. aurum* at different concentrations, it was found that N-acetyltyramine was present. N-acetyltyramine is a possible quorum sensing inhibitor (Reina et al., 2019). Hydroxamate siderophores (desferrioxamines) (Figure 5.6) were also detected through spectral matching in the R-35 co-culture with *M. aurum* samples. Both of these metabolites are known bacterial natural products (Martinez et al., 2001; Driche et al., 2022).

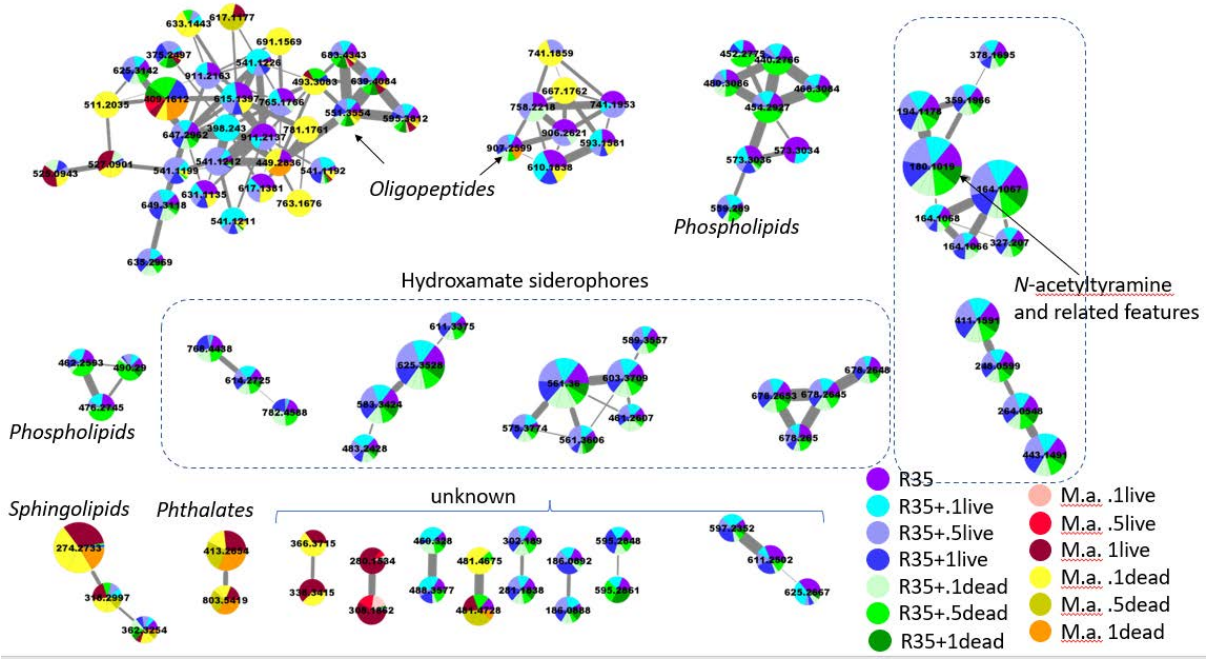


Figure 5.6: Annotated molecular network cluster for extracts from the direct contact co-culture of R-35 and *M. aurum* A+ at different concentrations.

5.3.5 Genome data – Quast analysis, TYGS results, Proksee, CARD analysis, and antiSMASH

5.3.5.1 Bioinformatic analyses of the assembled genome of R-35

Strain R-35 showed the best activity in all three co-culture experiments performed; for that reason, the genome of R-35 was sequenced and analyzed. The genome was assembled into 587 contigs with a total size of about 7.6 Mbp (Table 5.4). The overall GC content of the genome was found to be 72.18% which is consistent with a high GC mol% content of actinobacteria (Barka et al*.,* 2015:2; Seshadri et al., 2022). The TYGS report confirmed that strain R-35 belongs to a previously identified species, *Streptomyces griseoincarnatus.* This bacterium was first identified by Sajid et al*.* (2011) and was isolated from saline-concentrated soil. MS and NMR analyses of extracts from this type strain, showed promising antimicrobial activity against *S. aureus, Bacillus subtilis*, and *Streptomyces viridochromogenes* (Sajid et al., 2011).

Table 5.4: General information on the R-35 assembled genome.

Assembly parameter	R-35 scaffolds
# contigs (>= 0 bp)	587
# contigs (>= 1000 bp)	534
# contigs (>= 5000 bp)	351
# contigs (>= 10000 bp)	242
# contigs (>= 25000 bp)	79
# contigs (>= 50000 bp)	22
Total length (>= 0 bp)	7635181
Total length (>= 1000 bp)	7595584
Total length (>= 5000 bp)	7076918
Total length (>= 10000 bp)	6304525
Total length (>= 25000 bp)	3706660
Total length (>= 50000 bp)	1747247
# contigs	587
Largest contig	151946
Total length	7635181
GC (%)	72.18
N50	24150
N90	6095
auN	36777.1
L50	84
L90	314
# N's per 100 kbp	0.00

Complete QUAST and TYGS reports can be found in Appendix F.

5.3.5.2 Proksee and CARD analyses

CARD RGI analyses showed a large number of vancomycin- and tetracycline-resistant genes in the *S. griseoincarnatus* strain R-35 genome (Figure 5.7). Possessing these resistant genes is one of the most effective tools that microorganisms can possess in an antagonistic environment. This allows for protection against competitive organisms that are likely to produce these compounds as a defense mechanism (Munita & Arias, 2016).

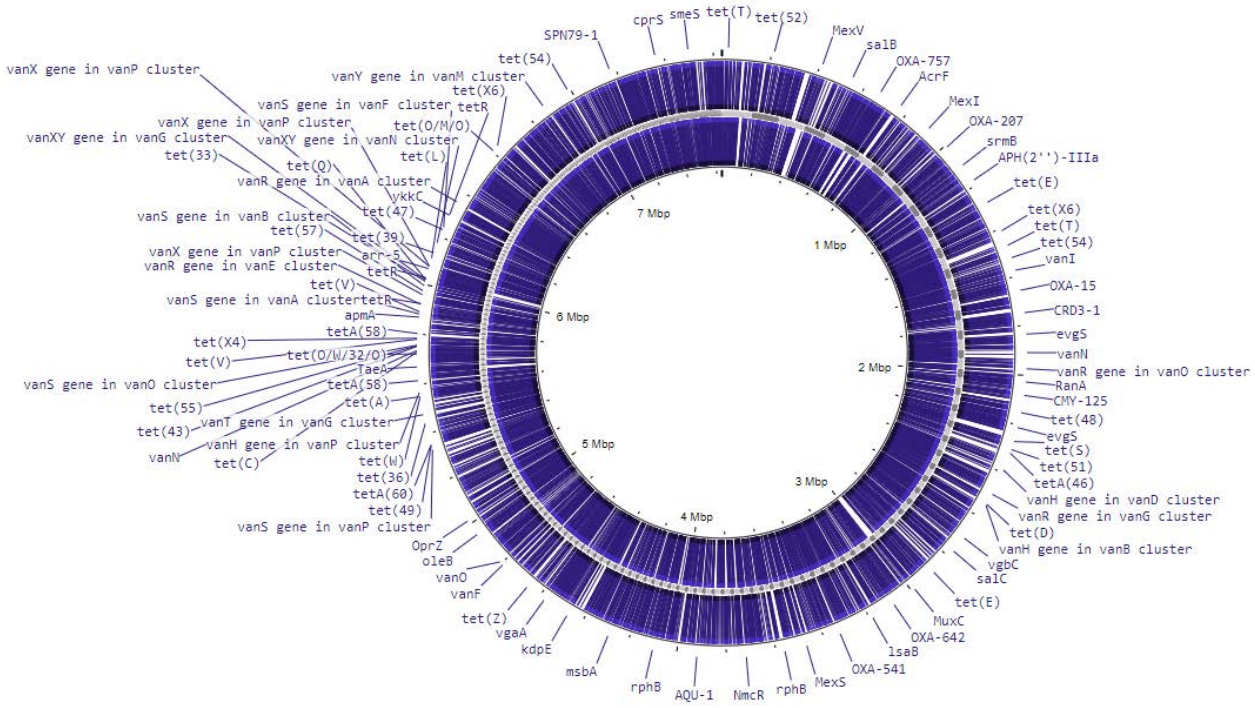
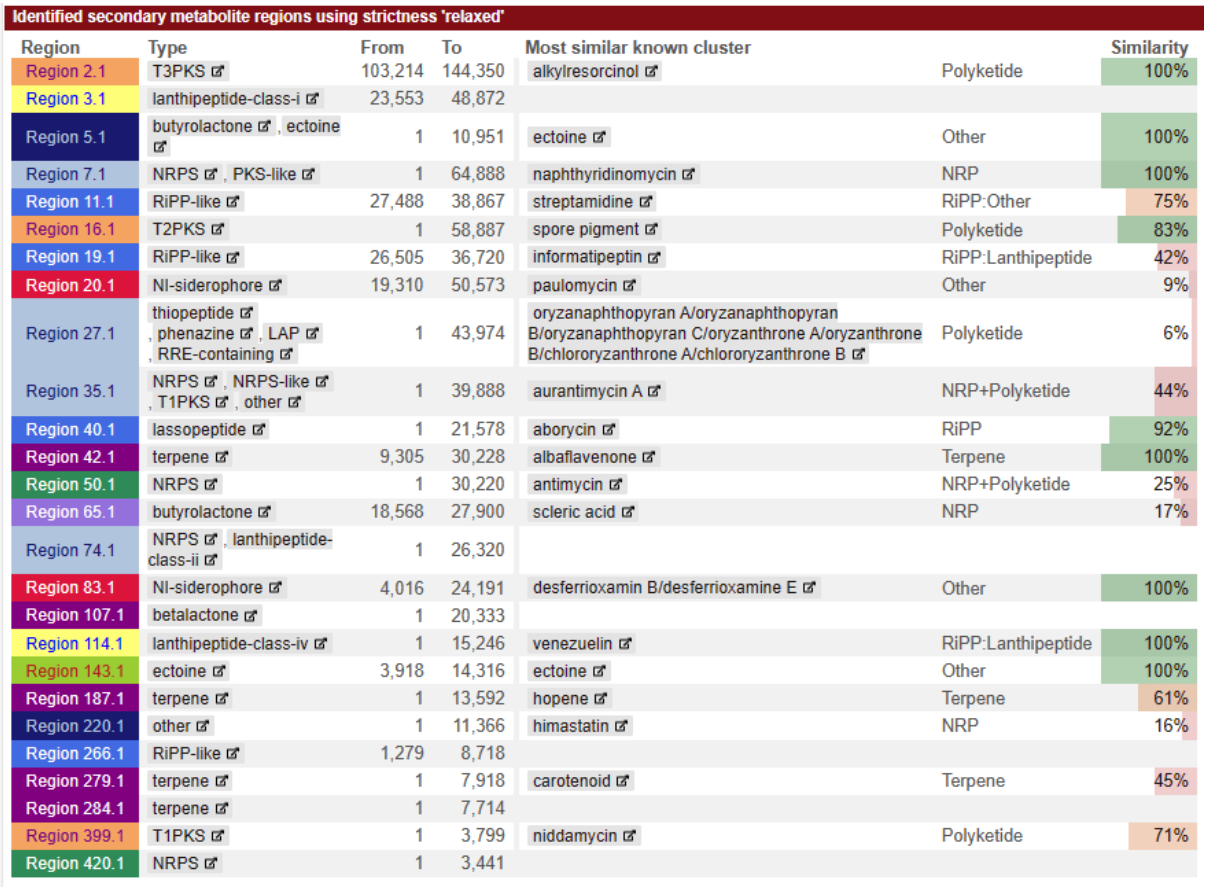


Figure 5.7: Proksee generated circular chromosome and CARD analysis indicating the abundance of antibiotic resistance genes to vancomycin and tetracycline in the *S. griseoincarnatus* strain R-35 genome.

5.3.5.3 antiSMASH

A microorganism's secondary metabolism comprises of biosynthetic pathways that can produce various compounds of interest. This includes antibiotics and other pharmaceutical compounds. Gene clusters such as polyketide synthetases (PKSs), antibiotic synthases, and non-ribosomal peptide synthetases (NRPSs) from bacterial genomes are mined for new compound discovery (Giang et al., 2020:715). The Contigs.FASTA file for the *S. griseoincarnatus* strain R-35 genome was submitted to the antiSMASH server (version 7.1.0). Twenty-six gene clusters were predicted. The predicted gene clusters included T3PKS, NRPS, and T2PK, among others (Figure 5.8).



Identified secondary metabolite regions using strictness 'relaxed'

Region	Type	From	To	Most similar known cluster		Similarity
Region 2.1	T3PKS	103,214	144,350	alkylresorcinol	Polyketide	100%
Region 3.1	lanthipeptide-class-i	23,553	48,872			
Region 5.1	butyrolactone , ectoine	1	10,951	ectoine	Other	100%
Region 7.1	NRPS , PKS-like	1	64,888	naphthyridinomycin	NRP	100%
Region 11.1	RiPP-like	27,488	38,867	streptamidine	RiPP:Other	75%
Region 16.1	T2PKS	1	58,887	spore pigment	Polyketide	83%
Region 19.1	RiPP-like	26,505	36,720	informatipeptin	RiPP:Lanthipeptide	42%
Region 20.1	NI-siderophore	19,310	50,573	paulomycin	Other	9%
Region 27.1	thiopeptide , phenazine , LAP , RRE-containing	1	43,974	oryzanaphthopyran A/oryzanaphthopyran B/oryzanaphthopyran C/oryzanthrone A/oryzanthrone B/chlororyzanthrone A/chlororyzanthrone B	Polyketide	6%
Region 35.1	NRPS , NRPS-like , T1PKS , other	1	39,888	aurantimycin A	NRP+Polyketide	44%
Region 40.1	lassopeptide	1	21,578	aborycin	RiPP	92%
Region 42.1	terpene	9,305	30,228	albaflavenone	Terpene	100%
Region 50.1	NRPS	1	30,220	antimycin	NRP+Polyketide	25%
Region 65.1	butyrolactone	18,568	27,900	scleric acid	NRP	17%
Region 74.1	NRPS , lanthipeptide-class-ii	1	26,320			
Region 83.1	NI-siderophore	4,016	24,191	desferrioxamin B/desferrioxamine E	Other	100%
Region 107.1	betalactone	1	20,333			
Region 114.1	lanthipeptide-class-iv	1	15,246	venezuelin	RiPP:Lanthipeptide	100%
Region 143.1	ectoine	3,918	14,316	ectoine	Other	100%
Region 187.1	terpene	1	13,592	hopene	Terpene	61%
Region 220.1	other	1	11,366	himastatin	NRP	16%
Region 266.1	RiPP-like	1,279	8,718			
Region 279.1	terpene	1	7,918	carotenoid	Terpene	45%
Region 284.1	terpene	1	7,714			
Region 399.1	T1PKS	1	3,799	niddamycin	Polyketide	71%
Region 420.1	NRPS	1	3,441			

Figure 5.8: Biosynthetic gene clusters of *S. griseoincarnatus* strain R-35 as predicted by the antiSMASH online tool.

The Region 7.1 predicted NRP gene cluster shows 100% similarity to the genes involved in naphthyridinomycin biosynthesis from *Streptomyces lusitanus* (Figure 5.9). Naphthyridinomycin has been reported to inhibit the growth of *E. coli* even at low concentrations (Zmijewski et al., 1982:789). Hybrid gene clusters NRP+Polyketide in region 7.1 show 37% similarity to antimycin biosynthetic gene clusters from *Streptomyces argillaceus*. Antimycin is an antibiotic that binds to a domain of cytochrome bH that slows down cytochrome bc 1 and prohibits the transport of electrons from the heme bH center to ubiquinone (Maruf et al., 2014). Furthermore, 31% similarity to splenocin biosynthetic gene clusters from *Streptomyces* sp. CNQ431 was observed (Figure 5.9).

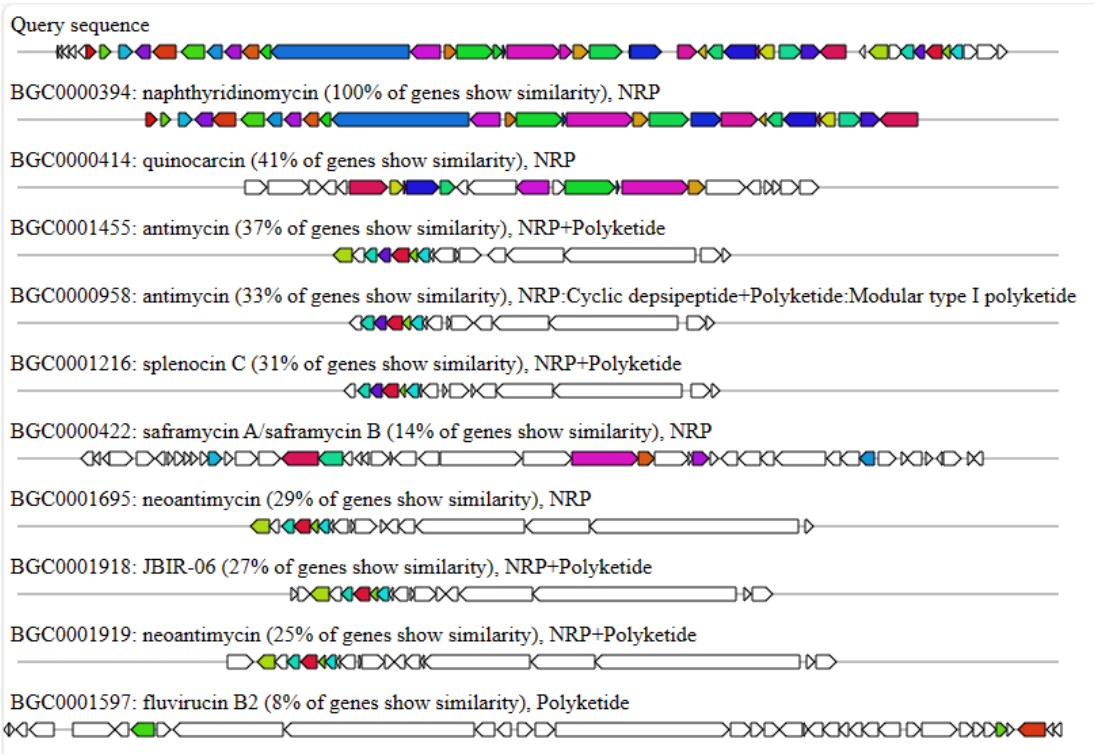


Figure 5.9: Region 7.1 shows sequence similarities to NRP biosynthetic gene clusters detected in the genomes of various *Streptomyces* species.

The Region 16.1 predicted polyketide gene cluster showed 100% similarity to curamycin biosynthetic gene clusters from *Streptomyces cyaneus* (Figure 5.10). Curamycin was initially derived from *Streptococcus cura-coi* that displayed activity against Gram-positive bacteria namely *S. aureus, Streptococcus pyogens, Bacillus subtills, Streptococcus faecalis, Streptococcus haemolyticus, Neissera gonorrhea, Bacillus polymyxa* and *Streptococcus agalactiae* (Cataldi et al., 1962).

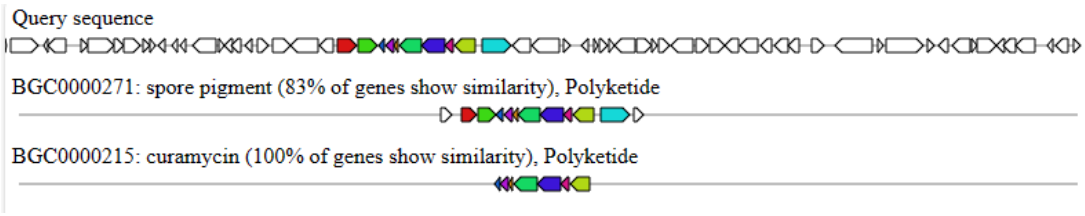


Figure 5.10: Region 16.1 show sequence similarities to polyketide biosynthetic gene clusters from *Streptomyces cyaneus.*

The Region 35.1 predicted gene cluster consisted of a hybrid NRP+Polyketide gene cluster with 44% similarity to aurantimycin A biosynthetic gene clusters from *Streptomyces auratiacus* JA4570 (Figure 5.11). Aurantimycin has been found to display activity against both Gram-positive and Gram-negative bacteria, but it has shown narrow-spectrum activity against *E. coli* and *Pseudomonas* species (Gräfe et al., 1995:124).

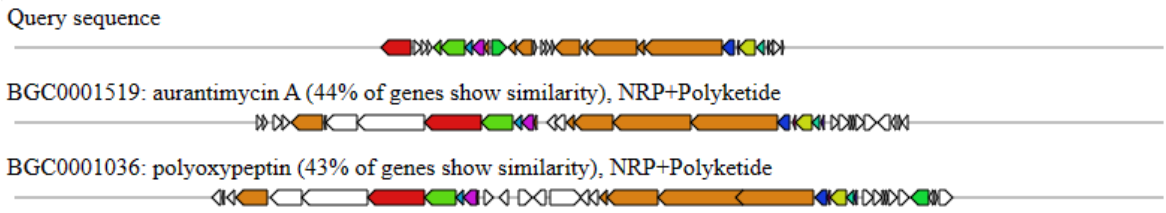


Figure 5.11: Region 35.1 with a predicted hybrid NRP+ Polyketide gene cluster with sequence similarities to gene clusters found in the genomes of *Streptomyces aurantiacus* and *Streptomyces* sp. MK498-98F14.

The gene cluster at region 42.1 (terpene) is predicted to have 100% similarity to a biosynthetic gene cluster from *Streptomyces coelicolor* A3(2) which encodes for albaflavenone (Figure 5.12). Albaflavenone has been reported to display antimicrobial activity against *B. subtilis* ATCC 6633, *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *Candida albicans* and *Candida parapsilosis* (Zheng et al., 2016:774).

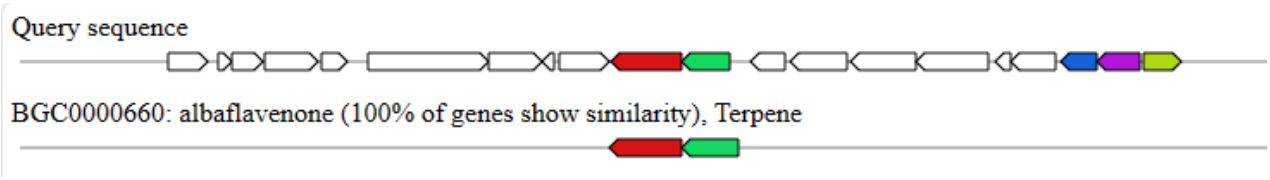


Figure 5.12: Region 42.1 Terpene predicted biosynthetic gene cluster indicating 100% similarity to biosynthetic gene clusters from the genome of *Streptomyces coelicolor.*

The Region 50.1 predicted gene cluster showed 25% similarity to the antimycin biosynthetic gene cluster from *Streptomyces argillaceus* and 26% similarity to the antimycin biosynthetic gene cluster from *Streptomyces* sp. S4 (Figure 5.13). Antimycin consists of a minimum of four compounds that are closely related in structure. It has been reported that antimycins exhibits antifungal activity against a large number of pathogenic fungi (Rieske, 1967). The cluster also exhibited 16% similarity to JBIR biosynthetic gene clusters from a *Streptomyces* sp. JBIR is a β-lactamase inhibitor from the antimycin family (Nishimura et al*.,* 2021:4415). Independent NRPs also showed 17% similarity to neoantimycin biosynthetic gene clusters from *Streptomyces orinoci* (Figure 5.13).

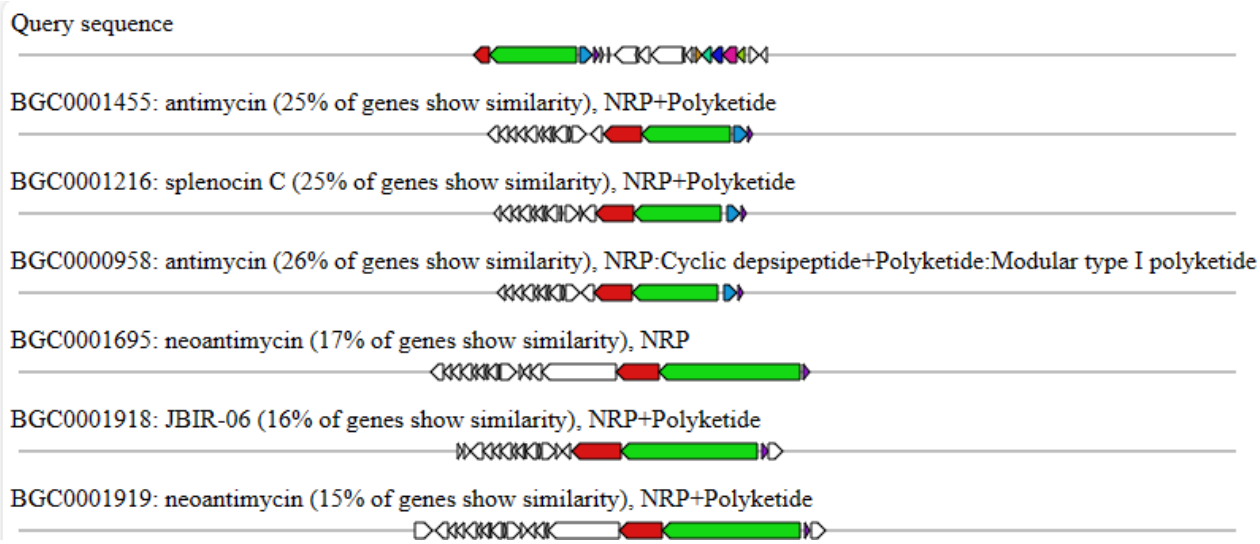


Figure 5.13: Region 50.1 predicted gene cluster showed similarities to hybrid NRP+Polyketide biosynthetic gene clusters and independent NRPs from various *Streptomyces* species.

Region 83.1 (Figure 5.14) shows 100% similarity to desferrioxamine biosynthetic gene clusters from five species. Desferrioxamine B from *Streptomyces coelicolor, Streptomyces griseus* subsp. *griseus* NBRC 13350, and a *Streptomyces* sp. Desferrioxamine E from *Streptomyces coelicolor* A3(2) and *Streptomyces* sp. ID 38640. Desferrioxamine from *Streptomyces argillaceus,* and 75% similarity to desferrioamine E biosynthetic gene clusters from *Pantaoea agglomarans*. Desferrioxamine is a chelating agent that helps reduce iron overload in patients (Bannerman et al., 1962:1574). Desferrioxamine can easily enter the cell or make it easier for other substances to do so. They are the best candidates to assist antibiotic transport into cells (Martinez et al., 2001:421).

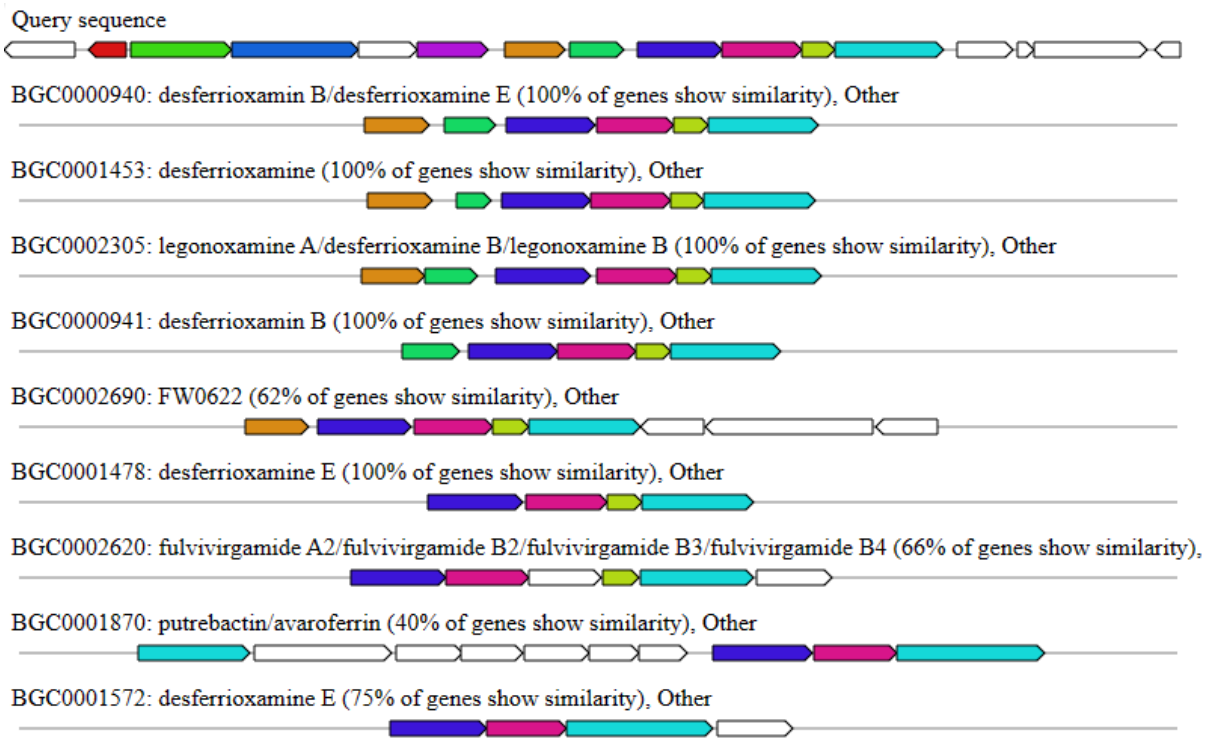


Figure 5.14: Region 83.1 showing similarity to desferrioxamine biosynthetic gene clusters from *Strepmyces coelicolor, Streptomyces griseus, Streptomyces argillaceus, Streptomyces* sp. ID 38640 and *Pantaoea agglomarans.*

Bacteria rely on electron transfer for the final stage of cellular respiration. Through a series of reactions, ATP is formed within the cytoplasmic membrane, and this initiates the oxidative phosphorylation of ADP to ATP. For this process to take place, bacteria use multiple cytochrome oxidases, non-heme iron components, and various cytochrome and flavins (Jurtshuk, 1996). Antimycin is the metabolite that is seen the most on the anitiSMASH data generated. This metabolite is also observed to be present in the MS-MS data generated (Figure 5.3C). Antimycin functions by permeating the cellular membrane and slowing down or inhibiting electron transfer, causing an interruption in cellular respiration (Hosotani et al., 2005:460). Although studies have confirmed antimycin as an antifungal antibiotic, it could be that this antifungal antibiotic also had an effect on the results obtained from this study due to its ability to infiltrate the cell membrane and disrupt cytochrome complex in an enzyme and thus the respiration cycle of the pathogenic bacteria (Huang et al., 2005:574-575), especially if present along with hydroxamate siderophores (see section 5.3.4). This could possibly explain the activity seen for R-35 in the cross-streak method and also the activity seen for extracts prepared from R-35 when co-cultured with *M. aurum* at different concentrations. This means that there could be novel antimycins present that have activity against the ESKAPE organisms, or activity against these test strains is due to novel compounds that were not identified in this

study. Additional chemical analyses could be conducted on the extracts to determine which compound inhibited the growth of the test strains.

5.4 References

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Chapter 6

Conclusions and Recommendations for future studies

The Table Mountain National Park is located within one of South Africa's most biodiverse regions, the Cape Floristic Region. Marine habitats within the park have not been explored for its actinobacterial diversity. It is, therefore, not surprising that metabarcoding analyses of sediment samples obtained from the tidal pool indicated a high actinobacterial diversity with many unknown species present. However, the limitations of this study lie in the use of actinobacterial-specific primers for metabarcoding, which could result in PCR bias. This can be overcome by sequencing the complete metagenome. Metabarcoding of a single sampling event also only provides information on the microbial community present at the time of sampling. Whether the diversity remains the same or similar can only be determined by doing multiple sampling events over a period of time, but this aspect was outside the scope of this study.

Various media and isolation techniques were used to isolate actinobacteria from marine sediment samples. Sixty isolates were obtained with twenty strains selected for further screening. The top eight bioactive strains were identified to belong to the genus *Streptomyces*, a genus often represented in both terrestrial and marine isolation studies. The limited set of isolation media used during this study could have led to the limited isolation diversity. The actinobacteria isolated in this study are known to utilise the media components used during this study, whereas actinobacteria that cannot utilise the components in the isolation media selected for growth will not be isolated. This could be overcome by using more types of isolation media, but this leads to elevated costs and is time-consuming. One of the challenges that could possibly be overcome would be to use seawater collected from the sampling site in the isolation media, ensuring that the environmental conditions during strain isolation are closer to that of the sampling site, thereby mimicking the site conditions, i.e., the pH and incubation temperature. One challenge faced was that even though antibiotics and antifungals were added to the isolation media, there was still contamination of the isolation plates, mainly by fungi. If there were colonies on the contaminated plate, the colony was picked and streaked onto fresh media to try and get pure cultures. An alternative selective isolation process may need to be implemented to access pure cultures from marine sediment samples, e.g., using the dilution approach where the standard dilution protocol is taken beyond the 10^{-3} dilution (up to 10^{-10}) and plates incubated for longer time periods.

Twenty isolates were selected for bioactivity screening on both solid media (cross-streak) and in liquid culture (mono-cultures). It was not surprising that the isolates displayed different activity profiles. More activity was seen from the eight marine isolates in the cross-streak test

than in mono-cultures. Strain R-21 had very good activity when cross-streaked on ISP2 agar with activity against eight test strains, excluding *E. coli* and *K. pneumoniae.* The strongest activity was seen from strains R-30 and R-35 as these isolates had good activity when cross-straked on all media types but had the best activity against all ten ESKAPE strains when cultured on ISP2 agar. The activity of the mono-cultures was not as strong, since the activity observed in the mono-culture exhibited very weak activity against *P. aeruginosa.* It is hypothesised that this test strain made use of its efflux pump defence mechanism to expel toxins from its cellular membrane before complete inhibition could occur. Although less activity was seen in the mono-cultures, R-21, R-30, and R-35 still produced strong activity against *S. aureus* ATCC 29213 and *A. baumannii* ATCC 19606. Strain R-21 only produced good activity in ISP2 at T_2 and T_3, whereas R-30 and R-35 produced good activity at T_0 in ISP2 and ISP2+, T_2 in ISP2 and T_3 in ISP2 and ISP2+ against these test strains. Co-culture experiments were therefore performed using only ISP2 media as this showed the best activity by isolates R-30 and R-35 in the mono-cultures.

The great value of using a co-culture technique was seen when different approaches were taken. When strains R-30 and R-35 were co-cultured together, the R-30 extracts had activity against more test strains than those of the mono-culture extracts. The R-30 co-culture extracts showed good activity against *P. aeruginosa, S. aureus* ATCC 29213, *A. baumannii* ATCC 19606, and both *E. faecalis* test strains at T_1. At T_1, R-35 also showed good activity against both *E. faecalis* strains compared to the mono-cultures of R-35 at T_1. Co-culture with alive or 'dead' *Mycobacterium aurum* A+ showed an increase in activity by strain R-35 at T_3. This co-culture with *Mycobacterium aurum* A+ alive or 'dead' showed an increase in activity by strain R-35 where at T_3 it was seen that extracts exhibited activity against *S. aureus* ATCC 29213, *A. baumannii* 19606, and both *E. faecalis* isolates. This indicated that co-culture with *M. aurum* had a positive effect on antimicrobial compound production. The strongest activity was seen when R-35 was co-cultured with different *M. aurum* concentrations (0.1%, 0.5%, and 1%). Strain R-35 showed very good activity against eight test strains, but no activity was observed against *P. aeruginosa* and *K. pneumoniae.* Based on these results, whole genome sequencing was performed on strain R-35 and it was found to be a strain of *Streptomyces griseoincarnatus.* Previous literature had indicated that *S. griseoincarnatus* produces compounds that inhibit the growth of *S. aureus* and other pathogenic bacteria. MS/MS analysis of the extracts prepared from the R-30 and R-35 non-contact co-culture set-up showed the presence of surfactin-related compounds, antimycins, plipastatin, flavonoids, and aminolipids which all display antimicrobial activity based on previous literature. MS/MS data of the extracts obtained from the direct contact co-culture and *M. aurum* also showed the presence of eight compounds that could not be identified with MS/MS analyses. The presence of eight unidentified compounds

makes it clear that there are many more potential compounds to be isolated from streptomycetes

For extract preparation, washing the beads and cells with water possibly washed away water-soluble compounds, but if the washing step is removed, this could result in the extraction of media components that could inhibit the bioactivity of the extracts. To overcome this, extract preparation for each strain would need to be optimised. Molecular networking is also limited to the compounds that are already in the GNPS database. A bigger picture of the compounds present in the extracts will only be possible once more compounds are loaded to the molecular networking database and analyses are focused on fractionated samples of the extracts. Even though this is a limitation with regard to the compounds observed, this application is useful in determining optimal growth conditions and growth time required for the production of potentially active compounds and could guide future scale-up studies.

Future studies could include a more in-depth analysis of the compounds isolated, fractionation studies, and identifying which bioactive compound or compounds had activity against both *A. baumannii* strains, both *S. aureus* strains, *E. coli, E. cloacae* and both *E. faecalis* test strains. Optimising the production of these compounds and identifying the biosynthetic gene clusters responsible for the production of these metabolites could lead to the identification of compounds that could be used to fight infections and diseases caused by antibiotic-resistant strains as these pathogens pose a major threat to human health and wellness.

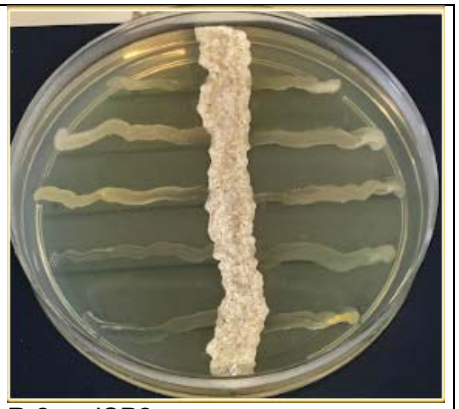
Appendix A: Images of the marine environment at the time of sampling at TMNP2 showing the presence of various marine macroalgae, fish, and cephalopod

Appendix B: Cross-streak: Isolates with activity against ESKAPE organisms and control plates


R-2 on M19 agar

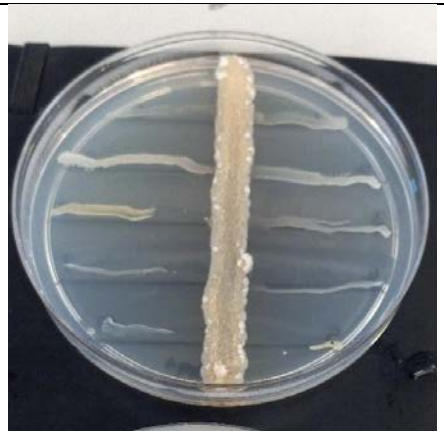

R-2 on M19+ agar


R-2 on ISP2

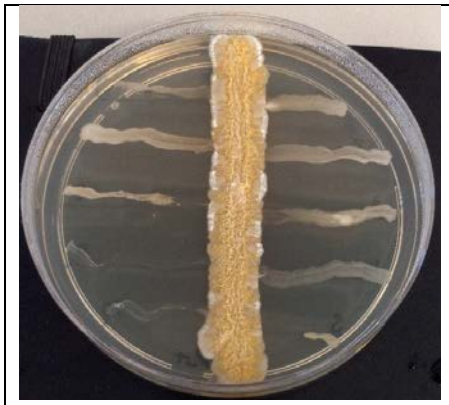
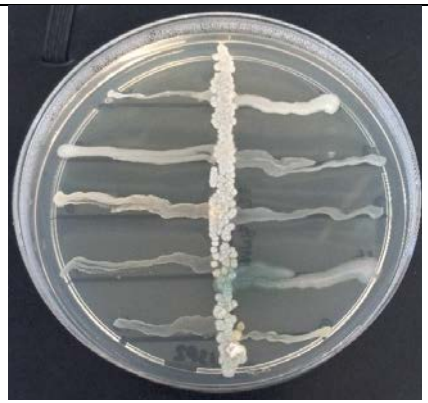
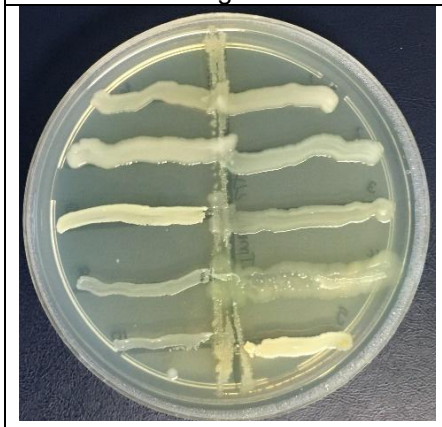

R-2 on ISP2+ agar


R-6 on ISP2+ agar


R-21 on M19 agar


R-21 on M19+ agar


R-21 on ISP2 agar

 R-21 on ISP2+ agar	 R-23 on M19 agar	 R-23 on ISP2 agar	 R-23 on ISP2+ agar
 R-24 on M19+ agar	 R-24 on ISP2 agar	 R-24 on ISP2+ agar	 R-30 on M19 agar


R-30 on M19+ agar


R-30 on ISP2 agar

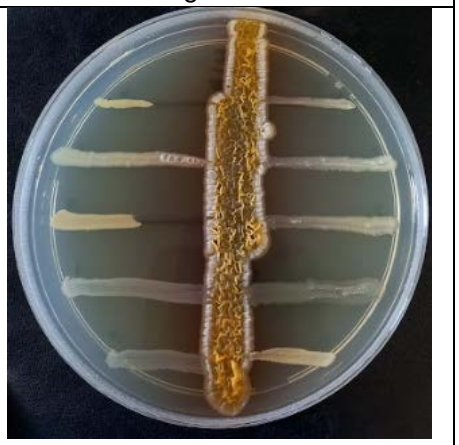

R-30 on ISP2+ agar

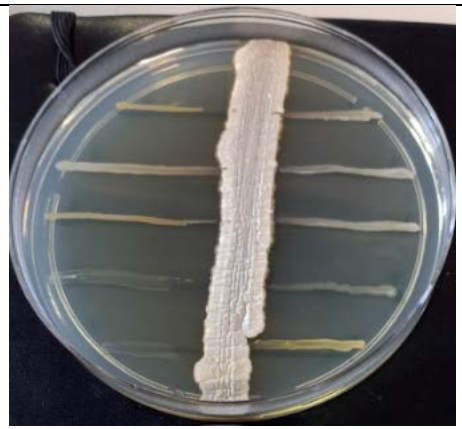
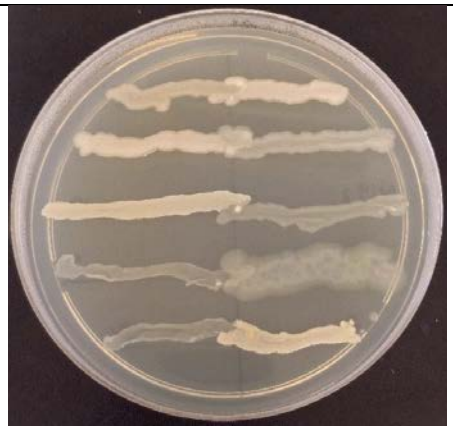
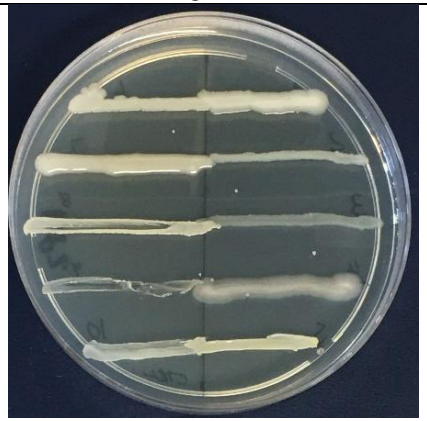

R-35 on M19 agar


R-35 on M19+ agar

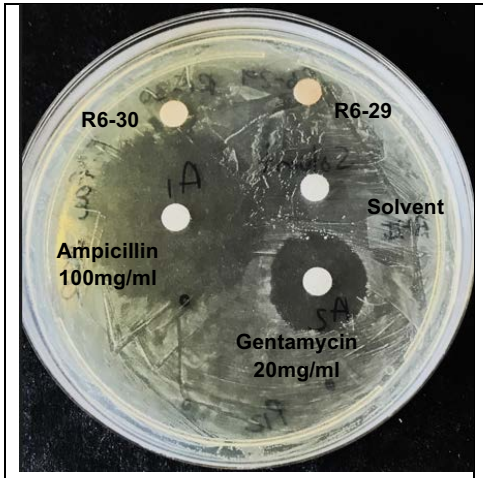

R-35 on ISP2 agar


R-35 on ISP2+ agar

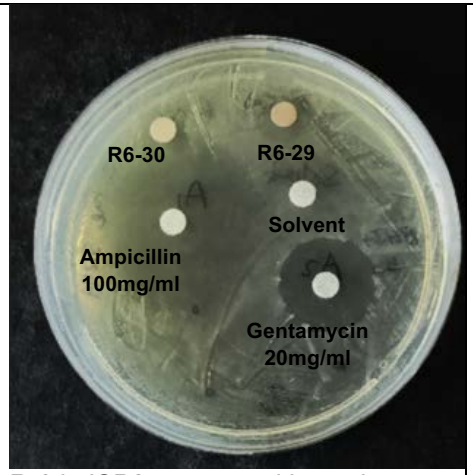

R-J on M19 agar

 R-J on ISP2 agar	 R-J on ISP2+ agar	 Test strains on M19 agar	 Test strains on M19+ agar
 Test strains on ISP2 agar	 Test strains on ISP2+ agar		

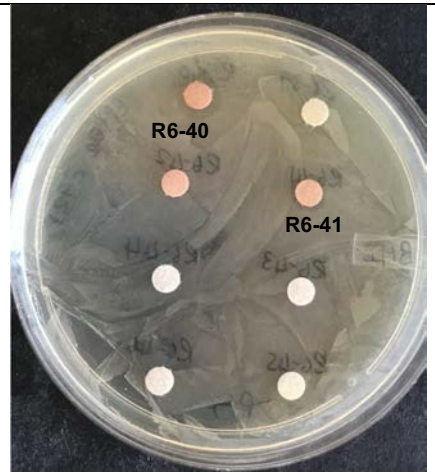
Appendix C: Filter disc test - activity of mono-cultures



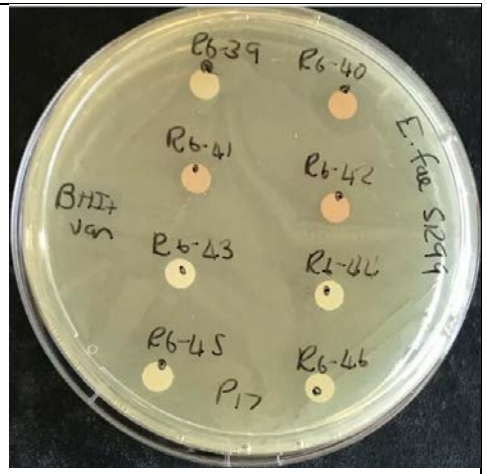
R-6 in ISP2+ extract with weak activity against *E. faecalis* ATCC 29212 at T_2



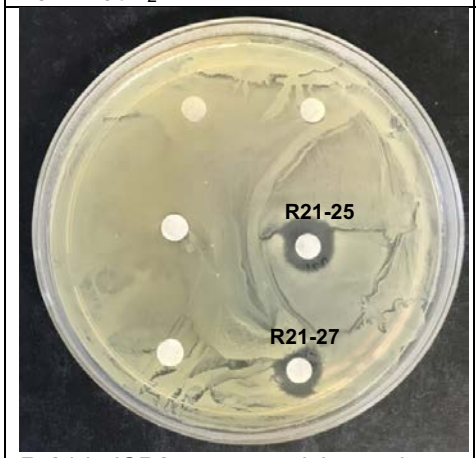
R-6 in ISP2+ extract with weak activity against *E. faecalis* ATCC 51299 at T_2



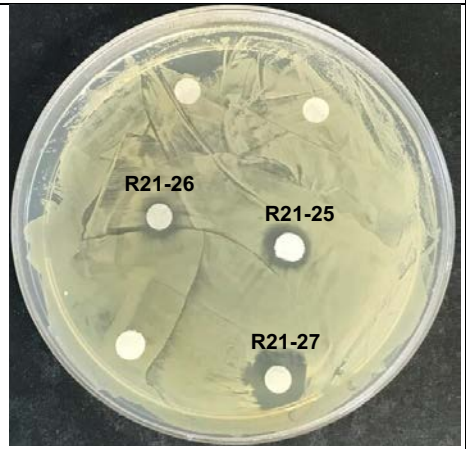
R-6 in ISP2+ extract with weak activity against *E. faecalis* ATCC 29212 at T_3



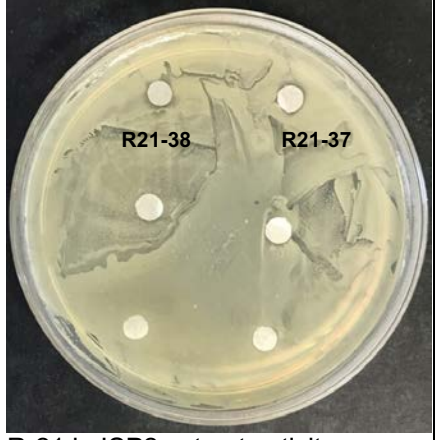
R-6 in ISP2+ extract with weak activity against *E. faecalis* ATCC 51299 at T_3
ISP2: R6-40 to R6-42



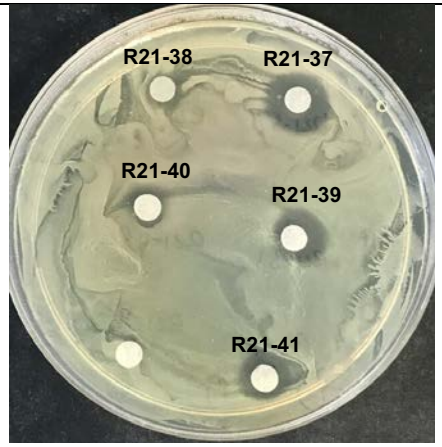
R-21 in ISP2 extract activity against *S. aureus* ATCC 29213 at T_2



R-21 in ISP2 extract activity against *A. baumannii* ATCC 19606 at T_2

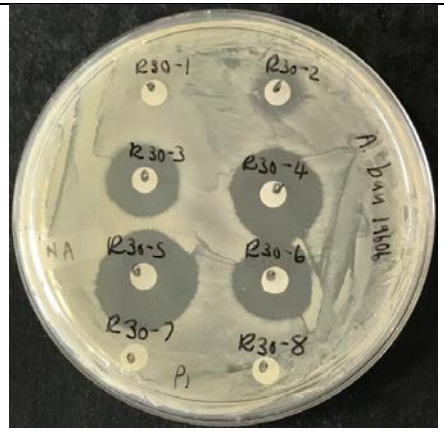


R-21 in ISP2 extract activity against *S. aureus* ATCC 29213 at T_3



R-21 in ISP2 and ISP2+ extract: Activity against *A. baumannii* ATCC 19606 at T_3
ISP2: R21-37 to R21-39
ISP2+: R21-40 to R21-41

<table>
<tr>
<td> 

Extract from T_0 with activity against S. aurues ATCC 29213
ISP2: R30-1 to R30-3
ISP2+: R30-4 to R30-6</td>
<td> 

Extract from T_0 with activity against A. baumannii ATCC 19606
ISP2: R30-1 to R30-3
ISP2+: R30-4 to R30-6</td>
<td> 

Extract from T_0 with activity against A. baumannii ATCC 19606
ISP2: R30-25 to R30-27
ISP2+: R30-28 to R30-30</td>
<td> 

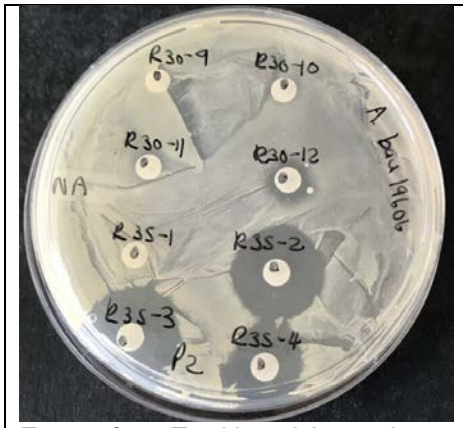
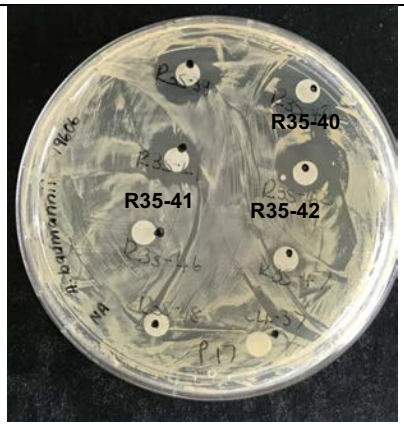
Extract from T_3 with activity against S. aureus ATCC 29213
ISP2: R30-25 to R30-27
ISP2+: R30-28 to R30-30</td>
</tr>
<tr>
<td> 

Extract from T_3 with activity against A. baumannii ATCC 19606
ISP2: R30-37 to R30-39
ISP2+: R30-40 to R30-42</td>
<td> 

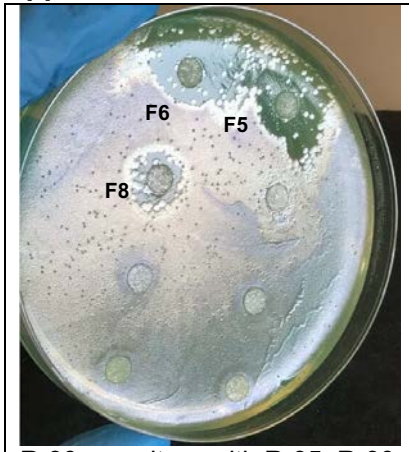
Extract from T_3 with activity against A. baumannii ATCC 19606 at
R-30 M19+: R30-46 to R30-48
R-35 ISP2: R35-37 and R35-38</td>
<td> 

Extract from T_0 with activity against S. aureus ATCC 29213 at
ISP2: R35-1 to R35-3
ISP2+: R35-4</td>
<td> 

Extract from T_2 with activity against S. aureus ATCC 29213
ISP2: R35-25 to R35-27</td>
</tr>
</table>

 Extract from T_0 with activity against *A. baumannii* ATCC 19606 ISP2: R35-1 to R35-3 ISP2+: R35-4 to R35-6	 Extract from T_2 with activity against *A. baumannii* ATCC 19606 ISP2+: R35-4 to R35-6	 Extract from T_2 with activity against *A. baumannii* ATCC 19606 ISP2+: R35-28 to R35-30 M19+: R35-36	 Extract from T_3 with activity against *A. baumannii* ATCC 19606 ISP2+: R35-40 to R35-42 M19+: R35-39

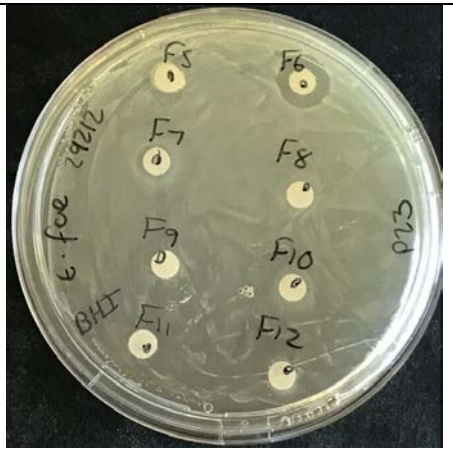
Appendix D: Filter disc test – the activity of co-culture extracts



R-30 co-culture with R-35: R-30 and R-35 activity against *P. aeruginosa* ATCC 27853 at T_1
R-30: F5 and F6
R-35: F8

R-30 co-culture with R-35: R-30 and R-35 activity against S. *aureus* ATCC 29213 at T_1
R-30: F6
R-35: F7

R-30 co-culture with R-35: R-30 and activity against *A. baumannii* ATCC 19606 at T_1 and R-35 at T_2
R-30: F5 and F6
R-35: F10



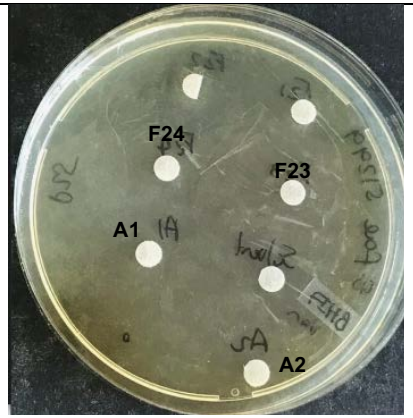
R-30 co-culture with R-35: R-30 and R-35 activity against *E. faecalis* ATCC 29212 at T_1
R-30: F5 and F6
R-35: F7

R-30 co-culture with R-35: R-30 and R-35 activity against *E. faecalis* ATCC 51299 at T_1
R-30: F6
R-35: F7

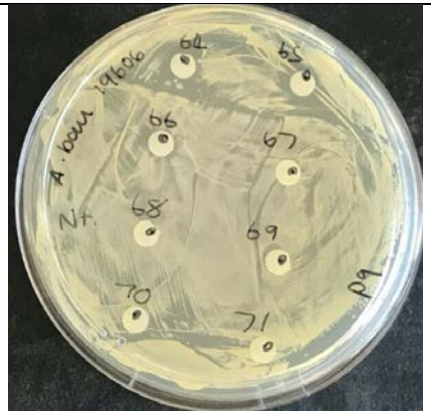
R-30 co-culture R-35: R-35 activity against *A. baumannii* ATCC 19606 at T_1
Mono-culture of R-35
R-35 co-culture: F21 and F22
R-35 Mono-culture: F23 and F24

R-35 mono-culture activity against *S. aureus* ATCC 29213 at T_3
R-35 monoculture: F23 and F24
A1 – Ampicillin 100mg/mL
A2 – Gentamycin 20mg/mL

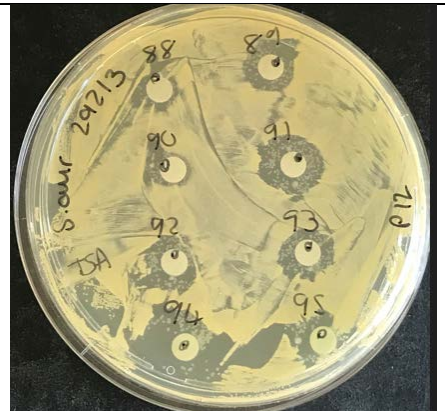
R-35 mono-culture activity against *E. faecalis* ATCC 29212 at T_3
R-35 monoculture: F23 and F24
A1 – Ampicillin 100mg/mL
A2 – Gentamycin 20mg/mL



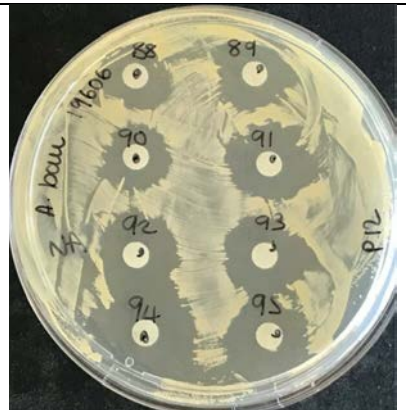
R-35 mono-culture activity against *E. faecalis* ATCC 51229 at T_3
R-35 monoculture: F23 and F24
A1 – Ampicillin 100mg/mL
A2 – Gentamycin 20mg/mL



R-35 co-culture with *M. aurum* A+ alive: R-35 activity against *A. baumannii* ATCC 19606 at T_2 and R-35 at T_2
R-35 plus *M. aurum*: 64 and 65



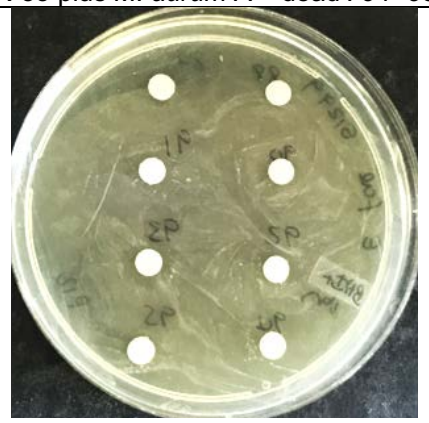
R-35 co-culture with *M. aurum* A+ alive and 'dead' and R-35 mono-culture': R-35activity against *S. aureus* ATCC 29213 at T_2 and R-35 at T_3
R-35 mono-culture: 88 – 90
R-35 plus *M. aurum* A+ alive: 91 - 93
R-35 plus *M. aurum* A+ 'dead': 94 -95



R-35 co-culture with *M. aurum* A+ alive and 'dead' and R-35 mono-culture': R-35activity against *A. baumannii* ATCC 19606 at T_2 and R-35 at T_3
R-35 mono-culture: 88 – 90
R-35 plus *M. aurum* A+ alive: 91 - 93
R-35 plus *M. aurum* A+ 'dead': 94 -95

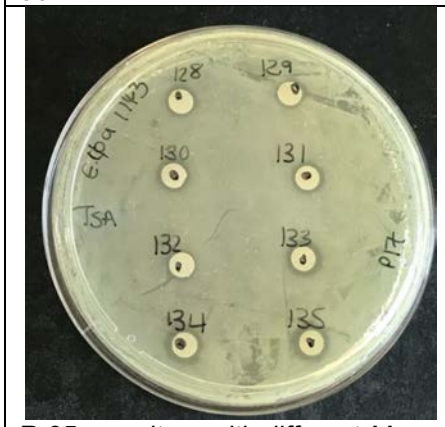
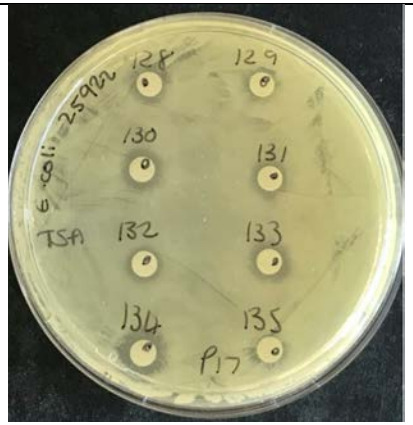
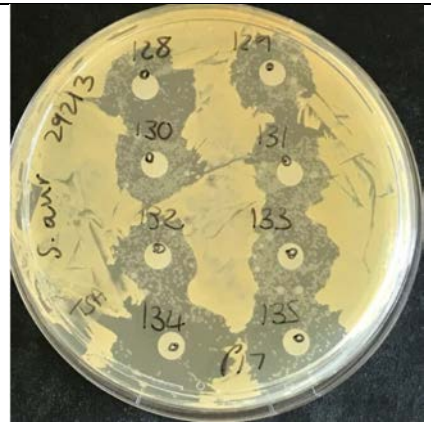
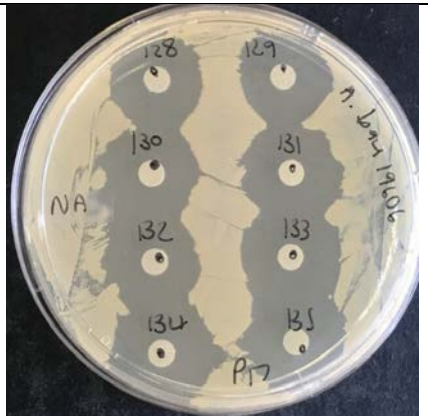
R-35 co-culture with *M. aurum* A+ alive and 'dead' and R-35 mono-culture': R-35activity against *E. faecalis* ATCC 29212 at T_2 and R-35 at T_3
R-35 mono-culture: 88 - 90

R-35 co-culture with *M. aurum* A+ alive and 'dead' and R-35 mono-culture': R-35activity against *E. faecalis* ATCC 29212 at T_2 and R-35 at T_3
R-35 mono-culture: 88 – 90



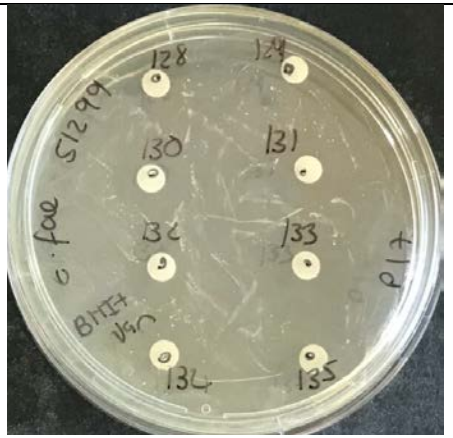
R-35 co-culture with *M. aurum* A+ alive
R-35 activity against *E. faecalis* ATCC 51299 at T_2 and R-35 at T_3
R-35 mono-culture: 88 – 90
R-35 plus *M. aurum* A+ alive: 91 - 93

R-35 co-culture with different *M. aurum* A+ alive concentrations and R-35 mono-cultures activity against *A. baumannii* ATCC BAA-1605
R-35 mono-culture: 128 and 129

R-35 plus *M. aurum* A+ alive: 91 - 93 R-35 plus *M. aurum* A+ 'dead': 94 - 96	R-35 plus *M. aurum* A+ alive: 91 - 93		R35 + 0.1% *M. aurum* A+ alive: 130 - 132 R35 + 0.5% *M. aurum* A+ alive: 133 - 135
 R-35 co-culture with different *M. aurum* A+ alive concentrations and R-35 mono-cultures activity against *E. cloacae* ATCC BAA-1143 R-35 mono-culture: 128 and 129 R35 + 0.1% *M. aurum* A+ alive: 130 - 132 R35 + 0.5% *M. aurum* A+ alive: 133 - 135	 R-35 co-culture with different *M. aurum* A+ alive concentrations and R-35 mono-cultures activity against *E. coli* ATCC BAA-1143 R-35 mono-culture: 128 and 129 R35 + 0.1% *M. aurum* A+ alive: 130 - 132 R35 + 0.5% *M. aurum* A+ alive: 133 - 135	 R-35 co-culture with different *M. aurum* A+ alive concentrations and R-35 mono-cultures activity against *S. aureus* ATCC 29212 R-35 mono-culture: 128 and 129 R35 + 0.1% *M. aurum* A+ alive: 130 - 132 R35 + 0.5% *M. aurum* A+ alive: 133 - 135	 R-35 co-culture with different *M. aurum* A+ alive concentrations and R-35 mono-cultures activity against *A. baumannii* ATCC 19606 R-35 mono-culture: 128 and 129 R35 + 0.1% *M. aurum* A+ alive: 130 - 132 R35 + 0.5% *M. aurum* A+ alive: 133 - 135

R-35 co-culture with different *M. aurum* A+ alive concentrations and R-35 mono-cultures activity against *S. aureus* ATCC 33591
R-35 mono-culture: 128 and 129
R35 + 0.1% *M. aurum* A+ alive: 130 - 132
R35 + 0.5% *M. aurum* A+ alive: 133 - 135

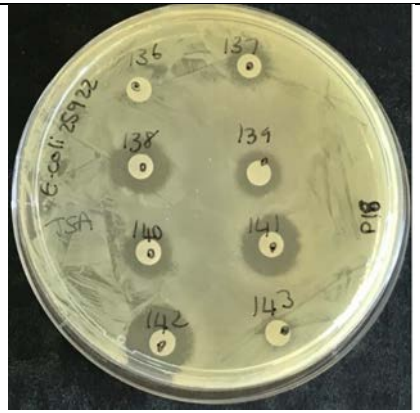
R-35 co-culture with different *M. aurum* A+ alive concentrations and R-35 mono-cultures activity against *E. faecalis* ATCC 29212
R-35 mono-culture: 128 and 129
R35 + 0.1% *M. aurum* A+ alive: 130 - 132
R35 + 0.5% *M. aurum* A+ alive: 133 - 135



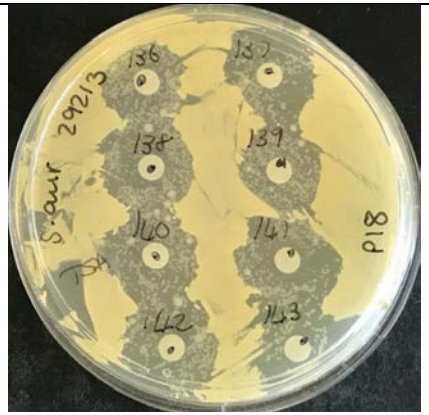
R-35 co-culture with different *M. aurum* A+ alive concentrations and R-35 mono-cultures activity against *E. faecalis* ATCC 51299
R-35 mono-culture: 128 and 129
R35 + 0.1% *M. aurum* A+ alive: 130 - 132
R35 + 0.5% *M. aurum* A+ alive: 133 - 135

R-35 co-culture with different *M. aurum* A+ (alive and 'dead') concentrations activity against *A. baumannii* ATCC BAA-1605
R-35 + 1% *M. aurum* A+ alive: 136 and 138
R35 + 0.1% *M. aurum* A+ 'dead': 139 - 141
R35 + 0.5% *M. aurum* A+ 'dead': 142 and 143

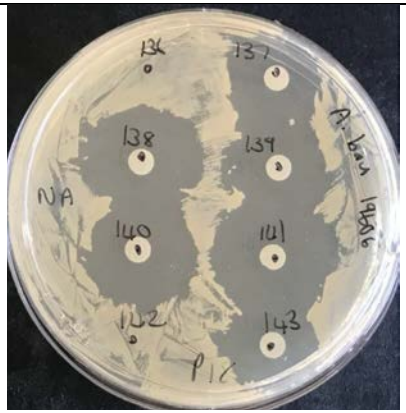
R-35 co-culture with different *M. aurum* A+ (alive and 'dead') concentrations activity against *E. cloacae* ATCC BAA-1143



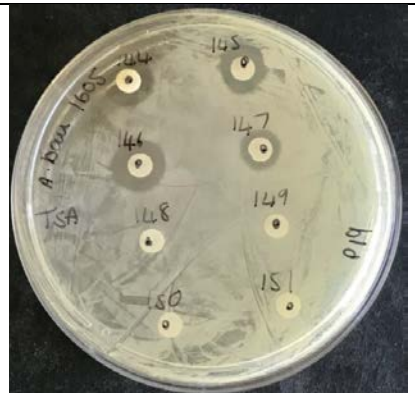
R-35 co-culture with different *M. aurum* A+ (alive and 'dead') concentrations against *E. coli* ATCC 25922

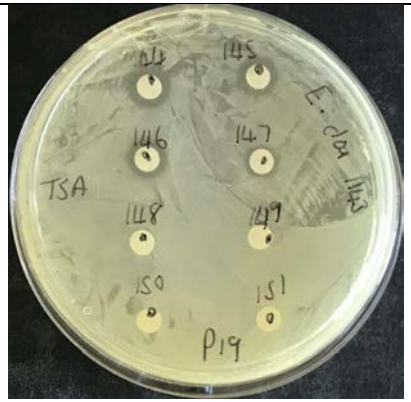
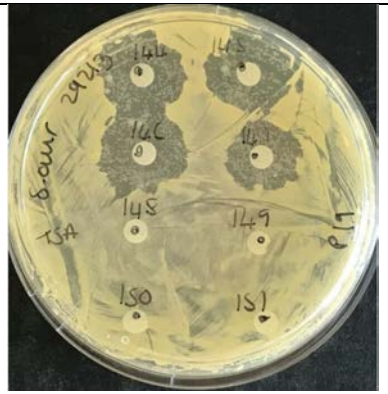
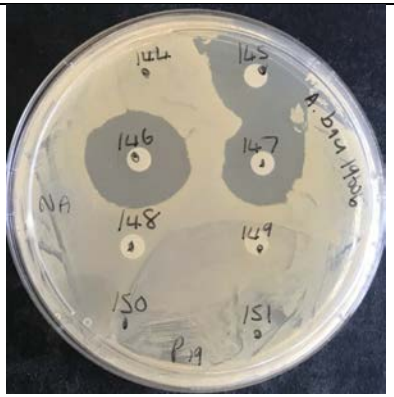
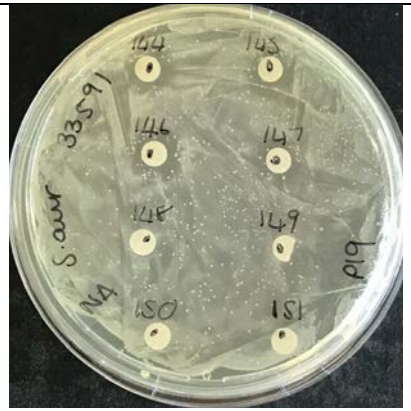
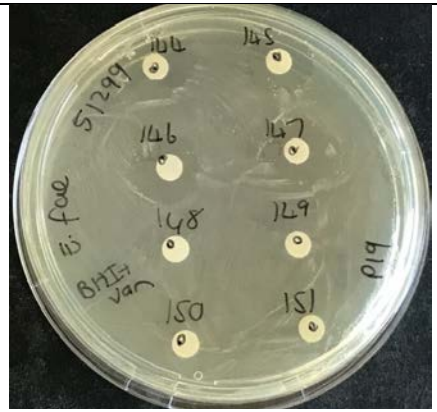


R-35 co-culture with different *M. aurum* A+ (alive and 'dead')



R-35 co-culture with different *M. aurum* A+ (alive and 'dead') concentrations activity against *A. baumannii* ATCC 19606

R-35 + 1% *M. aurum* A+ alive: 136 and 138 R35 + 0.1% *M. aurum* A+ 'dead': 139 - 141 R35 + 0.5% *M. aurum* A+ 'dead': 142 and 143	R-35 + 1% *M. aurum* A+ alive: 136 and 138 R35 + 0.1% *M. aurum* A+ 'dead': 139 - 141 R35 + 0.5% *M. aurum* A+ 'dead': 142 and 143	concentrations activity against *S. aureus* ATCC 29213 R-35 + 1% *M. aurum* A+ alive: 136 and 138 R35 + 0.1% *M. aurum* A+ 'dead': 139 - 141 R35 + 0.5% *M. aurum* A+ 'dead': 142 and 143	R-35 + 1% *M. aurum* A+ alive: 136 and 138 R35 + 0.1% *M. aurum* A+ 'dead': 139 - 141 R35 + 0.5% *M. aurum* A+ 'dead': 142 and 143
 R-35 co-culture with different *M. aurum* A+ (alive and 'dead') concentrations activity against *S. aureus* ATCC 33591 R-35 + 1% *M. aurum* A+ alive: 136 and 138 R35 + 0.1% *M. aurum* A+ 'dead': 139 - 141 R35 + 0.5% *M. aurum* A+ 'dead': 142 and 143	 R-35 co-culture with different *M. aurum* A+ (alive and 'dead') concentrations activity against *E. faecalis* ATCC 29212 R-35 + 1% *M. aurum* A+ alive: 136 and 138 R35 + 0.1% *M. aurum* A+ 'dead': 139 - 141 R35 + 0.5% *M. aurum* A+ 'dead': 142 and 143	 R-35 co-culture with different *M. aurum* A+ (alive and 'dead') concentrations against *E. faecalis* ATCC 51299 R-35 + 1% *M. aurum* A+ alive: 136 and 138 R35 + 0.1% *M. aurum* A+ 'dead': 139 - 141 R35 + 0.5% *M. aurum* A+ 'dead': 142 and 143	 R-35 co-culture with different *M. aurum* A+ 'dead' concentrations activity against *A. baumannii* ATCC BAA-1605 R-35 + 0.5% *M. aurum* A+ 'dead': 144 R35 + 1% *M. aurum* A+ 'dead': 145 - 147 Controls: 148 - 150

 R-35 co-culture with different *M. aurum* A+ 'dead' concentrations activity against *E. cloacae* ATCC BAA-1143 R-35 + 0.5% *M. aurum* A+ 'dead': 144 R35 + 1% *M. aurum* A+ 'dead': 145 - 147 Controls: 148 - 150	 R-35 co-culture with different *M. aurum* A+ 'dead' concentrations activity against *E. coli* ATCC 25922 R-35 + 0.5% *M. aurum* A+ 'dead': 144 R35 + 1% *M. aurum* A+ 'dead': 145 - 147 Controls: 148 - 150	 R-35 co-culture with different *M. aurum* A+ 'dead' concentrations activity against *S. aureus* ATCC 29213 R-35 + 0.5% *M. aurum* A+ 'dead': 144 R35 + 1% *M. aurum* A+ 'dead': 145 - 147 Controls: 148 - 150	 R-35 co-culture with different *M. aurum* A+ 'dead' concentrations activity against *A. baumannii* ATCC 19606 R-35 + 0.5% *M. aurum* A+ 'dead': 144 R35 + 1% *M. aurum* A+ 'dead': 145 - 147 Controls: 148 - 150
 R-35 co-culture with different *M. aurum* A+ 'dead' concentrations activity against *S. aureus* ATCC 33591 R-35 + 0.5% *M. aurum* A+ 'dead': 144	 R-35 co-culture with different *M. aurum* A+ 'dead' concentrations activity against *E. faecalis* ATCC 29212 R-35 + 0.5% *M. aurum* A+ 'dead': 144	 R-35 co-culture with different *M. aurum* A+ 'dead' concentrations activity against *E. faecalis* ATCC 51299 R-35 + 0.5% *M. aurum* A+ 'dead': 144	

R35 + 1% *M. aurum* A+ ‘dead’: 145 - 147 Controls: 148 - 150	R35 + 1% *M. aurum* A+ ‘dead’: 145 - 147 Controls: 148 - 150	R35 + 1% *M. aurum* A+ ‘dead’: 145 - 147 Controls: 148 - 150	

Appendix E: Molecular networking of mono-cultures and co-cultures for R-30 and R-35

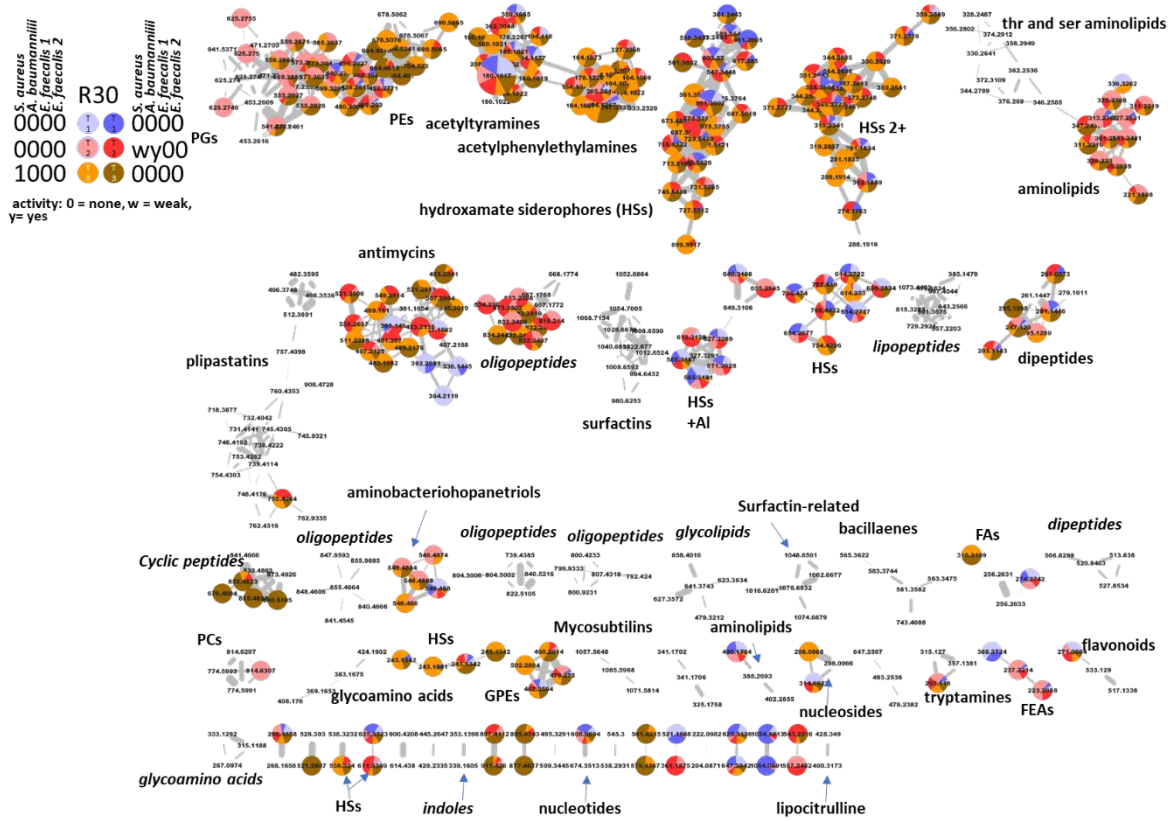


Figure E1: Molecular network showing only the abundances for compounds of R-30

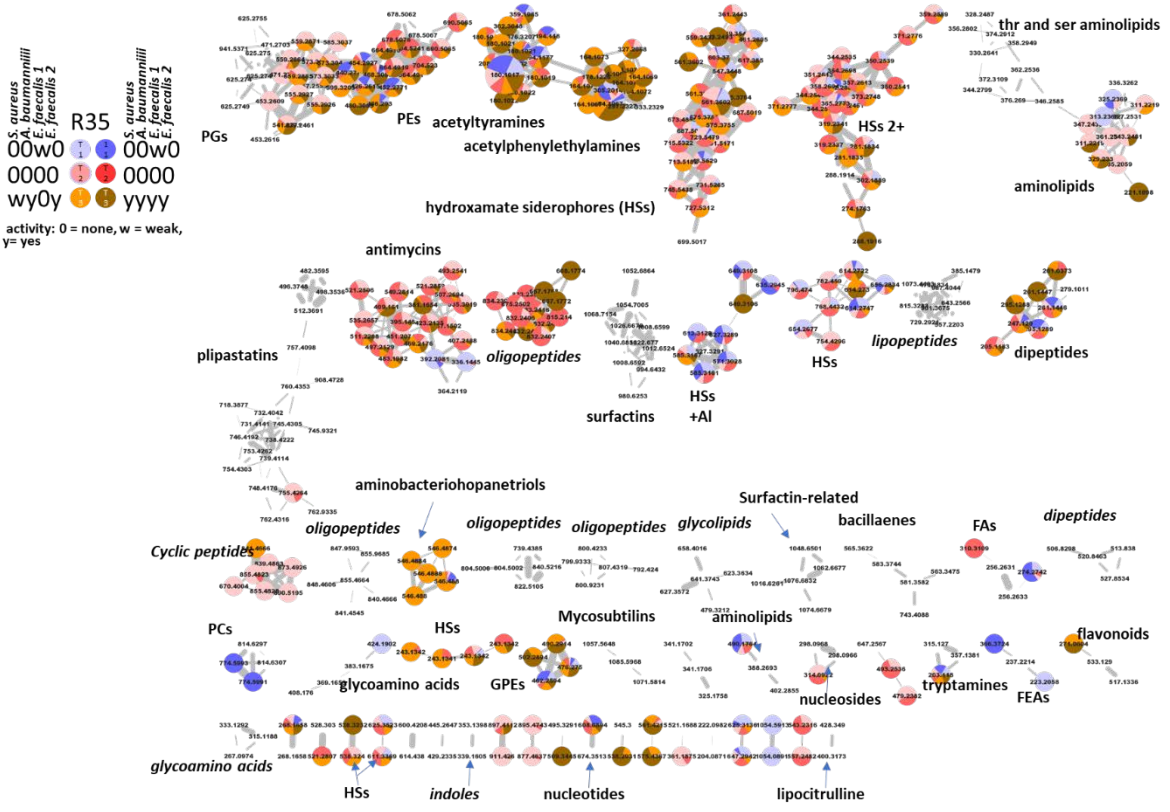


Figure E2: Molecular network showing only the abundance for compounds of R-35.

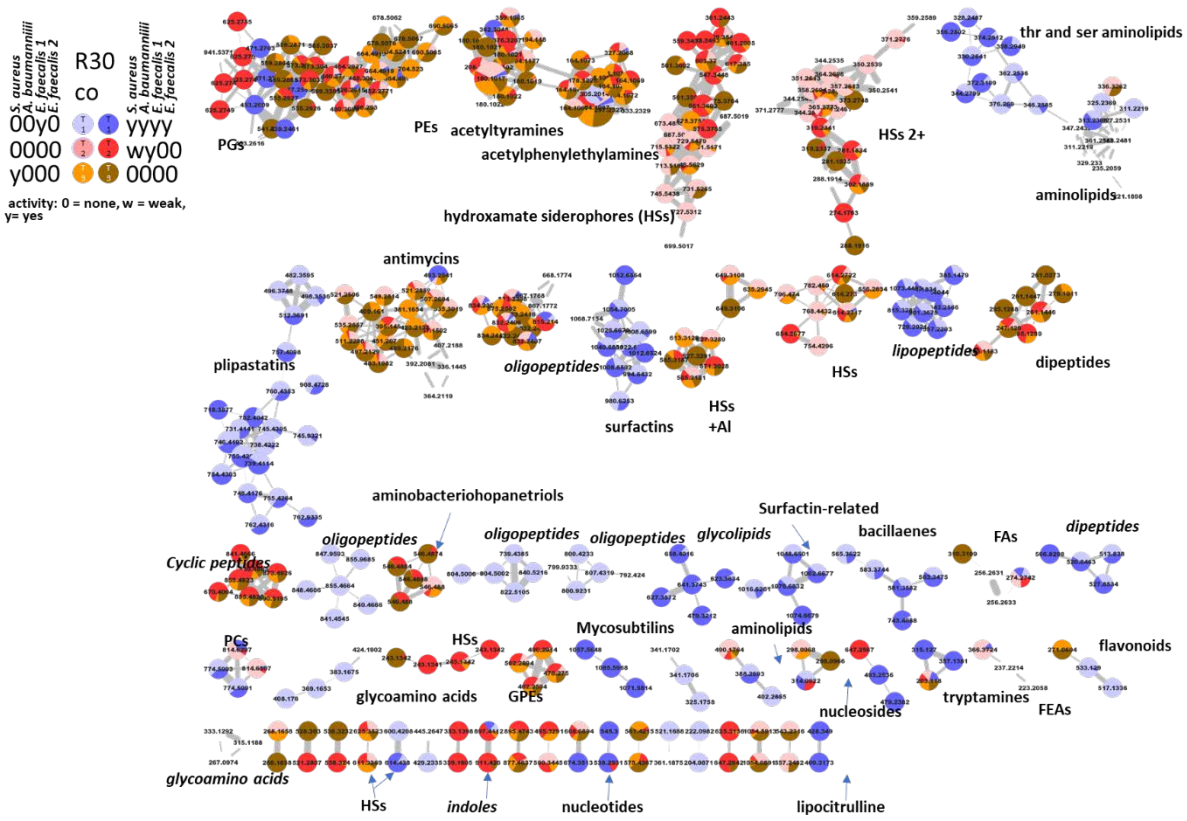


Figure E3: Molecular Networking showing the abundance for compounds from R-30 co-cultures.

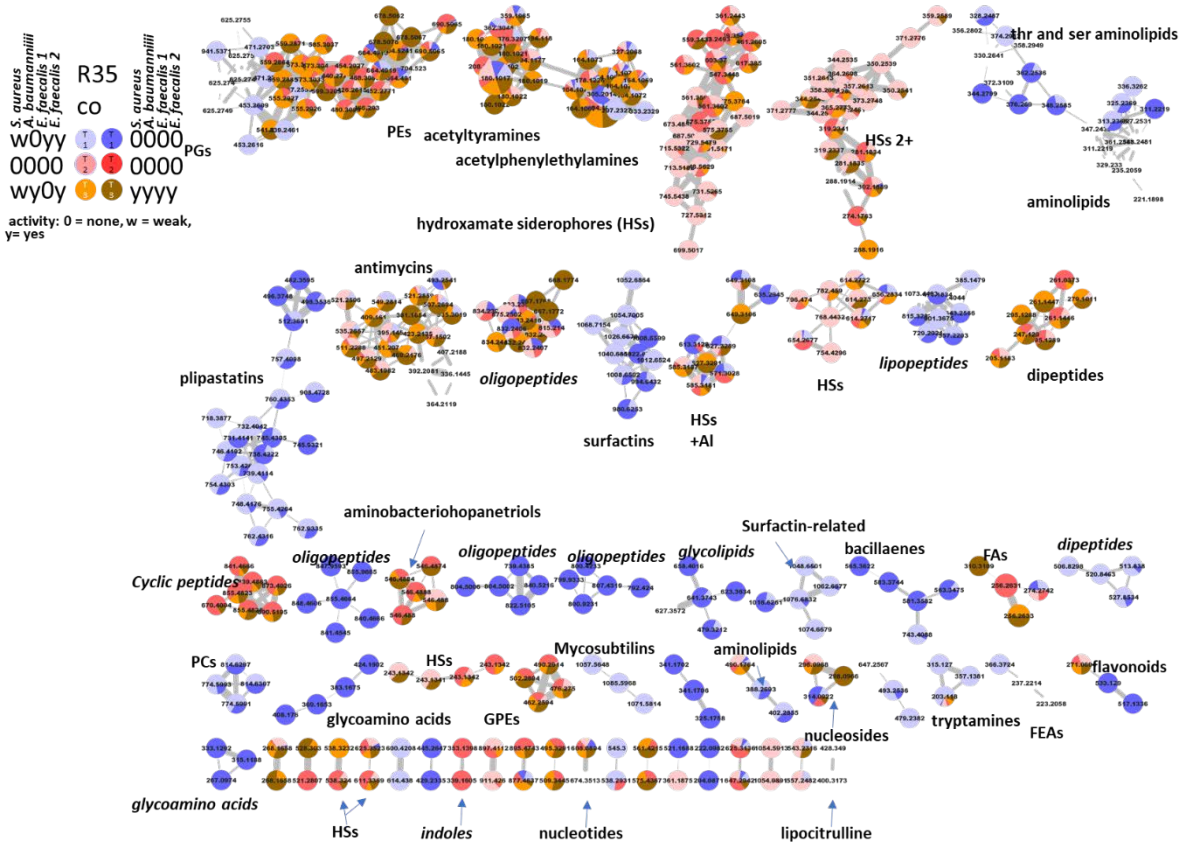


Figure E4: Molecular network only showing the abundance of compounds from R-35 co-culture.

Appendix F: Complete QUAST and TYGS reports for the R-35 genome sequence

QUAST

Quality Assessment Tool for Genome Assemblies by CAB

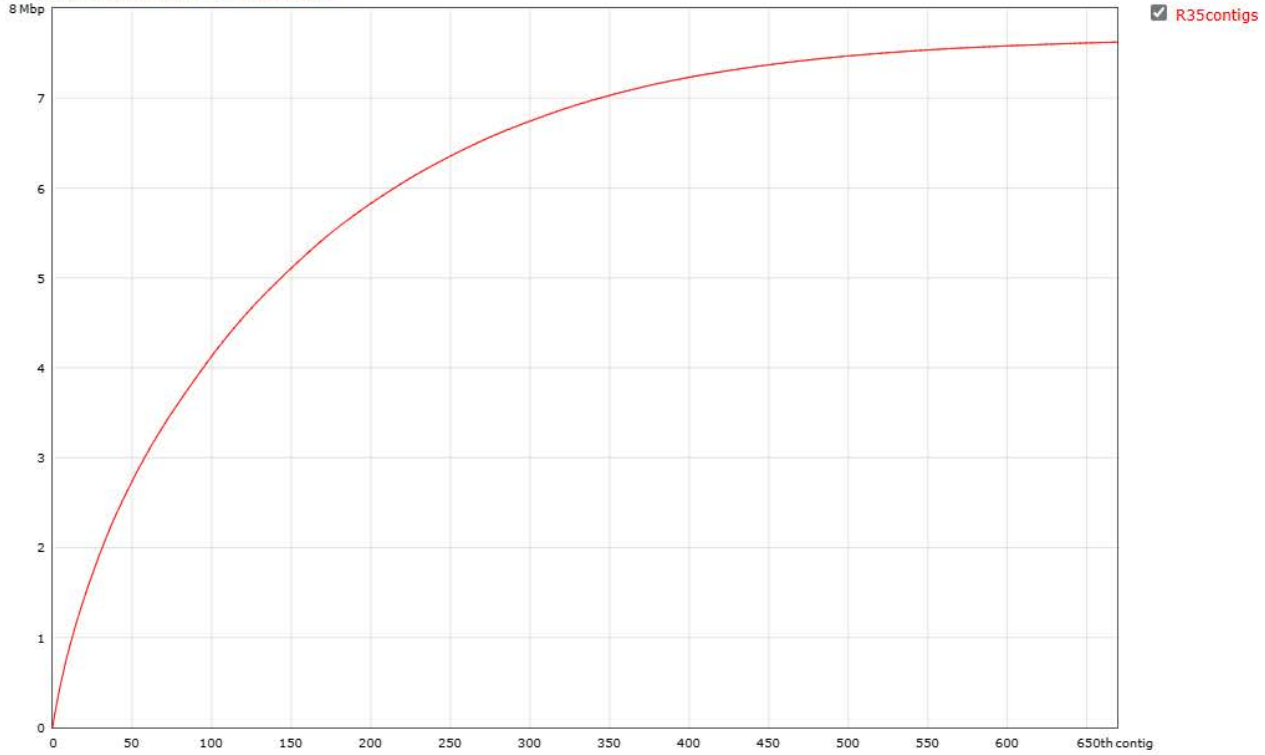
13 November 2023, Monday, 19:41:26

View in Icarus contig browser

All statistics are based on contigs of size >= 500 bp, unless otherwise noted (e.g., "# contigs (>= 0 bp)" and "Total length (>= 0 bp)" include all contigs).

Statistics without reference	R35contigs
# contigs	669
# contigs (>= 0 bp)	2269
# contigs (>= 1000 bp)	565
# contigs (>= 5000 bp)	340
# contigs (>= 10000 bp)	231
# contigs (>= 25000 bp)	87
# contigs (>= 50000 bp)	24
Largest contig	109 807
Total length	7 623 139
Total length (>= 0 bp)	7 818 256
Total length (>= 1000 bp)	7 551 115
Total length (>= 5000 bp)	6 980 460
Total length (>= 10000 bp)	6 174 152
Total length (>= 25000 bp)	3 811 975
Total length (>= 50000 bp)	1 654 570
N50	25 062
N90	5951
auN	32 446
L50	87
L90	319
GC (%)	72.23
Per base quality	
# N's per 100 kbp	0
# N's	0

Plots: Cumulative length Nx GC content



Contigs are ordered from largest (contig #1) to smallest.

TYPE (STRAIN) GENOME SERVER

JOB SUMMARY

PRINT DATE:	2023-11-13 19:02:56 +0100
JOB ID:	edd10028-9005-44f2-9dd6-7b6cabb9d76b
RESULT PAGE:	https://tygs.dsmz.de/user_results/show?guid=edd10028-9005-44f2-9dd6-7b6cabb9d76b

Table 1: Phylogenies

Publication-ready versions of both the genome-scale GBDP tree and the 16S rRNA gene sequence tree can be customized and exported either in SVG (vector graphic) or PNG format from within the phylogeny viewers in your TYGS result page. For publications the **SVG format is recommended** because it is lossless, always keeps its high resolution and can also be easily converted to other popular formats such as PDF or EPS. Please follow the link provided above!

Table 2: Identification

The below list contains the result of the TYGS species identification routine.

Explanation of remarks that might occur in the below table:

remark [R1]: The TYGS type strain database is automatically updated on an almost daily basis. However, if a particular type strain genome is not available in the TYGS database, this can have several reasons which are detailed in the FAQ. You can request an extended 16S rRNA gene analysis via the 16S tree viewer found in your result page to detect **not yet genome-sequenced** type strains relevant for your study.

remark [R2]: > 70% dDDH value (formula d_4) and (almost) minimal dDDH values for gene-content formulae d_0 and d_6 indicate a potentially unreliable identification result and should thus be checked via the 16S rRNA gene sequence similarity. Such strong deviations can, in principle, be caused by sequence contamination.

remark [R3]: G+C content difference of > 1 % indicates a potentially unreliable identification result because within species G+C content varies no more than 1 %, if computed from genome sequences (PMID: 24505073).

Strain	Conclusion	Identification result	Remark
'R35contigs'	belongs to known species	*Streptomyces griseoincarnatus*	

Table 3: Pairwise comparisons of user genomes vs. type-strain genomes

The following table contains the pairwise dDDH values between your user genomes and the selected type-strain genomes. The dDDH values are provided along with their confidence intervals (C.I.) for the three different GBDP formulas:

- formula d_0 (a.k.a. GGDC formula 1): length of all HSPs divided by total genome length
- formula d_4 (a.k.a. GGDC formula 2): sum of all identities found in HSPs divided by overall HSP length
- formula d_6 (a.k.a. GGDC formula 3): sum of all identities found in HSPs divided by total genome length

Note: Formula d_4 is independent of genome length and is thus robust against the use of incomplete draft genomes. For other reasons for preferring formula d_4, see the FAQ.

Query	Subject	d_0	C.I. d_0	d_4	C.I. d_4	d_6	C.I. d_6	Diff. G+C Percent
'R35contigs.fasta'	*Streptomyces griseoincarnatus* JCM 4381	84.8	[81.1 - 87.9]	84.0	[81.2 - 86.4]	87.6	[84.6 - 90.1]	0.06
'R35contigs.fasta'	*Streptomyces variabilis* JCM 4422	85.8	[82.1 - 88.8]	84.0	[81.3 - 86.5]	88.4	[85.4 - 90.8]	0.18
'R35contigs.fasta'	*Streptomyces matensis* JCM 4277	69.6	[65.7 - 73.2]	60.5	[57.6 - 63.3]	69.9	[66.4 - 73.1]	0.45
'R35contigs.fasta'	*Streptomyces griseorubens* JCM 4383	67.1	[63.3 - 70.8]	60.2	[57.4 - 63.0]	67.7	[64.3 - 70.9]	0.2
'R35contigs.fasta'	*Streptomyces griseorubens* DSM 40160	67.7	[63.8 - 71.4]	60.2	[57.4 - 63.0]	68.2	[64.8 - 71.4]	0.31
'R35contigs.fasta'	*Streptomyces althioticus* JCM 4344	67.7	[63.8 - 71.3]	60.2	[57.4 - 63.0]	68.2	[64.8 - 71.4]	0.33
'R35contigs.fasta'	*Streptomyces werraensis* JCM 4860	68.1	[64.2 - 71.8]	56.8	[54.1 - 59.6]	67.7	[64.3 - 70.9]	0.41
'R35contigs.fasta'	*Streptomyces viridodiastaticus* JCM 4536	63.2	[59.5 - 66.8]	54.0	[51.3 - 56.7]	62.8	[59.4 - 66.0]	0.35
'R35contigs.fasta'	*Streptomyces albogriseolus* JCM 4616	61.2	[57.5 - 64.7]	54.0	[51.3 - 56.7]	60.9	[57.7 - 64.1]	0.17
'R35contigs.fasta'	*Streptomyces cellulosae* JCM 4462	57.9	[54.3 - 61.4]	52.2	[49.6 - 54.9]	57.6	[54.4 - 60.8]	0.16
'R35contigs.fasta'	*Streptomyces malachitofuscus* JCM 4493	45.3	[41.9 - 48.7]	32.1	[29.7 - 34.7]	41.5	[38.5 - 44.5]	0.55
'R35contigs.fasta'	*Streptomyces griseoflavus* JCM 4479	43.9	[40.5 - 47.3]	31.5	[29.1 - 34.0]	40.2	[37.2 - 43.2]	0.09
'R35contigs.fasta'	*Streptomyces paradoxus* DSM 43350	31.5	[28.1 - 35.1]	27.0	[24.6 - 29.4]	29.4	[26.5 - 32.5]	0.79
'R35contigs.fasta'	*Streptomyces collinus* DSM 40129	31.1	[27.8 - 34.7]	27.0	[24.7 - 29.5]	29.1	[26.2 - 32.2]	0.83
'R35contigs.fasta'	*Streptomyces abyssomicinicus* NBRC 110469T	21.1	[17.8 - 24.7]	24.1	[21.8 - 26.6]	20.5	[17.8 - 23.6]	0.95
'R35contigs.fasta'	*Streptomyces koyangensis* VK-A60	20.9	[17.6 - 24.5]	22.8	[20.5 - 25.2]	20.2	[17.5 - 23.2]	0.85

Table 4: Strains in your dataset

Joint dataset of automatically determined closest type strains (if this mode was chosen), manually selected type strains (if selected accordingly) and the provided user strains, if provided (marked in yellow).

Strain	Authority	Other deposits	Synonyms	Base pairs	Percent G+C	No. proteins	Goldstamp	Bioproject accession	Biosample accession	Assembly accession	IMG OID
Streptomyces collinus DSM 40129	Lindenbein 1952	BCRC 11465; CCRC 11465; AS 4.1623; CGMCC 4.1623; KCTC 9713; NRRL B-5412; NRRL ISP-5129; ICMP 12539; CBS 482.68; ATCC 19743; DSM 2012; JCM 4361; IFO 12759; NBRC 12759; VKM Ac-710; RIA 1024; UNIQEM 130	*Streptomyces collinus*	8858480	71.4	7966	Gp0325167				2867977797

Strain	Authority	Other deposits	Synonyms	Base pairs	Percent G+C	No. proteins	Goldstamp	Bioproject accession	Biosample accession	Assembly accession	IMG OID
Streptomyces griseorubens DSM 40160	(Preobrazhenskaya et al. 1957) Pridham et al. 1958	BCRC 12104; CCRC 12104; NRRL B-3982; NRRL ISP-5160; NCIMB 9846; CBS 505.68; ATCC 19767; ATCC 19909; DSM 40160; JCM 4383; IFO 12780; NBRC 12780; VKM Ac-1894; INA 6124/54; NCIB 9846; RIA 1047; UNIQEM 153	*Actinomyces griseorubens*; *Streptomyces griseorubens*	7658110	72.5	6883	Gp0505850				2913524612
Streptomyces abyssomicinicus NBRC 110469T	Komaki et al. 2020	CHI39	*Streptomyces abyssomicinicus*	7911144	73.1	6697		PRJDB4097	SAMD00035864	GCA_008974245	

Strain	Authority	Other deposits	Synonyms	Base pairs	Percent G+C	No. proteins	Goldstamp	Bioproject accession	Biosample accession	Assembly accession	IMG OID
Streptomyces paradoxus DSM 43350	Goodfellow et al. 1986	BCRC 12521; BCRC (formerly CCRC) 12521; CCRC 12521; IFM 1160; KCTC 9118; NRRL B-3457; NRRL B-3483; ATCC 15813; JCM 3052; IFO 14887; IFO (now NBRC) 14887; NBRC 14887; VKM Ac-645; IMET 43491; INMI 3180; KCC 3052; KCC A-52; PCM 2310; RIA 655	*Actinosporangium violaceum*; *Streptomyces paradoxus*	8580274	71.4	7866	Gp0456270				2861534081
Streptomyces koyangensis VK-A60	Lee et al. 2005	DSM 41864; JCM 14915; NBRC 100598; KCCM 10555	*Streptomyces koyangensis*	7220839	73.0	5988	Gp0365742	PRJNA486241	SAMN09842183	GCA_003428925	

Strain	Authority	Other deposits	Synonyms	Base pairs	Percent G+C	No. proteins	Goldstamp	Bioproject accession	Biosample accession	Assembly accession	IMG OID
Streptomyces matensis JCM 4277	Margalith et al. 1959	NRRL B-2576; NRRL ISP-5188; NCIMB 9826; CBS 919.68; ATCC 23935; DSM 40188; JCM 4268; JCM 4651; IFO 12889; NBRC 12889; HAMBI 1048; IMET 42065; NCIB 9826; RIA 1142; RIA 570	*Streptomyces matensis*	7325 782	72.6	6731		PRJDB10510	SAMD00245408	GCA_014649175	
Streptomyces griseoincarnatus JCM 4381	(Preobrazhenska ya et al. 1957) Pridham et al. 1958 emend. Komaki 2021	9673/55; LMG 19316; BCRC 11481; CCRC 11481; BCRC 118481; AS 4.1409; CGMCC 4.1409; NRRL B-5313; NRRL ISP-5274; NCIMB 9825; CBS 838.68; ATCC 23623; ATCC 23917; DSM 40274; IFO 12871; NBRC 12871; INA 9673; INA 9673/55; ISP 5274; NCIB 9825; RIA 1192	*Actinomyces griseoincarnatus*; *Streptomyces griseoincarnatus*	7388 604	72.2	6759		PRJDB10510	SAMD00245431	GCA_014649575	

Strain	Authority	Other deposits	Synonyms	Base pairs	Percent G+C	No. proteins	Goldstamp	Bioproject accession	Biosample accession	Assembly accession	IMG OID
Streptomyces werraensis JCM 4860	Wallhäusser et al. 1964	BCRC 12038; CCRC 12038; NRRL B-5317; NRRL ISP-5486; CBS 437.67; CBS 705.72; ATCC 14424; DSM 40486; DSM 40745; IFO 13404; NBRC 13404; RIA 1365	*Streptomyces werraensis*	7294 408	72.6	6499		PRJDB10510	SAMD00245533	GCA_014656175	

Strain	Authority	Other deposits	Synonyms	Base pairs	Percent G+C	No. proteins	Goldstamp	Bioproject accession	Biosample accession	Assembly accession	IMG OID
Streptomyces albogriseolus JCM 4616	Benedict et al. 1954 emend. Komaki 2021	40003; 7-A; BCRC 12230; CCRC 12230; CIP 104424; CIP 104428; KCTC 9675; NRRL B-1305; NRRL ISP-5003; NCIMB 9604; CBS 614.68; ATCC 23875; DSM 40003; JCM 4004; IFO 3413; IFO 3709; IFO 12834; NBRC 3413; NBRC 3709; NBRC 12834; VKM Ac-1200; HUT 6045; ISP 5003; NCIB 9604; RIA 1101	*Streptomyces albogriseolus*	8052 128	72.3	7414		PRJDB10510	SAMD00245488	GCA_014650475	

Strain	Authority	Other deposits	Synonyms	Base pairs	Percent G+C	No. proteins	Goldstamp	Bioproject accession	Biosample accession	Assembly accession	IMG OID
Streptomyces variabilis JCM 4422	(Preobrazhenskaya et al. 1957) Pridham et al. 1958	BCRC 11488; CCRC 11488; NRRL B-3984; NRRL ISP-5179; CBS 568.68; ATCC 19815; ATCC 19930; DSM 40179; IFO 12825; NBRC 12825; VKM Ac-1854; IMET 42059; RIA 1095; UNIQEM 202	*Actinomyces variabilis*; *Streptomyces variabilis*	7339325	72.4	6699		PRJDB10510	SAMD00245451	GCA_014649935	
Streptomyces malachitofuscus JCM 4493	(ex Preobrazhenskaya et al. 1964) Preobrazhenskaya and Terekhova 1986	NRRL B-12273; NRRL ISP-5332; CBS 881.69; ATCC 25471; DSM 40332; IFO 13059; NBRC 13059; VKM Ac-1850; INA 739; KCC S-493; RIA 1251	*Streptomyces malachitofuscus*	7614656	71.6	6912		PRJDB10510	SAMD00245459	GCA_014650055	

Strain	Authority	Other deposits	Synonyms	Base pairs	Percent G+C	No. proteins	Goldstamp	Bioproject accession	Biosample accession	Assembly accession	IMG OID
Streptomyces griseoflavus JCM 4479	(Krainsky 1914) Waksman and Henrici 1948	LMG 19344; BCRC 12232; CCRC 12232; AS 4.1454; CGMCC 4.1454; NRRL B-5312; NRRL ISP-5456; CBS 409.52; CBS 689.69; ATCC 25456; DSM 40456; IFO 13044; NBRC 13044; VKM Ac-993; IMET 43530; RIA 1236	*Actinomyces griseoflavus*; *Streptomyces griseoflavus*	7531045	72.3	6910		PRJDB10510	SAMD00245456	GCA_014649995	

Strain	Authority	Other deposits	Synonyms	Base pairs	Percent G+C	No. proteins	Goldstamp	Bioproject accession	Biosample accession	Assembly accession	IMG OID
Streptomyces althioticus JCM 4344	Yamaguchi et al. 1957 emend. Komaki 2021	BCRC 13686; CCRC 13686; AS 4.1608; CGMCC 4.1608; KCTC 9752; NRRL B-3981; NRRL ISP-5092; CBS 463.68; ATCC 19724; DSM 40092; IFO 12740; IFO 15956; NBRC 12740; NBRC 15956; VKM Ac-705; ISP 5092; RIA 1005; UNIQEM 120	*Streptomyces althioticus*	7625043	72.5	6991		PRJDB10510	SAMD00245418	GCA_014649355	

Strain	Authority	Other deposits	Synonyms	Base pairs	Percent G+C	No. proteins	Goldstamp	Bioproject accession	Biosample accession	Assembly accession	IMG OID
Streptomyces griseorubens JCM 4383	(Preobrazhenskaya et al. 1957) Pridham et al. 1958	BCRC 12104; CCRC 12104; NRRL B-3982; NRRL ISP-5160; NCIMB 9846; CBS 505.68; ATCC 19767; ATCC 19909; DSM 40160; JCM 4383; IFO 12780; NBRC 12780; VKM Ac-1894; INA 6124/54; NCIB 9846; RIA 1047; UNIQEM 153	*Actinomyces griseorubens*; *Streptomyces griseorubens*	7668551	72.4	6981		PRJDB10510	SAMD00245433	GCA_014649615	
Streptomyces cellulosae JCM 4462	(Krainsky 1914) Waksman and Henrici 1948	LMG 19315; BCRC 12087; CCRC 12087; AS 4.1411; CGMCC 4.1411; KCTC 9703; NRRL B-2889; NRRL ISP-5362; CBS 122.18; CBS 670.69; ATCC 25439; DSM 40362; IFO 13027; NBRC 13027; VKM Ac-829; RIA 1219	*Actinomyces cellulosae*; *Streptomyces cellulosae*	7878110	72.0	7330		PRJDB10510	SAMD00245454	GCA_014655195	

Strain	Authority	Other deposits	Synonyms	Base pairs	Percent G+C	No. proteins	Goldstamp	Bioproject accession	Biosample accession	Assembly accession	IMG OID
Streptomyces viridodiastaticus JCM 4536	(Baldacci et al. 1955) Pridham et al. 1958	BCRC 12458; CCRC 12458; NRRL B-5622; NRRL ISP-5249; CBS 660.69; ATCC 25518; DSM 40249; IFO 13106; NBRC 13106; VKM Ac-1749; RIA 1298	*Actinomyces viridodiastaticus*; *Streptomyces viridodiastaticus*	7674156	72.5	7029		PRJDB10510	SAMD00245473	GCA_014655475	
R35contigs.fasta				7818256	72.2	8354					

Methods, Results and References

The genome sequence data were uploaded to the Type (Strain) Genome Server (TYGS), a free bioinformatics platform available under https://tygs.dsmz.de, for a whole genome-based taxonomic analysis [1]. The analysis also made use of recently introduced methodological updates and features [2]. Information on nomenclature, synonymy and associated taxonomic literature was provided by TYGS's sister database, the List of Prokaryotic names with Standing in Nomenclature (LPSN, available at https://lpsn.dsmz.de) [2]. The results were provided by the TYGS on 2023-11-12. The TYGS analysis was subdivided into the following steps:

Determination of closely related type strains

Determination of closest type strain genomes was done in two complementary ways: First, all user genomes were compared against all type strain genomes available in the TYGS database via the MASH algorithm, a fast approximation of intergenomic relatedness [3], and, the ten type strains with the smallest MASH distances chosen per user genome. Second, an additional set of ten closely related type strains was determined via the 16S rDNA gene sequences. These were extracted from the user genomes using RNAmmer [4] and each sequence was subsequently BLASTed [5] against the 16S rDNA gene sequence of each of the currently 19412 type strains available in the TYGS database. This was used as a proxy to find the best 50 matching type strains (according to the bitscore) for each user genome and to subsequently calculate precise distances using the Genome BLAST Distance Phylogeny approach (GBDP) under the algorithm 'coverage' and distance formula d_5 [6]. These distances were finally used to determine the 10 closest type strain genomes for each of the user genomes.

Pairwise comparison of genome sequences

For the phylogenomic inference, all pairwise comparisons among the set of genomes were conducted using GBDP and accurate intergenomic distances inferred under the algorithm 'trimming' and distance formula d_5 [6]. 100 distance replicates were calculated each. Digital DDH values and confidence intervals were calculated using the recommended settings of the GGDC 4.0 [2,6].

Phylogenetic inference

The resulting intergenomic distances were used to infer a balanced minimum evolution tree with branch support via FASTME 2.1.6.1 including SPR postprocessing [7]. Branch support was inferred from 100 pseudo-bootstrap replicates each. The trees were rooted at the midpoint [8] and visualized with PhyD3 [9].

Type-based species and subspecies clustering

The type-based species clustering using a 70% dDDH radius around each of the 16 type strains was done as previously described [1]. The resulting groups are shown in Table 1 and 4. Subspecies clustering was done using a 79% dDDH threshold as previously introduced [10].

Results

Type-based species and subspecies clustering

The resulting species and subspecies clusters are listed in Table 4, whereas the taxonomic identification of the query strains is found in Table 1. Briefly, the clustering yielded 11 species clusters and the provided query strains were assigned to 1 of these. Moreover, user strains were located in 1 of 11 subspecies clusters.

Figure caption SSU tree

Figure 1. Tree inferred with FastME 2.1.6.1 [7] from GBDP distances calculated from 16S rDNA gene sequences. The branch lengths are scaled in terms of GBDP distance formula d_5. The numbers above branches are GBDP pseudo-bootstrap support values > 60 % from 100 replications, with an average branch support of 54.1 %. The tree was rooted at the midpoint [8].

Figure caption genome tree

Figure 2. Tree inferred with FastME 2.1.6.1 [7] from GBDP distances calculated from genome sequences. The branch lengths are scaled in terms of GBDP distance formula d_5. The numbers above branches are GBDP pseudo-bootstrap support values > 60 % from 100 replications, with an average branch support of 84.6 %. The tree was rooted at the midpoint [8].

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