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Antibiotics from *Streptomyces* spp. isolated from Namibian desert sand

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DECLARATION OF ORIGINALITY

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- I dedicate this dissertation to my mom (Francina Nkgowana Nxumalo), for being my number one support. I am truly grateful for all you have done for me. You are my number one fan, the only person who has always supported and believed in me. Thank you for holding my hand when things got tough during my studies.
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"*Rejoice always, pray continually, and give thanks in all circumstances; for this is God's will for you in Christ Jesus*". Thessalonians 5:16-18.

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LIST OF ABBREVIATIONS

TLC – Thin layer chromatography

NMR - Nuclear Magnetic Resonance

GC-MS – Gas Chromatography – Mass spectrometry

RNA – Ribonucleic acid

DNA - Deoxyribonucleic acid

GC content – Guanine Cytosine content

HGT- Horizontal Gene Transfer

WHO – World Health Organization

MRSA – Multi-Resistant *Staphylococcus aureus*

MHA – Muller Hinton Agar

PCR – Polymerase Chain Reaction

dNTP – deoxy-ribonucleotide triphosphate

NF H_20 – Nuclear free water

ddH_20 – Double Distilled water

INT - p-Iodonitrotetrazolium violet

MTT - 3-(4, 5-dimethyldiazol-2-yl)-2, 5 diphenyltetrazolium bromide

UV light – Ultra Violet Light

R_f – Retention Factor

ABSTRACT

The discovery of antibiotics has been one of the major breakthroughs in the history of medicine for the treatment of microbe-related infections. Soil-borne microorganisms have been known to potentially produce clinically important antibiotics. However, many studies have shown that most deadly disease-causing bacteria have acquired the ability to resist the actions of these antibiotics and this phenomenon is known as antibiotic resistance. The aim of the study was to discover antibiotics from sand collected from inside fairy circles in the Namib desert. The bacteria were isolated using the serial dilution method and pure cultures were made from a 1 X 10^{-4} plate. These bacteria were subjected to primary screening in which the bacteria resembling the *Streptomyces* morphological features were tested against non-*Streptomyces* bacteria (also isolated from the soil) and the four known pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Achromobacter xylosoxidans* and *Proteus vulgaris*) for antagonistic activity.

The *Streptomyces* resembling bacteria together with the bacteria that were susceptible were identified using the 16S rRNA sequencing method. Three bacteria were isolated and identified as *Streptomyces parvulus* strain R5-344, *Kocuria turfanensis* strain HO-9042 and uncultured *Microbacterium* sp. Clone 1c05. For the extraction and purification of the active compounds from the bacteria, a crude extract was made using ethyl acetate and spotted on TLC plates to separate the compounds. A direct TLC-bio-autography method was performed to show which compounds were antibacterial. The active section was scraped off from the TLC plate (R_f = 0.76) and extracted with ethyl acetate. The extract was analysed by ^{1}H NMR and GC-MS for identification of the compounds. The comparison of the ^{1}H NMR spectrum of the control and the active sample showed

new peaks at chemical shifts of 4.33 ppm, 4.75 ppm and 0.90 ppm. And lastly, with the GC-MS analysis, three compounds were tentatively identified in the active fraction; one compound showed 92 % similarity to 2-methyloctacosane (Retention time (RT) = 16.420 minutes), the second compound showed 92 % similarity to acetic acid n-octadecyl ester (RT = 20.355 minutes) and the third one showed 87 % similarity to octacosyl acetate (RT = 22.185 minutes).

CHAPTER 1: GENERAL BACKGROUND

1.1. Background of the study

The discovery of antibiotics is considered as one of the major breakthroughs in medicine for the treatment of microbe-related infections (Aminov, 2010). The first true antibiotic, penicillin, was accidentally discovered in 1928 by Sir Alexander Fleming and was produced from a fungal species called *Penicillium chrysogenum*. The agent was only introduced in the 1940s as a treatment for TB infections (Tan & Tatsumura, 2015). He discovered the growth of *P. chrysogenum* on the plate he was growing a bacterium (*Staphylococcus aureus*) on and after a few days realized that it was inhibiting the growth of the bacterium. He concluded the fungus had the ability to produce a compound with antibacterial properties and with further research discovered that the fungus could also kill other bacteria, like *Streptococcus*, *Meningococcus* and *Diphtheria* species. The discovery and introduction of penicillin paved a way for the development and discovery of many clinically important antimicrobial agents from both bacteria and fungi. These antibiotics possess different properties depending on which types of organisms/species are treated. For example, microorganisms can produce compounds that have antifungal; antiviral, and anti-parasitic (e.g. nematodes) activity (George et al., 2010; Procópio et al., 2012; Etebu & Arikekpar, 2016). Most of the clinically important antibiotics are produced by microorganisms found in the soil (Nesme & Simonet, 2015). The current study was focused on discovering antibacterial antibiotics produced by soil bacteria.

The production of antibiotics in microorganisms can be triggered by changes in the environment, which then act as a form of defence mechanism to eliminate the competition (Procópio et al., 2012). Antibiotics, particularly produced by bacteria, can be grouped based on their modes of

action or activity spectrum. The classification based on the spectrum of activity indicates the ability of the antibiotic to inhibit or kill one type of bacterial group or broad range of bacterial groups. These groups are Gram-negative and Gram-positive bacteria. When an antibiotic can kill or inhibit both type of bacterial groups, it is said to have broad-spectrum activity and when it only targets one type of bacterial group, is said to have a narrow spectrum activity (Amyes, 2010). The grouping of antibiotics that is based on modes of action involves the ability of the bacterium to either kill or minimize the growth of the susceptible species (Etebu & Arikekpar, 2016). When an antibacterial agent can kill the competition, the agent is called bactericidal and if it only inhibits the growth, it is bacteriostatic. There are several antibiotics that are produced by *Streptomyces* bacteria and such antibiotics include classes from beta-lactam, tetracycline, macrolides, glycosides, and aminoglycosides (Etebu & Arikekpar, 2016). Figure 1.1 show examples of these classes based on their mechanisms of action.

A good antibiotic may be difficult to be chemically synthesised but not impossible. There are several characteristics that an antibiotic should have to be clinically effective. Some of the properties include: its ability to be harmless to the person using the drug without causing severe side effects and allergic reactions but can kill the targeted microorganism; having a long shelf life and being less prone to resistance and most importantly be affordable and easily produced (Finch et al., 2010; Hossain & Rahmann, 2014). The clinical value of antibiotics can be determined by what is called selective toxicity. Selective toxicity means the antibiotic must be able to be active against harmful microorganisms but not to human cells (Scholar & Pratt, 2000; Matsuzaki, 2009).

It is possible that the target sites for the antibiotic on the microorganism might not be present in humans and therefore in this way the antibiotic will not have any deleterious effects (Scholar & Pratt, 2000; Pommerville, 2010).

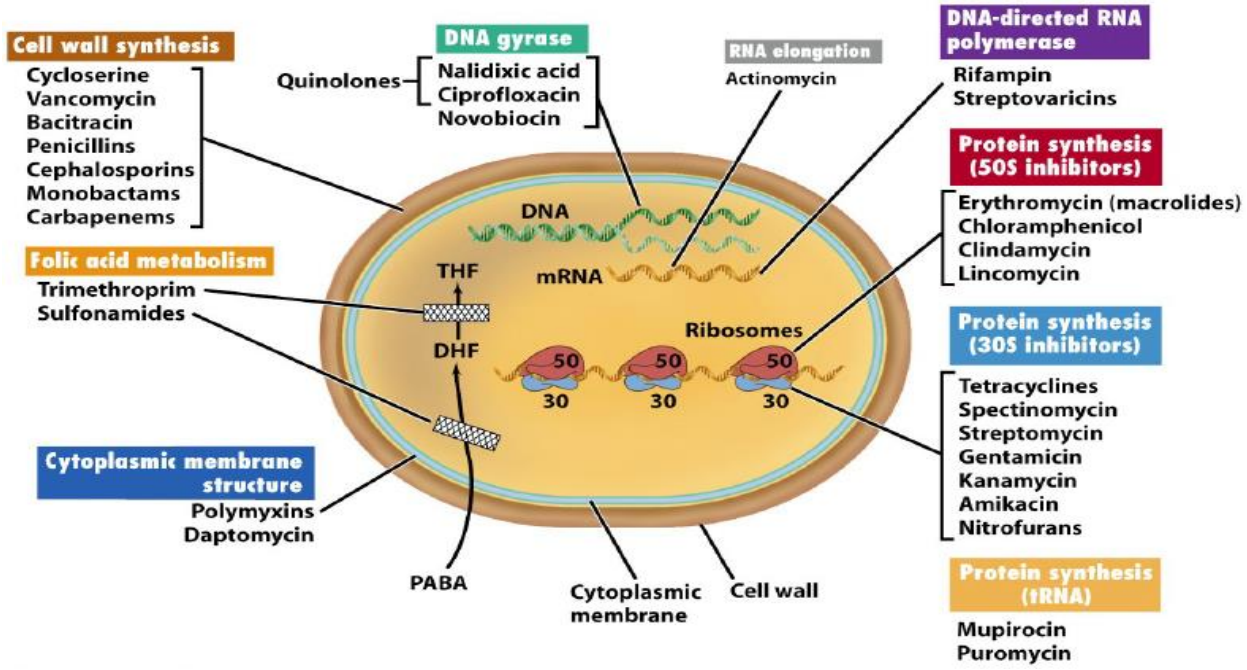

Figure 1.1: Mechanisms of action of antibiotics employed by bacteria to fight against competition. These modes of action include inhibition of cell wall, proteins, RNA elongation and disruption of the bacterial membrane (Bbosa et al., 2014).

Bacterial and human cells are structurally and chemically dissimilar. Bacterial cells have cell walls made up of peptidoglycan, whereas human cells do not contain a cell wall, meaning a given antibiotic will have to specifically target the peptidoglycan in bacteria, therefore killing the bacteria without causing any harm to the human (Scholar & Pratt, 2000).

1.2. Soil microorganisms as a source of antibiotics

Many soil microorganisms play an essential role in maintaining a plant's growth and protecting them from pathogenic soil-borne microorganisms. They are equally important for fixing nitrogen, breaking down of compounds and many other related activities (Raaijmakers et al., 2002; Seipke et al., 2012). Most importantly, soil microorganisms are known to be most abundant and produce many of the clinically and agriculturally important antibiotics (Ceylan et al., 2008; George et al., 2010; Hossain & Rahman, 2014; Nesme & Simonet, 2015). These antibiotic-producing soil microorganisms include *Streptomyces*, *Bacillus* and *Penicillium* species (Forsberg et al., 2012; Sandhya et al., 2015). These bacteria and fungi have a way to insulate themselves from being harmed by their own antibiotics and to only target the other bacteria in the same environment (Seipke et al., 2012). Other important secondary metabolites produced by soil microorganisms that also play major roles in fighting off plant pathogens are volatile compounds, sideosphores, toxins and enzymes (Raaijmakers et al., 2012; Paul, 2014). For an exceedingly long time, microorganisms have been the causative agents of many human related infections and initially there was no known treatment of such infections. Antibiotics can be produced either naturally or synthetically, however synthetic antibiotics are still rarely produced compared to the natural ones (Fair & Tor, 2014). Semi-synthetic antibiotics are often produced in the laboratory by manipulating natural antibiotics that have previously decreased in effectiveness due to microorganisms gaining resistance (Fischbach & Walsh, 2009; Asha et al., 2011). Soil microorganisms are not studied as much as the free-living ones because the frequency at which these bacterial cells form colonies is found to be low and therefore making it difficult to grow under the laboratory conditions (Davis et al., 2005). The study done by Davis et al. (2005), looked at various factors affecting the growth of these soil

bacteria and the factors include the size of the inoculums, the period in which the bacteria are incubated and the media in which they are grown. Therefore, to secure more colonies, the incubation period of soil microorganisms, including slow growers, ranges between 7 to 14 days.

Streptomyces spp. are Gram-positive bacteria that inhabit the soil and have been known to represent the most antibiotic-producing bacterial species in the history of science (Ceylan et al., 2008; George et al., 2010; Hossain & Rahman, 2014). Like most other bacteria they are known to be non-photosynthetic, meaning that their source of energy is derived from the oxidation of organic compounds (Hasani et al., 2014). *Streptomyces* spp. are also characterized on the richness of their guanine and cytosine content in their DNA sequences, ranging from 35 % to 75 % (Ceylan et al., 2008; Wu et al., 2012). They grow under moderate temperatures of between 10 °C – 37 °C, whereas some thrive at high temperature that range from 45 °C – 55 °C (Hasani et al., 2014). Although *Streptomyces* spp. play a crucial role in medicine and agriculture, there are a few that pose a threat to the health of humans and plants (Seipke et al., 2012). Those that cause diseases in plants are more abundant than those in humans. The first bacterial antibiotic, streptomycin, was discovered by Waksman in 1943 produced by *S. griseus* (Seipke et al., 2012). Streptomycin is a bactericidal and effective aminoglycoside antibiotic against Gram-positive bacteria. Most of the antibiotics that are found today are from *Streptomyces* spp. and almost all of them have become ineffective over the years (Procópio et al., 2012). The ineffectiveness of these antibiotics is the result of susceptible pathogens acquiring ways to resist the actions of the antibiotics (George et al., 2010). This study focused on isolating *Streptomyces* spp. that have the potential of producing antibacterial antibiotics.

1.3. Antibiotic resistance

The problem of antibiotic resistance has remained a substantive issue over the years and with research it was discovered that resistance to antibiotics can occur suddenly after the introduction of the antibiotic. Penicillin was initially given to patients in 1941 and followed by its resistance in 1942. Another instance was seen with methicillin that was introduced in 1960 and methicillin-resistant bacteria were reported in 1961 (Landecker, 2015). Some of the bacteria have become resistant to more than one antibiotic, and therefore are called multi-resistant bacteria. Managing such conditions is even more difficult to deal with. Bacteria are quick adaptors to external environments, especially if the newly introduced environment limits their chances of survival. Their ability to adapt to such external environments enables them to enhance/activate survival mechanisms that will allow them to grow better and the possible way of achieving this is by adjusting how they regulate their gene expression (Kiel et al., 2010; Guo & Gross, 2014). According to Hottes et al. (2013) the loss of function mutations in certain bacterial genes, particularly those targeted by the changes in the environment, represent possible ways of how the bacterium can avoid harsh environmental challenges like temperature, lack of nutrients, etc. Interestingly, these mutations that give resistance in some bacteria are thought to have existed long before the bacterium was ever exposed to an antibiotic. So, this proves that therefore, bacterial resistance has been around long before the discovery of antibiotics (Davies & Davies, 2010).

Another conventional way in which bacteria become resistant is through horizontal gene transfer (HGT) (Figure 1.2). HGT is a process in which one organism acquires new genetic material from another organism rather from parent to offspring (Heuer & Smalla, 2007; Wiedenbeck & Cohan,

2011). Of the three different modes of HGT, conjugation is the most common in which bacteria become resistant to antibiotics (Davies & Davies, 2010; Giedraitienė et al., 2011; Greenwood, 2012). Conjugation is when one bacterial cell directly transfers its plasmid DNA to another bacterial cell (Levy, 2002; Heuer & Smalla, 2007; Giedraitienė et al., 2011; Greenwood, 2012; Arber, 2014). Transduction occurs when a bacteriophage transfers DNA material from one bacterium to another whereas in transformation one bacterial cell takes up external DNA from another bacterial cell (Havarstein, 1998; Tenover, 2006). Horizontal gene transfer can occur within either the same or different genera (Havarstein, 1998).

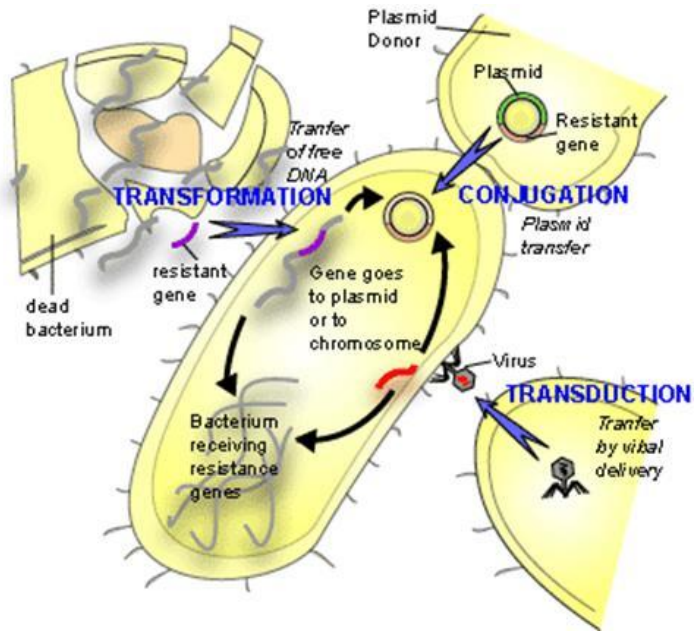

Figure 1.2: Bacteria use different methods of horizontal gene transfer as a mechanism of preventing the activity of antibiotics. These methods include transformation, conjugation and transduction (Tadakamalla & Evans, 2014).

Resistance acquired due to plasmid transfer occurs more rapidly than resistance that involves genes on a chromosome, this is because chromosomal mutations are exceedingly rare and if they do occur, they occur at a very moderate rate (Giedraitienė et al., 2011). For chromosomal mutations that results in resistance to take place, several genes are required to change before a desirable effect is achieved. Martinez & Baquero (2000) and Giedraitienė et al. (2011) mentioned that for *Escherichia coli* to become resistant to quinolone, about seven nucleotides in the gyrA gene had to be changed to be quinolone resistant and in the parC gene, changes in only three nucleotides conferred quinolone resistance. This therefore means that for the mutation to occur in the gyrA gene is less likely than on the parC gene (Martinez & Baquero, 2000; Giedraitienė et al., 2011).

Another highly studied concept that plays a role in the development of resistance is biofilm production (Figures 1.3 and 1.4). Many bacteria are likely to grow in biofilms which consist of a community of bacterial and attached cells at the surface (Vu et al., 2009). These communities of bacterial cells 'work together' and can be from the same or different species. The bacteria produce polysaccharides, proteins, and amino acids which provide them with support and protection from invaders or stressors (Hughes & Anderson, 2017). Biofilms are known to play a role in the antibiotic resistance of most bacteria (e.g. *Staphylococcus epidermis* and *Pseudomonas aeruginosa*) and it was also confirmed that when a sensitive strain was grown in bacterial biofilm, it became resistant to the exposed antibiotic faster than when it was grown alone (Stewart, 2002; Wu et al., 2015). But once the bacterium was removed from the bacterial biofilms, the bacterium immediately restored its susceptibility to the antibiotic (Stewart, 2002). Once these biofilms have formed, it becomes a challenge to remove them and therefore being the reason why they are not easily killed by antibiotics or immune response (Wu et al., 2015). In cystic fibrosis, *P. aeruginosa* (and other infection-causing pathogens) has been seen to form biofilms during infection which

leads to the difficulties of treating the infection with the prescribed antibiotics (Skindersoe et al., 2008; Jain et al., 2013; Wu et al., 2015).

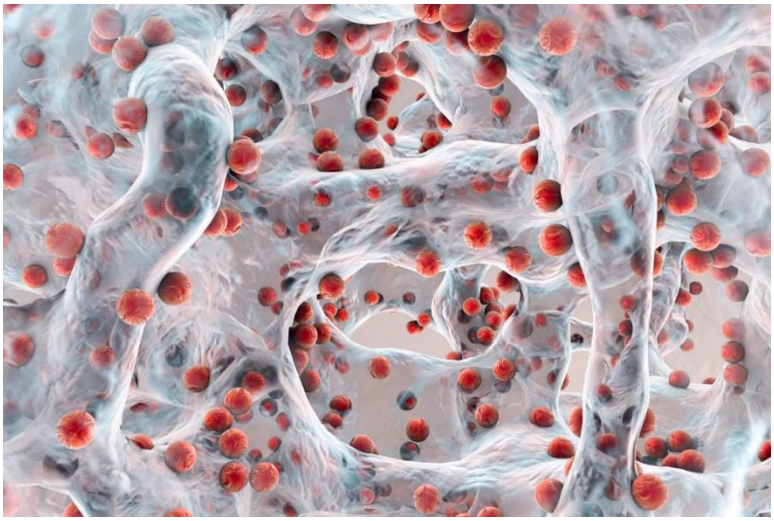

Figure 1.3: Bacterial biofilms formed by planktonic bacteria to protect themselves from the activity of antibiotic agents (Ebrey et al., 2004).

The production of antibiotics by bacteria is regulated by quorum sensing which is defined as a mode of communication between bacterial species to regulate their gene expression in response to an irregular population cell density. Other processes that are regulated by quorum sensing include bioluminescence, sporulation, competence, biofilm formation, and virulence factor secretion (Rutherford and Bossler, 2012). Quorum sensing is made possible by the production of small signalling molecules known as auto-inducers which are produced by members of the bacterial population. Population cell density controls the concentration the signalling molecules produced; at low cell density, the production of these molecules is low and not enough to trigger detection

and at high bacterial density, the molecules are produced in high amounts and therefore detection and response is possible (Rutherford and Bossler, 2012). The figure below explains how population cell density affects the effectiveness of the produced antibiotic. To ensure that the antibiotic produced will be having high inhibitory effects, the bacterial community must reach a certain concentration to be able to eradicate the targeted species. In Figure 1.4, the red line indicates the production of the antibiotic without being affected by population density.

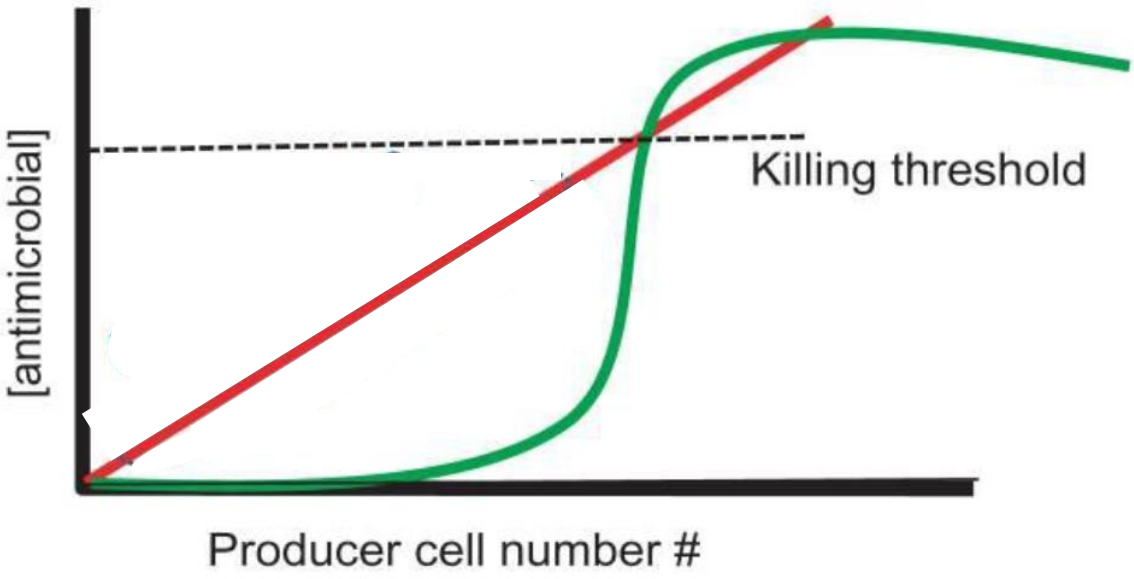

Figure 1.4: A graph showing how quorum sensing can mediate the production and effectiveness of antibiotics (Hibbing et al., 2010).

It is said that when the population size is low, the effectiveness of the antibiotics is less, and this therefore gives the targeted bacterial population time to alter their gene expression (or mutation) and being able to survive in the presence of that antibiotic. The green line shows how the

production of antibiotics is mediated by quorum sensing and this usually occurs when a population density is reached. The antibiotic is then produced and released to the environment and killing or inhibiting the targeted bacterial population without it being able to have enough time to protect them, thus it is less likely for them to gain resistance (Hibbing et al., 2010).

1.4. Economic impact of antibiotic resistance

Antibiotic resistance, particularly that of multi-drug resistant microorganisms has an enormous impact on human and animal health and on the economy of a country (Bennett, 2008; Hossain & Rahman, 2014). Many of these bacteria may have been resistant to one antibiotic but with the exposure of different antibiotics or even growing in the presence of other resistant strains have gained other resistance mechanisms. The World Health Organization has explained that the cost used to cover the people suffering from resistant infections is exceedingly higher than that for patients with non-resistant infections. This is because those resistant infections require longer hospitalization and more drugs and therefore leading to more deaths (WHO, 2016).

With many antibiotics becoming ineffective against life-threatening bacterial infections, there is a need to develop drugs that would help reduce these resistant strains (Hossain & Rahman, 2014). An example of an antibiotic used to treat such infections is colistin, also known as polymyxin E, produced by certain strains of *Bacillus polymyxa* (Falagas et al., 2005). The mode of action of this antibiotic is through the disruption of the bacterial cell membrane (Falagas et al., 2005; Gupta et al., 2009; Yahav et al., 2012). The antibiotic was initially discovered in the 1940s and it was later

determined that it produced major side effects (kidney toxicity) on patients and therefore its use was no longer supported (Cosgrove & Carmeli, 2003; Falagas et al., 2005; Gupta et al., 2009). After many years, colistin was re-introduced as last line of treatment to treat carbapenem-resistant Gram-negative bacteria (*Acinetobacter* spp., *Pseudomonas aeruginosa*, *Klebsiella* spp. and *Enterobacter* spp.) (Gupta et al., 2009; Yahav et al., 2012). It is evident that discovering new antibiotics that are 100 % reliable (e.g. no side effects or resistance) is going to be a challenge.

The major problems surrounding resistance are the factors that contribute to its increase. The inappropriate usage of antibiotics is a concern, whereby patients do not complete their treatment course; do not follow the guidelines specified by the physician (misuse or overuse); the fact that some antibiotics are commercially available at the pharmacy where the public can purchase without the doctor's prescription; using antibiotics for viral related infections (like the flu or colds); prolonged stays at the hospital are also classified as a way in which patients can be infected with hospital-acquired infections caused by multi-resistant strains (e.g. *Staphylococci*) (Levy, 2002; Institute of Medicine USA, 2013; Castro-Sánchez et al., 2016; WHO, 2016).

1.5. New antibiotics

The discovery and development of new antibiotics have been one of the most challenging problems. This contributes to the increase and demand to develop new antibiotics that do not confer resistance (Ceylan et al., 2008; Hamamoto et al., 2014). One example that was recently discovered but still in clinical trials is an antibiotic called teixobactin that was isolated from a Gram-negative

soil bacterium known as *Elefteria terrae* (Ling et al., 2015; Piddock, 2015). The bacterium producing this antibiotic was isolated from the soil with the use of an isolation chip (Ichip). This technique was used to isolate bacteria that cannot be grown under normal laboratory conditions. The antibiotic is known to be effective against Gram-positive bacteria like *Clostridium difficile*, *Bacillus anthraci* and the multi-drug resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Mycobacterium tuberculosis* (Arias and Murray, 2015; Ling et al., 2015). The effectiveness of the antibiotic was tested on mice infected with MRSA and *M. tuberculosis* and it was found that none of them conferred resistance against the antibiotic. Teixobactin is not effective against Gram-negative bacteria because these bacteria do not contain a target site to which the antibiotic acts on (Arias and Murray, 2015). Piddock (2015) stated that *Escherichia coli* was used as a test bacterium and was found to grow in the presence of the antibiotic. The reason behind the observation was that *E. coli* could have resisted the entrance of the antibiotic due to the thickness of the peptidoglycan it contains (which is thin in Gram-positive and was therefore able to penetrate). Another reason could have been that *E coli* might have utilized one of its resistant mechanisms to flush out the antibiotic (Piddock, 2015). In other words, the antibiotic has a narrow spectrum as it can kill only one group of bacteria. Figure 1.5 below shows the mode of action of teixobactin.

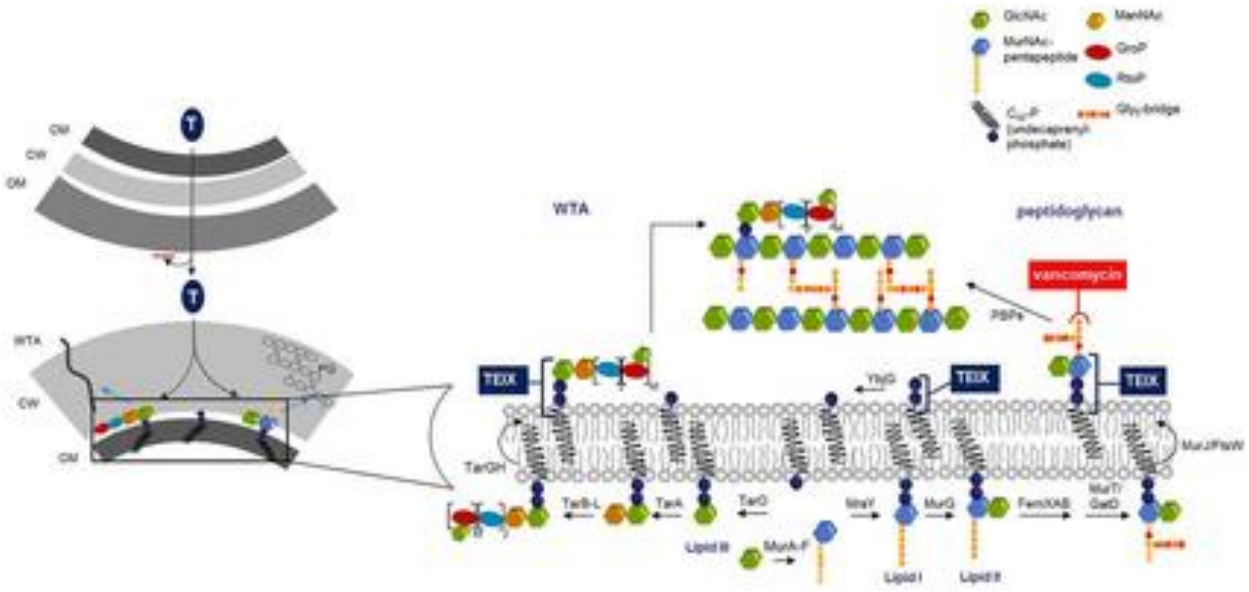

Figure 1.5: Mechanism of action of teixobactin by preventing the formation of the bacteria cell wall (Ling et al., 2015).

Teixobactin is known to prevent the formation of Gram-positive bacteria's peptidoglycan that is found in the cell walls. The antibiotic binds to the peptidoglycan fatty acids molecules lipid II and III which are of key importance in the formation of Gram-positive bacteria's cell walls and this binding therefore allows the antibiotic to kill the bacteria (Ling et al., 2015; Piddock, 2015).

1.6. Aims and objectives of the study

Motivation

The main aim of the study was to isolate and identify a new antibiotic from soil of the Namibian desert. The Namibian desert was chosen as a site of soil collection because of its well-known fairy circles which are known to inhibit plant growth, and perhaps microbial growth (Meyer et al., 2015). The soil from these fairy circles were chosen because there are no published reports on the potential of the microbial populations from Namibian fairy circles to produce antibiotics.

Problem statement

The problem of antibiotic resistance had become one of the major setbacks in the history of medicine. Therefore, the discovery of new antibiotics had been a challenge for many years and this was because bacteria were evolving rapidly and became resistant to almost all known antibiotics. Today, pharmaceutical companies have developed ways to manipulate the structures of old antibiotics which results in the altered mode of action that will make them unrecognizable by the previously resistant-strains.

Aims

- The main aim was to isolate a new *Streptomyces* strain from Namibian soil samples that possesses antagonistic activity against bacteria.

- Extract and identify the inhibitory compound produced by the isolated *Streptomyces* strain by using different biological and chemical methods.

Objectives

- Isolation of soil microorganisms by means of serial dilution method.
- Preliminary screening of selected *Streptomyces* spp. to produce antibiotics using the cross-streak method on Mueller Hinton agar media.
- Identification of soil isolates using 16S rRNA sequencing and a bio-editing program for editing the sequences.
- Extraction and purification of crude bacterial extract using ethyl acetate,
- Direct TLC bio-autography: testing the extract for antibacterial activity.
- Structure determination of antibacterial compound using ^{1}H NMR and GS-MS.

1.7. Hypothesis

A new *Streptomyces* strain will be isolated from the Namib soil that exhibit good antagonistic activity against selected bacteria.

1.8. References

1. Aminov, R. I. (2010). A Brief History of the Antibiotic Era: Lessons Learned and Challenges for the Future. Frontiers in Microbiology, 1: 134.
2. Amyes, S. (2010). Antibacterial Chemotherapy: Theory, Problems, and Practice, OUP Oxford.
3. Arber, W. (2014). Horizontal Gene Transfer among Bacteria and Its Role in Biological Evolution. Life: Open Access Journal, 4 (2): 217–224.
4. Arias, C. A., & Murray, B. E. (2015). A New Antibiotic and the Evolution of Resistance. The New England Journal of Medicine, 372 (12): 1168–1170.
5. Asha-Devi, N. K., Rajendran, R., & Karthik Sundaram, S. (2011). Isolation and characterization of bioactive compounds from marine bacteria. Indian Journal of Natural Products and Resources, 2 (1): 59-64.
6. Bbosa, G. S., Mwebaza, N., Odda, J., Kyegombe, D. B., & Ntale, M. (2014). Antibiotics/antibacterial drug use, their marketing and promotion during the post-antibiotic golden age and their role in emergence of bacterial resistance. Health, 6 (5): 410-425.
7. Bennett, P. M. (2008). Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. British Journal of Pharmacology, 153 (1): 347-357.
8. Castro-Sánchez, E., Moore, L. S. P., Husson, F., & Holmes, A. H. (2016). What are the factors driving antimicrobial resistance? Perspectives from a public event in London, England. BMC Infectious Diseases, 16 (1): 465.

9. Ceylan, O., Okmen, G., & Ugur, A. (2008). Isolation of soil *Streptomyces* as source antibiotics active against antibiotic-resistant bacteria. EurAsia Journal of Biosciences, 2: 73-82.
10. Cosgrove, S. E., & Carmeli, Y. (2003). The impact of antimicrobial resistance on health and economic outcomes. Antimicrobial resistance, 36: 1433-1437.
11. Davies, J., & Davies, D. (2010). Origins and Evolution of Antibiotic Resistance. Microbiology and Molecular Biology Reviews: MMBR, 74 (3): 417–433.
12. Davis, K. E. R., Joseph, S. J., & Hanssen, P. H. (2005). Effects of Growth Medium, Inoculum Size, and Incubation Time on Culturability and Isolation of Soil Bacteria. Applied and Environmental Microbiology, 71: 826–834.
13. Ebrey, R., Shea-Hamilton, M., Cairns, G., & Lappin-Scott, H. M. (2004). Biofilms and Hospital-Acquired Infections, p 294-313. In Ghannoum, M., and O'Toole, G., (ed), Microbial Biofilms. ASM Press, Washington, DC.
14. Etebu, E., & Arikekpar, I. (2016). Antibiotics: Classification and mechanisms of action with emphasis on molecular perspectives. International Journal of Applied Microbiology and Biotechnology Research, 4: 90-101.
15. Fair, R. J., & Tor, Y. (2014). Antibiotics and Bacterial Resistance in the 21st Century. Perspectives in Medicinal Chemistry, 6: 25–64.
16. Falagas, M. E., Kasiakou, S. K., & Saravolatz, L. D. (2005). Colistin: The Revival of Polymyxins for the Management of Multidrug-Resistant Gram-negative Bacterial Infections. Clinical Infectious Diseases, 40 (9): 1333–1341
17. Finch, R. G., Greenwood, D., Whitley, R. J., & Norrby, S. R. (2010). Antibiotic and Chemotherapy, E-Book, Elsevier Health Sciences.

18. Fischbach, M. A., & Walsh, C. T. (2009). Antibiotics for Emerging Pathogens. Science, 325: 1089-1094.

19. Forsberg, K. J., Reyes, A., Wang, B., Selleck, E. M., Sommer, M. O. A., & Dantas, G. (2012). The Shared Antibiotic Resistome of Soil Bacteria and Human Pathogens. Science, 337: 1107-1111.

20. George, M., George, G., & Hatha, A. A. M. (2010). Diversity and antibacterial activity of actinomycetes from wetland soil. The South Pacific Journal of Natural and Applied Sciences. 28: 52-57.

21. Giedraitienė, A., Vitkauskienė, A., Naginienė, R., & Pavilonis, A. (2011). Antibiotic Resistance Mechanisms of Clinically Important Bacteria. Medicina (Kaunas), 47 (3): 137–146.

22. Greenwood, D. (2012). Medical Microbiology, with student consult online access, 18: Medical Microbiology, Churchill Livingstone.

23. Guo, M. S., & Gross, C. A. (2014). Stress induced remodelling of the bacterial proteome. Current Biology: CB, 24 (10): R424–R434.

24. Gupta, S., Govil, D., Kakar, P. N., Prakash, O., Arora, D., Das, Govil, P., & Malhotra, A. (2009). Colistin and polymyxin B: A re-emergence. Indian Journal of Critical Care Medicine: Peer-Reviewed. Official Publication of Indian Society of Critical Care Medicine, 13 (2): 49–53.

25. Hamamoto, H., Urai, M., Ishii, K., Yasukawa, J., Paudel, A., Murai, M., Kaji, T., Kuranaga, T., Hamase, K., Katsu, T., Su, J., Adachi, T., Uchida, R., Tomoda, H., Yamada, M., Souma, M., Kurihara, H., Inoue, M., & Sekimizu, K. (2014). "Lysocin E is a new antibiotic that targets menaquinone in the bacterial membrane." Nature Chemical Biology, 11: 127.

26. Hasani, A., Kariminik, A., & Issazadeh, K. (2014). Streptomycetes: Characteristics and Their Antimicrobial Activities. International journal of Advanced Biological and Biomedical Research, 2 (1): 63-75.
27. Havarstein, L. S. (1998). Bacterial gene transfer by Natural genetic transformation. APMIS, 106: 43-46.
28. Heuer, H., & Smalla, K. (2007). Horizontal gene transfer between bacteria. Environmental Biosafety Research, 6 (1-2): 3-13.
29. Hibbing, M, E., Fuqua, C., Parsek, M. R., & Peterson, S. B. (2010). Bacterial competition: surviving and thriving in the microbial jungle. Nature Reviews Microbiology, 8: 15-25.
30. Hossain, N., & Rahman, M. (2014). Antagonistic activity of antibiotic producing *Streptomyces* sp. against fish and human pathogenic bacteria. Brazilian Archives of Biology and Technology, 57 (2): 233-237.
31. Hottes, A. K., Freddolino, P. L., Khare, A., Donnell, Z. N., Liu, J. C., & Tavazoie, S. (2013). Bacterial Adaptation through Loss of Function. PLoS Genetics, 9 (7): 1–13.
32. Hughes, D., & Anderson, D. I. (2017). Environmental and genetic modulation of the phenotypic expression of antibiotic resistance. FEMS Microbiology Reviews, 41: 374–391.
33. Institute of Medicine (US) Forum on Emerging Infections: Knobler, S. L., Lemon, S. M., Najafi, M., & Burroughs, T., Editors. The Resistance Phenomenon in Microbes and Infectious Disease Vectors: Implications for Human Health and Strategies for Containment: Workshop Summary. Washington (DC): National Academies Press (US). (2003). 5, Factors Contributing to the Emergence of Resistance. Available from: https://www.ncbi.nlm.nih.gov/books/NBK97126/

34. Jain, K., Parida, S., Mangwani, N., Dash, H. R., & Das, S. (2013). Isolation and characterization of biofilm-forming bacteria and associated extracellular polymeric substances from oral cavity. Annals of Microbiology, 63: 1553-1562.

35. Kiel, C., Yus, E., & Serrane, L. (2010). Engineering Signal Transduction Pathways. Cell, 140: 33 – 47.

36. Landecker, H. (2015). Antibiotic Resistance and the Biology of History. Body & Society, 1-34.

37. Levy, S. B. (2002). Factors impacting on the problem of antibiotic resistance. Journal of Antimicrobial Chemotherapy, 49: 25–30.

38. Ling, L. L., Schneider, T., Peoples, A. J., Spoering, A. L., Engels, I., Conlon, B. P., Mueller, A., Schäberle, T. F., Hughes, D. E., Epstein, S., Jones, M., Lazarides, L., Steadman, V. A., Cohen, D. R., Felix, C. R., Fetterman, K. A., Millett, W. P., Nitti, A. G., Zullo, A. M., Chen, C., & Lewis, K. (2015). A new antibiotic kills pathogen without detectable resistance. Nature, 517: 455–459.

39. Martinez, J. L., & Baquero, F. (2000). Mutation Frequencies and Antibiotic Resistance. Antimicrobial Agents and Chemotherapy, 44 (7): 1771–1777.

40. Meyer, J. J. M., Senejoux, F., Heyman, H. M., Meyer, N. L., & Meyer, M. A. (2015). The occurrence of triterpenoids from Euphorbia gummifera inside the fairy circles of Garub in Southern Namibian pro-desert. South African Journal of Botany, 98: 10-15.

41. Mutsuzaki, K. (2009). Control of cell selectivity of antimicrobial polypeptides. Biochimica et Biophysica Acta, 1788: 1687–1692.

42. Nesme, J., & Simonet, P. (2015). The soil resistome: a critical review on antibiotic resistance origins, ecology and dissemination potential in telluric bacteria. Environmental Microbiology, 17: 913–930.
43. Paul, E. A. (2014). Soil Microbiology, Ecology and Biochemistry, Elsevier Science.
44. Piddock, L. J. V. (2015). Teixobactin, the first of a new class of antibiotics discovered by iChip technology? Journal of Antimicrobial Chemotherapy, 70: 2679–2680.
45. Pommerville, J. C. (2010). Alcamo's Fundamentals of Microbiology. Jones & Bartlett Learning.
46. Procópio, R. E. d. L., Silva, I. R. d., Martins, M. K., Azevedo, J. L. d., & Araújo, J. M. d., (2012). Antibiotics produced by *Streptomyces*. Brazilian Journal of Infectious Diseases 16: 466-471.
47. Raaijmakers, J. M., Vlami, M., & de Souza, J. T. (2002). Antibiotic production by bacterial biocontrol agents. Antonie Van Leeuwenhoek, 81: 537-547.
48. Rutherford, S. T., & Bossler, B. L. (2012). Bacterial Quorum Sensing: It's Role in Virulence and Possibilities for Its Control. Cold spring harbor perspectives in medicine, 2: a012427.
49. Sandhya, M. V. S., Ramyakrishna, E., Divya, P., Kumar, A. P., Rajkumar, K., Yazein, E., & Burgula, S. (2015). Isolation of antibiotic producing bacteria from soil. International Journal of Applied Biology and Pharmaceutical Technology, 1: 46-51.
50. Scholar, E. M., & Pratt, W. B. (2000). The antimicrobial drugs. Oxford University Press.
51. Seipke, R. F., Kaltenpoth, M., & Hutchings, M. I. (2012). *Streptomyces* as symbionts: an emerging and widespread theme? FEMS Microbiology Review, 36: 862–876.
52. Skindersoe, M. E., Alhede, M., Phipps, R., Yang, L., Jensen, P. O., Rasmussen, T. B., Bjarnsholt, T., Tolker-Nielsen, T., Høiby, N., & Givskov, M. (2008). Effects of Antibiotics

on Quorum Sensing in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy, 52: 3648–3663.

53. Stewart, P. S. (2002). Mechanisms of antibiotic resistance in bacterial biofilms. International Journal of Medical Microbiology, 292: 107-113.
54. Tadakamalla, P., & Evans, J. (2014). Antibiotic Resistance: MRSA in Dentistry. International Dental Journal of Student's Research 2 (3): 4-7.
55. Tamvakis, I. (2014). The persistent enigma of the Namibian desert fairy circles. 10.13140/2.1.5040.4167.
56. Tan, S. Y., & Tatsumura, Y. (2015). Alexander Fleming (1881–1955): Discoverer of penicillin. Singapore Medical Journal, 56 (7): 366–367.
57. Tenover, F. C. (2006). Mechanisms of antimicrobial resistance in bacteria. American Journal of Infection Control, 34: S3-S10.
58. Vu, B., Chen, M., Crawford, R. J., & Ivanova, E. P. (2009). Bacterial Extracellular Polysaccharides Involved in Biofilm Formation. Molecules, 14 (7): 2535-2554
59. Wiedenbeck, J., & Cohan, F. M. (2011). Origins of bacterial diversity through horizontal gene transfer and adaptation to new ecological niches. FEMS Microbiological Reviews, 35 (5): 957-976.
60. World Health Organization. (2016). Antimicrobial resistance.
61. Wu, H., Moser, C., Wang, H. Z., Høiby, N., & Song, Z. J. (2015). Strategies for combating bacterial biofilm infections. International Journal of Oral Science, 7 (1): 1–7.
62. Wu, H., Zhang, Z., Hu, S., & Yu, J. (2012). On the molecular mechanism of GC content variation among eubacterial genomes. Biology Direct, 7 (2): 1-16.

63. Yahav, D., Farbman, L., Leibovici, L., & Paul, M. (2012). Colistin: new lessons on an old antibiotic. Clinical Microbiology and Infection, 18: 18–29.

CHAPTER 2: ISOLATION, IDENTIFICATION AND SCREENING OF *STREPTOMYCES* SPP. FOR ANTIBIOTIC ACTIVITY

2.1. Introduction

2.1.1. Screening for bacterial antibiotics production

The discovery and development of antibiotics is one of the most fascinating and complex studies in the medical industry. Microorganisms, particularly the soil-borne, are known to possess the potential to produce antibiotics that can be used as for human infections and in agriculture (Ceylan et al., 2008; Hossain & Rahman, 2014). The isolation and purification of these secondary metabolites are extremely lengthy procedures and, obtaining the tangible product to be used in treatments takes about 10 to 15 years. The very first step in the isolation and identification of novel antibiotics is testing the antagonistic activity of the bacteria involved. The standard method that enables such an activity is known as the cross-streak method and it involves the bacteria that possess the potential to produce antibiotics and those tested against it (Balouiri et al., 2016). The bacterium with the ability to produce antibiotic compounds will inhibit or suppress the growth of those bacteria tested against it and this can be shown in a Petri dish (Velho-Pereira & Kamat, 2011). The bacterium with the potential to produce antibiotics will be streaked at the centre on the agar plate and incubated for a week and the test bacteria are then later streaked perpendicular to the one at the centre. After a day or more of incubation, the bacterium at the centre might have excreted secondary metabolites in the solid media so preventing the growth of the susceptible test bacteria and this can be seen by zones of inhibition (Balouiri et al., 2016).

The bacterial samples (collected from soil or water or other sources) can be isolated using the serial dilution method. The method allows us to estimate the bacterial colony size, and this can be observed in terms of the number of colonies at a given dilution (Ben-David & Davidson, 2014).

2.1.2. Identification of bacteria by means of 16S rRNA sequencing

Several techniques are used to identify the genus and species of bacteria (Janda & Abbott, 2007). Identification and classification of organisms can be based either on phenotypic or genotypic features (Figure 2.1.). Those used to identify/classify bacteria based on their phenotypes (physical attributes) are known to be less reliable because it merely provides morphological information about the organism hence needs to be incorporated with the genotypic identification (Clarridge, 2004; Donelli et al., 2013). Methods that solely rely on phenotypic identification include Gram staining, colony morphology and biochemical tests.

The genotypic level of identification provides us with more molecular-based information that includes 16S rRNA sequencing and microarrays. The 16S rRNA sequencing involves the 16S rRNA gene that forms a part of a small ribosomal subunit of the ribosome. The bacterial ribosome is a large compartmental site producing proteins and is found in the mitochondria of all living cells. It is made up of two subunits which are the small ribosomal subunit (30S) and large ribosomal subunit (50S). The 30S subunit only composes of a 16S rRNA and the 50S consists of 5S RNA and 23S RNA subunits (Clemons et al., 1999). The 16S rRNA gene (Figure 2.2) is a small portion of a DNA that is present in all bacteria and archaea and has unique features for both identification and classification of these organisms (Janda & Abbott, 2007). The unique characteristics that

enables this gene to be used as an identification tool is based on its small size of 1 500 bp and therefore giving it the advantage of being easier and faster to sequence and analyse compared to the other bacterial genes.

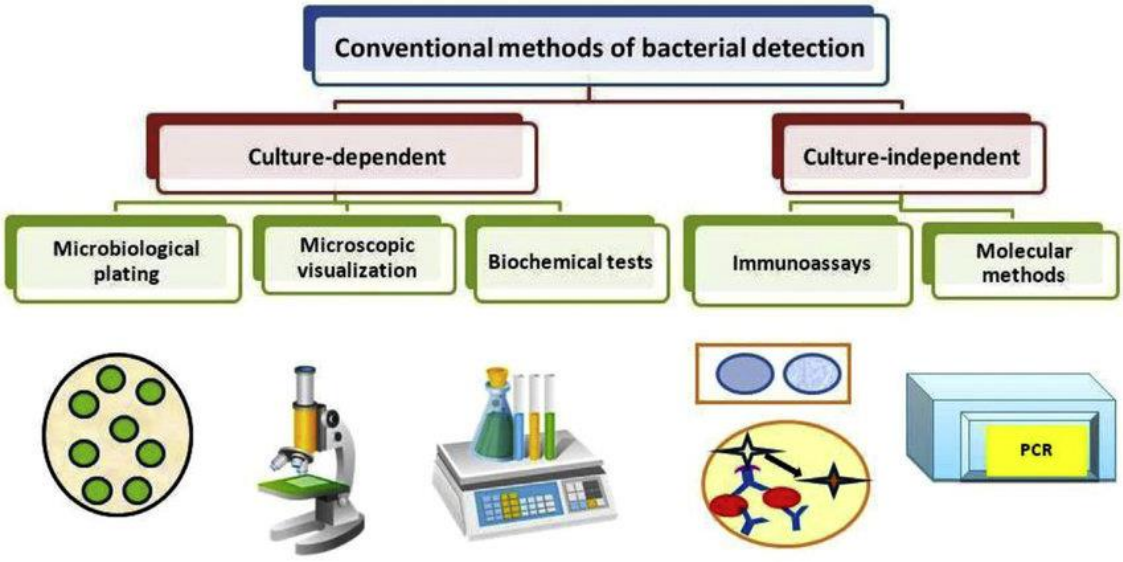

Figure 2.1: Different methods employed for identification of bacteria (Bhardwaj et al., 2017).

The gene is also known to be highly conserved and this is because the gene does not mutate over a long period of time. This gene is known as an orthologous gene meaning that it performs the same function throughout evolution or history and therefore enables us to identify different species that have a common ancestor. And for this reason, 16S rRNA sequencing is better known for its ability to identify newly isolated pathogenic bacteria and bacteria that cannot be cultured in the laboratory (Baker et al., 2003; Clarridge, 2004).

The primers that were used in this study were the two universal primers, the forward primer 27F and the reverse primer 1492R. The reason why these primers were considered as universal was because they are known to align very well to the highly conserved regions (Figure 2.2) of the 16S rRNA gene and these conserved regions are shared amongst many bacterial species (Frank et al., 2008).

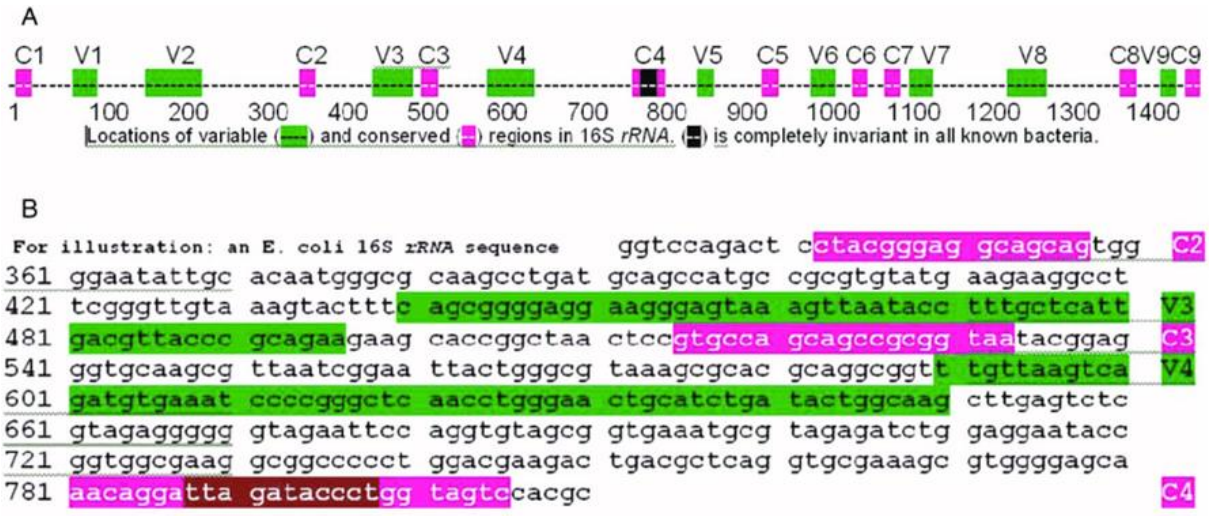

Figure 2.2: The 16S rRNA gene sequence of *E. coli* showing different sites of the conserved regions targeted by the universal primers. Conserved regions are highlighted in pink and variable regions are highlighted in green (Rom et al., 2011).

In the study conducted by Frank et al. (2008) they explained that the forward 27F primer binds the positions from 8 to 27 and the reverse primer 1492R amplifies from 1 492 to 1 507 and this was observed from the 16S rRNA of *E. coli*. However, it has been mentioned that these two primers were not as universal as the name suggests, and this was because they were unable to align to the 16S rRNA gene of some bacteria (Baker et al., 2003; Rom et al., 2011; Fredriksson et al., 2013).

This does not mean that the 27F and 1492R primers cannot be used for sequencing anymore but if they do not work, there are many other primers that are used and align effectively to the gene.

The soil used for isolation was collected from Namibian desert sand. The soil samples were collected from the outside and inside of fairy circles and from underneath dead *Euphorbia gummifera* plants (Meyer et al., 2015). The aim of this chapter was to isolate and identify *Streptomyces* spp. that have antibacterial activity against other bacteria. The selected test bacteria were the four known pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Proteus vulgaris* and *Achromobacter xylosoxidans*) and two bacteria isolated from the Namibian soil. The soil isolates were identified using the 16S rRNA sequencing technique.

2.2. Materials and methods

2.2.1. Isolation of soil isolates

2.2.1.1. Sample collection

Soil samples were collected from the inside and outside of fairy circles and from underneath dead *E. gummifera* plants in Garub, Namibia (coordinates 26.5953° S, 15.9436° E). The samples were stored into 250 ml plastic bottles for further studies.

2.2.1.2. Isolation of soil microbes

Soil microorganisms were isolated by means of the serial dilution method. The stock solution was prepared by diluting 1 g of each soil sample in 9 ml of distilled water and vigorously vortexing it for 10 seconds. From each stock solution, 1 ml was used to make the final volumes of 10^{-1} to 10^{-5} ml by serial dilution. An amount of 0.1 ml of each serial diluted sample was transferred into tryptic soy agar plates by means of the spread plate method (Figure 2.3). The plates were labelled and incubated for 7 days at 25 °C. The agar tryptic soy agar was prepared as follows: 15 g of tryptic soy agar was added into 1 000 ml of distilled water. The solution was autoclaved at 121 °C for 1 hour. The medium was left to cool off and poured into 30 Petri dishes until solid.

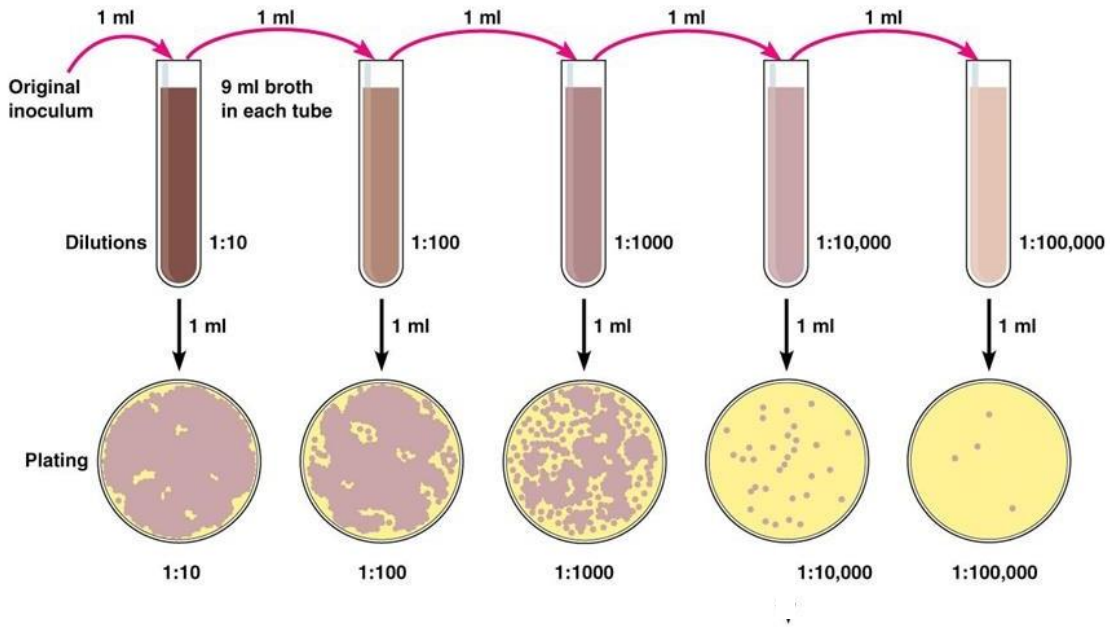

Figure 2.3: Serial dilution method used to isolate bacterial colonies from soil samples of the Namibian fairy circles (Society for General Microbiology, 2003).

2.2.2. Identification of soil isolates

2.2.2.1. DNA extraction

DNA extraction was conducted using the Quick-gDNA^{TM} Mini-prep kit purchased from Inqaba Biotech Ltd. Bacterial colonies were transferred into tubes containing 700 µl of genomic lysis buffer and thoroughly vortexed. The mixture was then transferred into a Zymo-spin 11C column in collection tubes and centrifuged at 10 000 rpm for 1 minute. After centrifugation, 200 µl of DNA pre-wash buffer was added to the spin column tubes and centrifuged at 10 000 rpm for 1 minute. The spin columns were transferred to clean 1.5 ml Eppendorf tubes (this was because the

spin column tubes were small and did not fit in the centrifuge that was used), 75 µl of DNA elution buffer was added to the spin columns, incubated at room temperature for 2-3 minutes and then centrifuged at 14 000 rpm for 30 seconds to elute the DNA. The concentrations and qualities of the extracted DNA were measured using a NanoDrop ™ 2 000 spectrophotometer. To ensure the presence of DNA, a 1.2 % agarose electrophoresis gel was prepared by heating 0.9 g agarose powder and 90 ml tris-acetate-EDTA (TAE) in beaker and adding 0.5 µg/ml ethidium bromide (EtBr). A volume of 5 µl of each DNA sample was run on agarose gel at 90 V and 400 mA for 40 minutes. The agarose gel was visualised under UV light using a Molecular Imager ® Gel Doc XR System that is equipped with the Image Lab ™ software.

2.2.2.2. Agarose gel electrophoresis

A master mix for one reaction tube was prepared in a 1.5 ml Eppendorf tube containing 2.5 µl of buffer, 2.0 µl of dNTPs, 2.0 µl of $MgCl_2$, 0.5 µl of forward primer (27F - 5'-AGAGTTTGATCCTGGCTCAG-3'), 0.5 µl of reverse primer (1492R - 5'-GGTTACCTTGTTACGACTT-3'), 16.85 µl of nuclease free water (NF-H_2O), 0.15 µl of Taq polymerase and 0.5 µl of extracted bacterial DNA (18.9 ng/ µl). The reactions were always mixed and kept on ice. The reactions were then transferred into PCR tubes and spun down in a mini-centrifuge at maximum rpm to remove bubbles. The tubes were placed in a PCR machine with the following PCR reaction cycles: initial denaturation at 94 °C for 3 minutes followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 1 minute, extension at 72 °C for 1 minute, and a final extension at 72 °C for 5 minutes. The positive control reaction contained the 16S rRNA gene from *Staphylococcus aureus* and the negative control reaction contained all the

reagents except the DNA sample. The primers were purchased at Inqaba Biotech Ltd and were diluted 10 times.

A 1.2 % agarose electrophoresis gel was prepared by heating 0.9 g agarose powder and 90 ml TDA in a beaker and added 0.5 µg/ml ethidium bromide (EtBr). A volume of 5 µl of PCR products were run on agarose gel at 90 V and 400 mA for 40 minutes. The agarose gel was visualised under UV light using a Molecular Imager ® Gel Doc XR System that is equipped with the Image Lab ™ software. The PCR products were cleaned by Sephadex G50 in which 3 g of Sephadex G50 powder was weighed and added into 450 ml double distilled water (ddH_2O). This was heated until a slurry was formed and cooled to room temperature. A volume of 650 µl of the slurry was added into an Eppendorf tube and span at 720/750/770 rcf for 2 minutes. The PCR reaction was carefully placed in the middle of the column in a new tube and spun for 2 minutes. The eluted liquid was a clean PCR product.

2.2.2.3. Sequencing PCR

A master mix for one reaction tube was prepared in a 1.5 ml Eppendorf tube containing 1.0 µl of big dye, 1.5 µl of sequencing buffer, 1.6 µl of forward primer (0.5 µM), 2.9 ul ddH_2O and 0.5 µl of bacterial DNA. The reactions were always mixed and kept on ice. The reactions were then transferred into PCR tubes and span down in a mini-centrifuge at maximum rpm to remove bubbles. The tubes were placed in a PCR machine with the following PCR reaction cycles: 25 cycles of denaturation at 96 °C for 10 seconds, annealing at 55 °C for 1 minute and extension at 60 °C for 4 minutes. The sequencing PCR products were cleaned using Sephadex G50 (method

described above). The clean sequencing PCR products were submitted to the Forestry and Agricultural Biotechnology Institute (FABI) at University of Pretoria for sequencing. The sequences were obtained and analysed in National Center for Biotechnology Information (NCBI) GenBank.

2.2.3. Screening of *Streptomyces* spp. isolates for antibacterial activity

The antagonistic activity of *Streptomyces* spp. were determined by using the cross-streak method described by Kumar et al. (2014) and this was done on Mueller Hinton agar (MHA) (Figure 2.4). A *Streptomyces* sp. was streaked at the centre of the plate and incubated at 25 °C for 7 days. Then after 7 days, a fresh culture of test organism (*Kocuria* sp. and *Microbacterium* sp.) was streaked perpendicular to the bacterium at the centre. The plates were incubated at 37 °C for 24 hours. The *Streptomyces* sp. was also tested against the four known pathogens (*E. coli*, *S. aureus*, *P. vulgaris* and *A. xylosoxidans*). The control plates were prepared without the *Streptomyces* sp. The sizes of inhibition zones were recorded.

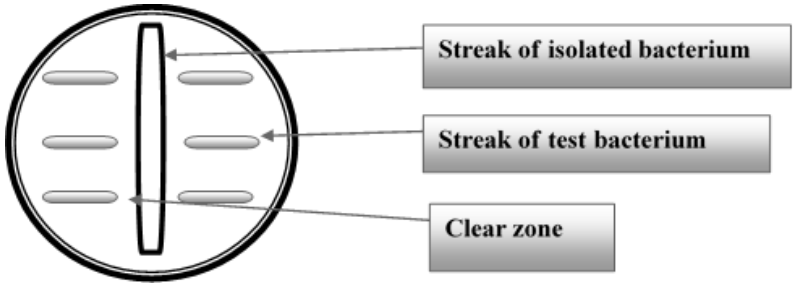

Figure 2.4: Screening of a *Streptomyces* sp. against two selected test soil isolates.

2.3. Results and discussion

2.3.1. Isolation of soil isolates by serial dilution

The soil samples that were used for isolation were collected from the inside and outside of fairy circles and underneath dead *E. gummifera* plants in Namibia. A serial dilution method was used to isolate the bacteria shown in Figure 2.5. Most plates from serial dilutions of the soil samples from the inside and outside contained bacteria that were forming biofilms and based on these outcomes, they were excluded from the study.

Figure 2.5: Serial dilution plate of the Namibian dead *E. gummifera* plant's soil showing different bacterial colonies.

The soil sample from the dead plants however, produced bacterial colonies of distinct species (Figure 2.5). This observation was solely based on the morphology (shape) and colour of the colonies. The medium that was used, Tryptic soy agar, gave good results and clearly supported the growth of many different bacteria. A study conducted by Oskay et al. (2004) mentioned that the growth and maintenance of soil microorganisms can be negatively affected by metabolites produced by plants which might impact the number of actinomycetes especially isolated from the soil. One bacterial species from the genus *Streptomyces* was isolated in pure cultures (Figure 2.6) and based on their potential to produce antibiotics, were used for further studies. Other soil bacteria (named A, B, C and D) that were isolated in this study were tested for susceptibility against the isolated *Streptomyces* species. These test bacteria were chosen because in the preliminary screening results the isolated *Streptomyces* species exhibited antagonistic activity against them (further discussed below).

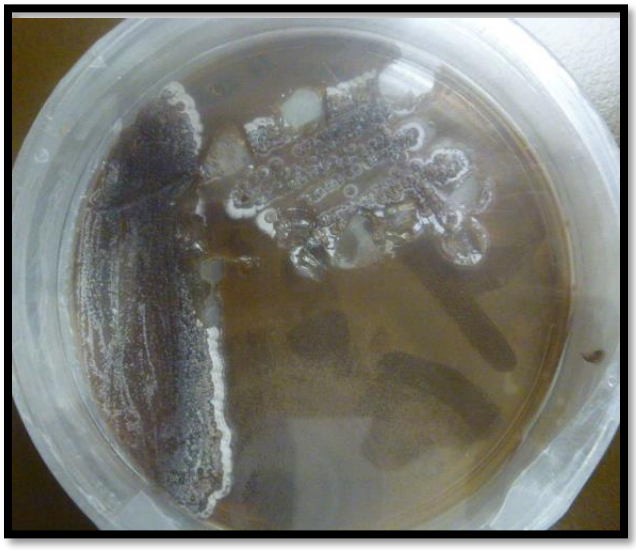

Figure 2.6: Pure culture of the isolated *Streptomyces* sp. used for the antibiotic study.

The colonies of the *Streptomyces* sp. were powdery white in colour and did not spread out like normal bacterial colonies. The other observation was they changed the medium from yellowish to a darker brownish colour (Figure 2.6). This change of colour is due to the release of pigments that are produced during secondary metabolites production (Procópio et al., 2012).

2.3.2. Antibacterial screening of isolated *Streptomyces* spp. against other non-*Streptomyces* soil isolates

Figure 2.7 shows how selected soil bacteria (A and B) grew individually without any competition (the presence of other bacteria) and these served as negative controls. The media that was used was Mueller Hinton agar which clearly supported the growth of all the bacteria that were used in this study.

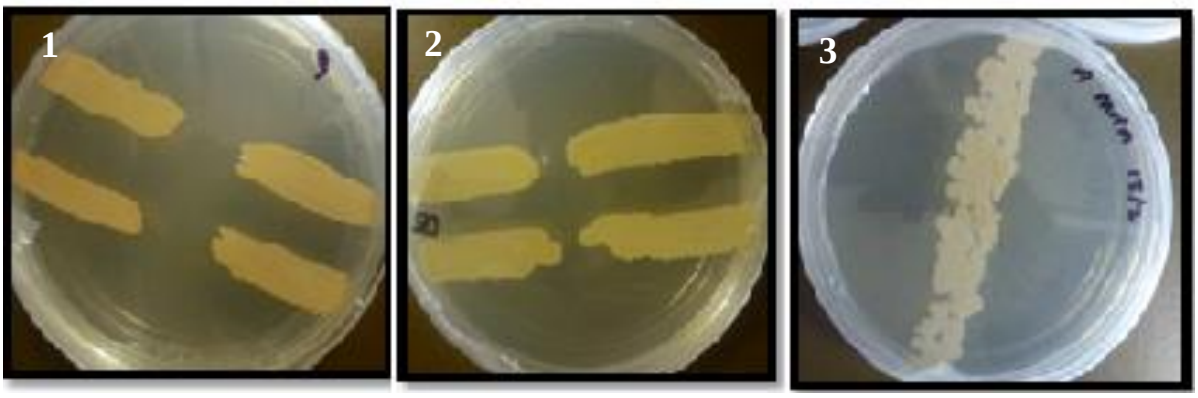

Figure 2.7: The plates showing negative controls of soil bacteria A (1) and B (2) grown on their own and the *Streptomyces* sp. (3).

A cross-streak method was performed and from the observation of the results (Figure 2.8), the *Streptomyces* sp. at the centre exhibited very good inhibition against soil bacteria A, B, C and D but less against four pathogens, *E. coli, S. aureus, P. vulgaris* and *A. xylosoxidans* (Appendix, Figure 5.1). These results showed that the *Streptomyces* sp. might possess the potential of producing antagonistic compound(s) that is active against especially bacteria A to D. Velho-Pereira & Kamat (2011), also conducted a similar study where they isolated 30 actinobacteria and 16 of them belonged to the *Streptomyces* genus. From these 30 isolates, 28 showed to have good antimicrobial activity against all of their eight tested microorganisms (five Gram-positive bacteria, two bacteria Gram-negative and one yeast species). Valli et al. (2012) were able to isolate 21 actinomycetes from a marine environment which were tested against 10 test organisms. Two of the 21 isolates were identified as *Streptomyces* species and they showed to have more antibacterial activity compared to the other isolates.

Oskay et al. (2004) isolated 50 actinomycetes from soil samples and 17 of them were antibacterial against their selected test bacteria which included Gram-positive and Gram-negative bacteria. Based on the morphological analysis of the isolated bacteria they concluded that seven of those 17 bacteria belonged to the genus of *Streptomyces*. George et al. (2010) also concluded that *Streptomyces* spp. had good antagonistic activity against some of their test microorganisms such as *E. coli* and *Salmonella typhimurium*. In another study, Hossain & Rahman (2014) isolated *Streptomyces* spp. from soil samples and tested them for antagonistic activity against several fish and human pathogenic bacteria. They reported that one of the *Streptomyces* sp. showed good results against all tested pathogens.

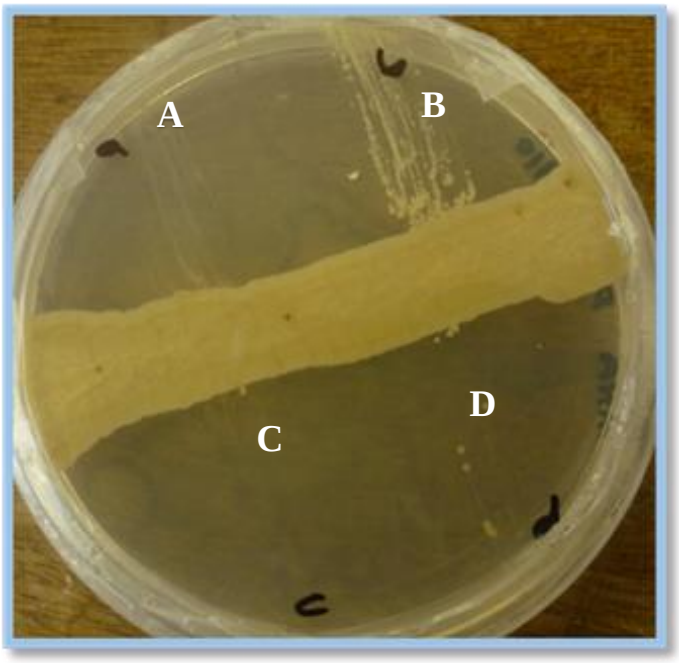

Figure 2.8: *Streptomyces* sp. (centre) tested against four isolated bacterial species (A, B, C and D) using the cross-streak method done on MHA medium.

It is believed that the distribution of secondary metabolites amongst organisms is dissimilar with the filamentous actinomycetes, unicellular bacteria (especially *Bacillus* and *Pseudomonas* species) and some other eukaryotic fungi having more diverse metabolites (Berdy, 2005). The *Streptomyces* genus is known to produce most of the commercially used antibiotics (Berdy, 2005; Ceylan et al., 2008; George et al., 2010) and with the antimicrobial activity results found in this study and other, it clearly shows that this genus is indeed exceptional.

2.3.3. Identification of soil isolates using 16S rRNA sequencing

After conducting PCR, the 16S rRNA gene was isolated and is represented by the bright white bands shown in Figure 2.9 and it is evident that all the isolated bacteria had the gene present. The presence of the gene indicates that the organisms are indeed of bacterial origin. The 16S rRNA gene that was used as a positive control was isolated from a *Staphylococcus aureus* (Appendix, Figure 5.3).

The two primers that were used for PCR were the frequently used primers, 27F (forward primer) and 1492R (reverse primer). Fedriksson et al. (2013) mentioned that these two primers were considered as good primers because they are known to amplify almost the whole of the 16S rRNA gene sequences of bacteria. The results clearly showed that the primers were able to align to the sequences of the gene of the bacteria. The PCR bands were clear, indicating no contamination from primers (Figure 2.9).

The sequences acquired from the sequencing facility were edited with the use of a Bio-Edit Sequence Alignment Editor (Figure 2.10). The bio-edit software is used to align and analyse DNA bases obtained from the subject sample and help construct a sequence for the sample to be identified. It can be seen from Figure 2.10 that the peaks or waves representing DNA bases with each base represented by a different colour. By making use of the given information, one can construct a sequence. There are, however, some unidentified bases which were depicted by the letter N, meaning that the sequencer could not distinguish between the four DNA bases at that peak/wave hence naming it N instead (Carridge, 2004).

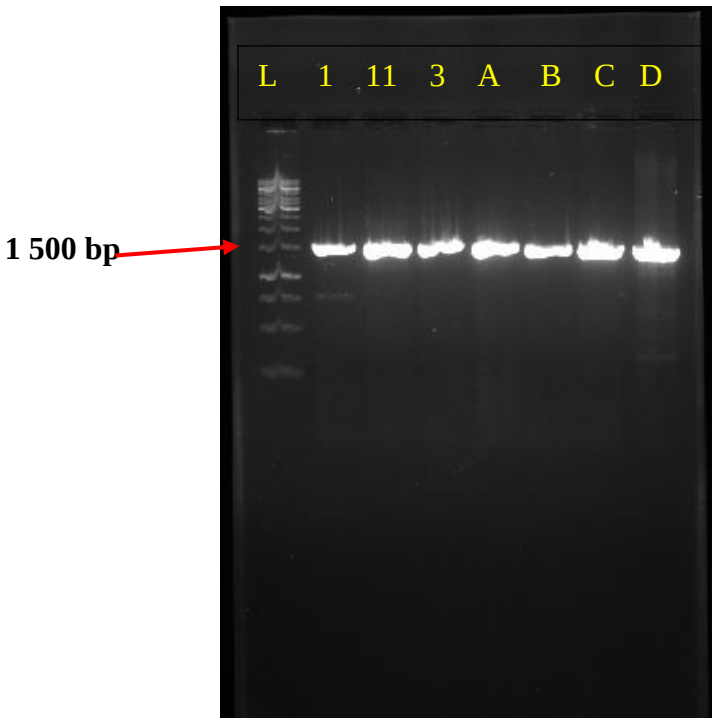

Figure 2.9: Gel electrophoresis analysis of the amplified 16S rRNA gene from the *Streptomyces* species and soil isolates.

The first few bases were represented by a string of N's and do not show any peaks or peaks are low and unorganized. After base 12, the string of N's continues until base 35 and these were excluded for sequencing as they are poorly resolved. Several N's can also be observed at the end of the sequence making it difficult to read the rest of the sequence. In this case, these unidentified bases were deleted therefore reducing the size of the gene sequence. After base 35, the sequence bases were identifiable, however in between this sequence, the letter N can still be seen. In the middle of the chromatogram, the peaks are long and organized and well resolved and therefore the sequence could be used. At some of the locations where there was the letter N, there were two or more peaks representing the identified DNA bases. By determining the height and the colour of

the peaks, the one that was higher was then inserted at that location. These overlaps were known to be caused when a primer has multiple alignment sites on the sequence which was observed when the PCR product on the gel electrophoresis had a few bands in one lane. To obtain a good DNA sequence, one must always make sure that the DNA that is used is properly cleaned and that all impurities such as primer dimers, salts, dNTPs, etc have been removed. To confirm all of this, a PCR product can be run on a gel. Carridge (2004) stated the reduced sequences can still be used for BLAST and obtain an accurate genus or species identification. It is therefore desirable to possess the complete gene (1 500 bp long), typically when trying to identify new species. The bio-edit software was used to construct sequences and were later subjected to BLAST, to enable the identification of the bacteria isolated.

After removing some of the unidentified bases from the sequence, 594 bp were recovered for sequencing. The sequence length from the database was 1 440 bp and this was used to align to the sequence. From the alignment results, it was shown that bacterium A shared 100 % identity to *Kocuria turfanensis* strain HO-9042 (Figure 2.11). The *Kocuria* genus belongs to the Gram-positive bacteria that produce bright orange colonies which grow under aerobic conditions and is known for their inability to form endospores (Zhou et al., 2008). It was reported to be susceptible to the action of antibiotics like ampicillin, carbenicillin and erythromycin and some species of this genus were found to be tolerant in up to 15 % of NaCl (Goswami et al., 2014; Dai et al., 2016). The *Kocuria* genus was previously grouped under the genus *Micrococcus* and separated by Stackebrandt et al. (1995). Goswami et al. (2014) mentioned that this genus can be found in different environments like air, soil, fermented sea food, mammalian skin, the rhizoplane, freshwater, seawater, marine sediment, and desert soil (Zhou et al., 2008).

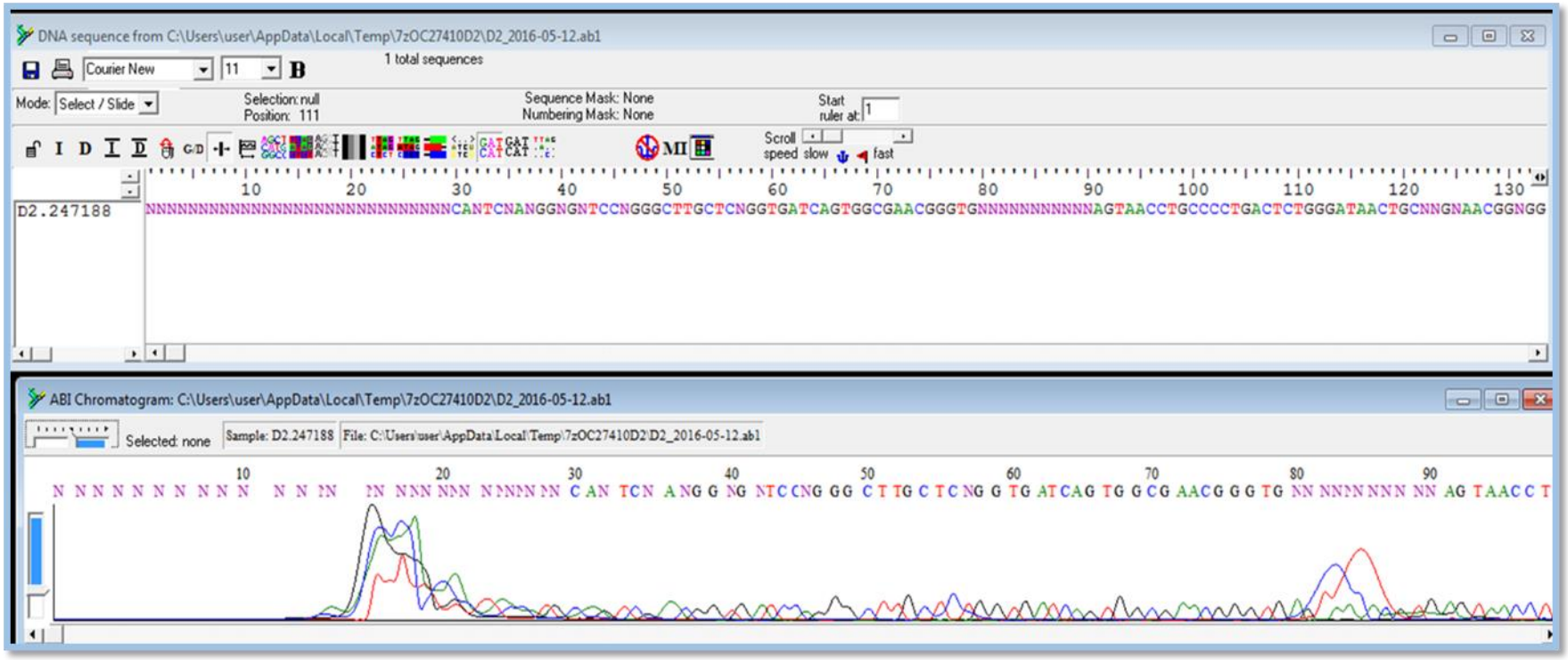

Figure 2.10: The chromatogram that was used to edit sequences of a given sample to help with the identification. The letters (A, G, C and T) depicted in different colours represents DNA bases and the letter N represents any of the bases.

Bacterium C showed to possibly be a bacterium known as an uncultured *Microbacterium* sp. clone 1c05 with 99 % identity (Figure 2.12). The bacteria of the genus *Microbacterium* are rod-shaped and exhibit yellow colonies in agar media (like bacterium C). They are found in soil, water, milk and cheese and were reported to be found in clinical specimen (Takeuchi & Hatano, 1998). Swerczek (2007) was studying diseases that are caused by bacteria in female horses when he found that some of the bacteria responsible for early and late fetal loss syndrome was caused by plant bacterial endophytes like *Microbacterium* spp. and *Cellumonas* spp. Kelly et al. (2009) reported *Microbacterium* sp. as one of the bacteria that caused spoilage in commercial milk samples collected from diary plants. Laffineur et al. (2003) isolated a bacterium species from the blood of a child with acute lymphoblastic leukaemia and with the use of 16S rRNA they identified the bacterium to belong to the genus *Microbacterium.* Since this strain differed phenotypically from other *Microbacterium* species it was classified as a new species and was named *M. paraoxydans.* One of the phenotypic characteristics that set it apart from other *M. paraoxydans* was that it could grow under 37 °C. In the current study, similar findings were observed with the *Microbacterium* sp. that was isolated.

The isolated *Streptomyces* sp. showed to most probably be *Streptomyces parvulus* strain R5-344 with a 100 % identity. A *S. parvulus* bacterium was previously isolated from soil samples and found to produce a polypeptide antibiotic called actinomycin D, which inhibits the synthesis of RNA of other bacteria. This strain has been proven to have antibacterial effects on pathogens such as *S. aureus, E. coli, P. aeruginosa* and *B. subtilis* (Shetty et al., 2014). As it was mentioned in the previous chapter, the isolated *Streptomyces* sp. in the current study inhibited four soil isolates effectively but the selected known pathogens less effectively.

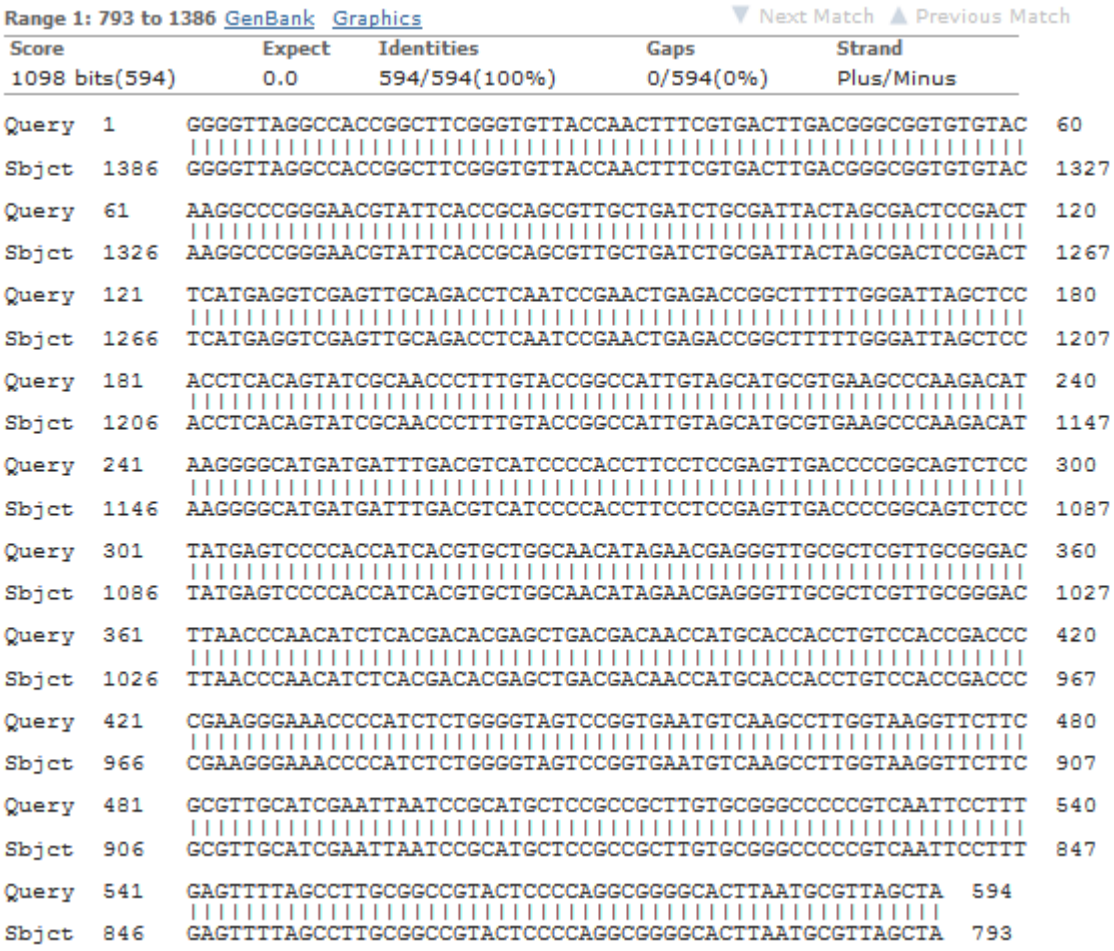

Figure 2.11: The diagram shows the alignment between the edited sequences of bacterium A (query) vs the sequence found on the database (subject). This alignment identified bacterium A as a *Kocuria turfanensis* HO-9042.

Figure 2.12: The diagram shows the alignment between the edited sequences of bacterium C (query) vs the sequence found on the database (subject). This alignment identified bacterium C as previously uncultured *Microbaterium* sp. clone 1c05.

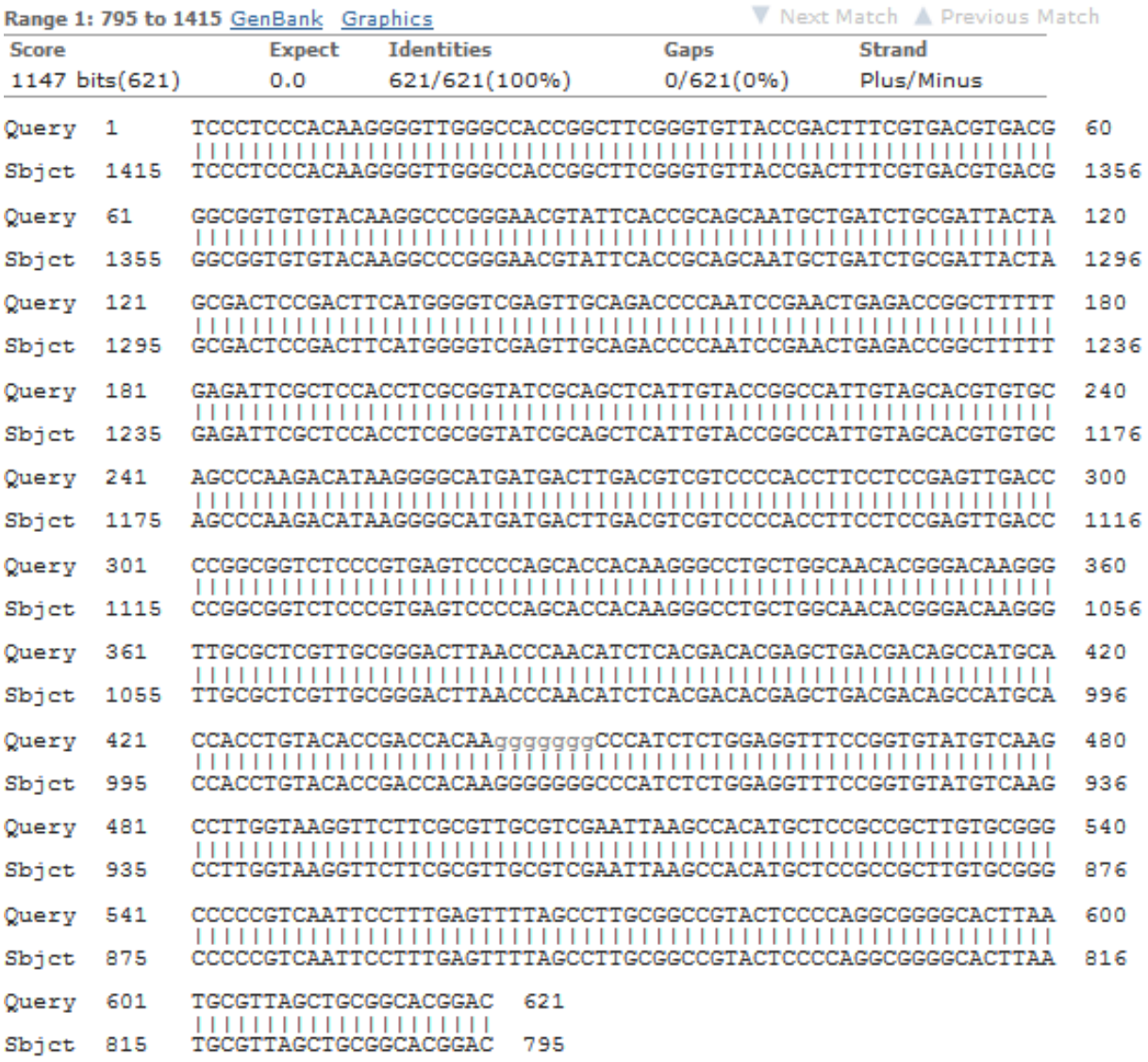

Figure 2.13: The diagram shows the alignment between the edited sequences of the *Streptomyces* bacterium (query) vs the sequence found on the database (subject). This *Streptomyces* sp. is most likely *Streptomyces parvulus* strain R5-344.

2.4. Conclusion

A *Streptomyces* species most likely, *S. parvulus* strain R5-344 was isolated from a dead *E. gummifera* plant's soil sample collected from the Namibian Desert. The bacterium was identified based on both morphology and with the use of 16S rRNA sequencing. This *Streptomyces* sp. has been shown to have inhibited the growth of bacteria A, B, C and D and to a lesser extent selected known pathogens, proving that it might have the ability in producing antibacterial compounds. Two of the isolated soil bacteria investigated in the study were identified as follows; bacterium A is most probably *Kocuria turfanensis* HO-9042 with a 100 % identity and bacterium C is probably an uncultured *Microbacterium* sp. clone 1c05 with a 99 % identity.

2.5. References

1. Baker, G. C., Smith, J. J., & Cowan, D. A. (2003). Review and re-analysis of domain-specific 16S primers. Journal of Microbiological Methods, 55 (3): 541-555.
2. Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). "Methods for in vitro evaluating antimicrobial activity: A review." Journal of Pharmaceutical Analysis, 6 (2): 71-79.
3. Ben-David, A., & Davidson, C. E. (2014). "Estimation method for serial dilution experiments." Journal of Microbiological Methods, 107: 214-221.
4. Berdy, J. (2005). Bioactive microbial metabolites: a personal view. Journal of Antibiotics, 58 (1): 1-26.

5. Bhardwaj, N., Bhardwaj, S. K., Nayak, M. K., Mehta, J., Kim, K-H., & Deep, A. (2017). Fluorescent nanobiosensors for the targeted detection of food-borne bacteria. Trends in Analytical Chemistry, 97: 120-135.
6. Ceylan, O., Okmen, G., & Ugur, A. (2008). Isolation of soil *Streptomyces* as source antibiotics active against antibiotic-resistant bacteria. EurAsia Journal of Biosciences, 2: 73-82.
7. Clarridge, J. E. (2004). Impact of 16S rRNA Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases. Clinical Microbiology Reviews, 17 (4): 840–862.
8. Clemons, W. M., May, J. L. C., Wimberley, B. T., McCutcheon, J. P., Capel, M. S., & Ramakrishnan, V. (1999). Structure of a bacterial 30S ribosomal subunit at 5.5 [thinsp] A resolution. Nature, 400 (6747): 833-840.
9. Dai, J., Yu, C., Huang, Z., Fan, M., Jia, Z., Li, X., Wang, Z., Li, Z., Yao, J., Li, P., Zheng, G., & Chen, X. (2016). "Genome sequencing and annotation of *Kocuria turfanensis* HO-9042, to promote explorating high-salt resistance associated gene resources." Journal of Biotechnology, 224: 51-52.
10. Donelli, G., Vuotto, C., & Mastromarino, P. (2013). Phenotyping and genotyping are both essential to identify and classify a probiotic microorganism. Microbial Ecology in Health and Disease, 24: 10.3402/mehd.v24i0.20105.
11. Frank, J. A., Reich, C. I., Sharma, S., Weisbaum, J. S., Wilson, B. A., & Olsen, G. J. (2008). Critical Evaluation of Two Primers Commonly Used for Amplification of Bacterial 16S rRNA Genes. Applied and Environmental Microbiology, 74 (8): 2461–2470.

12. Fredriksson, N. J., Hermansson, M., & Wilén, B. M. (2013). The Choice of PCR Primers Has Great Impact on Assessments of Bacterial Community Diversity and Dynamics in a Wastewater Treatment Plant. PLoS ONE 8 (10): e76431.
13. George, M., George, G., & Hatha, A. A. M. (2010). Diversity and antibacterial activity of actinomycetes from wetland soil. The South Pacific Journal of Natural and Applied Sciences, 28: 52-57.
14. Goswami, D., Pithwa, S., Dhandhukia, P., & Thakker, J. N. (2014). Delineating *Kocuria turfanensis* 2M4 as a credible PGPR: a novel IAA-producing bacterium isolated from saline desert. Journal of Plant Interactions, 9 (1): 566-576.
15. Hossain, N., & Rahman, M. (2014). Antagonistic activity of antibiotic producing *Streptomyces* sp. against fish and human pathogenic bacteria. Brazilian Archives of Biology and Technology, 57 (2): 233-237.
16. Janda, J. M., & Abbott, S. L. (2007). 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. Journal of Clinical Microbiology, 45 (9): 2761–2764.
17. Kelly, P., Woonton, B. W., & Smithers, G. W. (2009). 8 – Improving the sensory quality, shelf-life and functionality of milk. Functional and Speciality Beverage Technology, 170–231.
18. Kumar, P. S., Duraipandiyan, V., & Ignacimuthu, S. (2014). Isolation, screening and partial purification of antimicrobial antibiotics from soil *Streptomyces* sp. SCA 7. The Kaohsiung Journal of Medical Sciences, 30 (9): 435-446.
19. Laffineur, K., Avesani, V., Cornu, G., Charlier, J., Janssens, M., Wauters, G., & Delmée, M. (2003). Bacteremia Due to a Novel *Microbacterium* Species in a Patient with Leukemia and Description of *Microbacterium paraoxydans* sp. nov. Journal of Clinical Microbiology, 41 (5): 2242–2246.

20. Meyer, J. J. M., Senejoux, F., Heyman, H. M., Meyer, N. L., & Meyer, M. A. (2015). The occurrence of triterpenoids from *Euphorbia gummifera* inside the fairy circles of Garub in Southern Namibian pro-desert. South African Journal of Botany, 98: 10-15.
21. Microbiology, S. f. G. (2003). Basic Practical Microbiology: A Manual, Society for General Microbiology.
22. Oskay, M., Tamer, A. U., & Azeri, C. (2004). Antibacterial activity of some actinomycetes isolated from farming soils of Turkey. African Journal of Biotechnology, 3 (9): 441-446.
23. Procópio, R. E. d. L., Silva, I. R. d., Martins, M. K., Azevedo, J. L. d., & Araújo, J. M. (2012). Antibiotics produced by *Streptomyces*. Brazilian Journal of Infectious Diseases, 16: 466-471.
24. Rom, J. L., Karm, A. S., Sendler, E. D., & Kato, I. (2011). Strategy for microbiome analysis using 16S rRNA gene sequence analysis on the illuming sequencing platform. System Biology in Reproductive Medicine, 00: 1-9.
25. Shetty, P. R., Buddana, S. K., Tatipamula, V. B., Naga, Y. V., & Ahmad, J. (2014). Production of polypeptide antibiotic from *Streptomyces parvulus* and its antibacterial activity. Brazilian journal of microbiology. Brazilian Society for Microbiology, 45 (1): 303-312.
26. Stackebrandt, E., Koch, C., Oxana, G., & Schumann, P. (1995). Taxonomic Dissection of the Genus *Micrococcus*: *Kocuria* gen. nov., *Nesterenkonia* gen. nov., *Kytococcus* gen. nov., *Dermacoccus* gen. nov., and *Micrococcus* Cohn 1872 gen. emend. International Journal of Systematic Bacteriology, 45 (4): 682-692.
27. Swerczek, T. W. (2007). Chapter 24 – Bacterial Diseases of the Fetus and Placenta Associated with Fetal Loss in the Mare. Current Therapy in Large Animal Theriogenology (Second Edition), 183–188.

28. Takeuchi, M., & Hatano, H. (1998). Proposal of six new species in the genus *Microbacterium* and transfer of *Flavobacterium marinotypicum* ZoBell and Upham to the genus *Microbacterium* as *Microbacterium maritypicum* comb. nov. International Journal of Systematic of Bacteriology, 48: 973-982
29. Valli, S., Suvathi, S. S., Aysha, O. S., Nirmala, P., Vinoth, K. P., & Reena, A. (2012). Antimicrobial potential of Actinomycetes species isolated from marine environment. Asian Pacific Journal of Tropical Biomedicine, 2 (6): 467-473.
30. Velho-Pereira, S., & Kamat, N. M. (2011). Antimicrobial Screening of Actinobacteria using a Modified Cross-Streak Method. Indian Journal of Pharmaceutical Sciences, 73 (2): 223–228.
31. Zhou, G., Luo, X., Tang, Y., Zhang, L., Yang, O., Qiu1, Y., & Fang, C. (2008). "*Kocuria flava sp*. nov. and *Kocuria turfanensis sp*. nov., airborne actinobacteria isolated from Xinjiang, China." International Journal of Systematic and Evolutionary Microbiology, 58 (6): 1304-1307.

CHAPTER 3: IDENTIFICATION OF ANTIBACTERIAL COMPOUNDS FROM SOIL ISOLATED *STREPTOMYCES* SPP.

3.1. Introduction

Thin layer chromatography (TLC) is one of the most used chromatographic techniques in the detection of metabolites in a sample. This technique has been used over many years and still is important today because it is easy, quick and cheap to carry out (Rhee et al., 2001; Sherma & Fried, 2003; Suleiman et al. 2010; Balouiri et al., 2016; Grzelak et al., 2016). TLC was used as the starting point to analyse the metabolites that are found in the bacterial extracts of *Streptomyces*. The separated compounds were analysed with the use of a direct TLC bio-autography. Bio-autography is a biological assay that is used to identify active compounds from biological extracts (Suleiman et al., 2010; Jesionek et al., 2015). Apart from direct TLC bio-autography, there are also two other types of bio-autography (Figure 3.1).

Agar diffusion or contact bio-autography uses a developed TLC plate that is placed face down on an agar plate containing a fresh bacterial culture and this allows the antimicrobial compounds to diffuse through the agar plate. Agar overlay or immersion bio-autography involves poring an agar medium onto the TLC plate and this type of bio-autography is known to be the combination of the of direct and contact bio-autography (Rios et al., 1988; Islam et al., 2003; Suleiman et al., 2010; Choma & Grzelak, 2011; Dewanjeea et al., 2014). Of all the bioautographic methods, the direct TLC bio-autography is mostly used (Choma & Grzelak, 2011; Balouiri et al., 2016).

Bacteria can produce compounds that are harmful to other bacterial species and other microorganisms (Asha-Devi et al., 2011). These compounds (antibiotics) can be difficult to isolate as they are produced in small quantities. There are two ways in which antibiotic compounds can be isolated from its producer. The first method would be to extract the compound directly from the agar. This will be done by cutting the agar into smaller cubes and

extracting the compound using a suitable extraction solvent for few hours or days (Uzair et al., 2008). This method, however, would require many agar plates due the small concentration of the compound being produced by the producer.

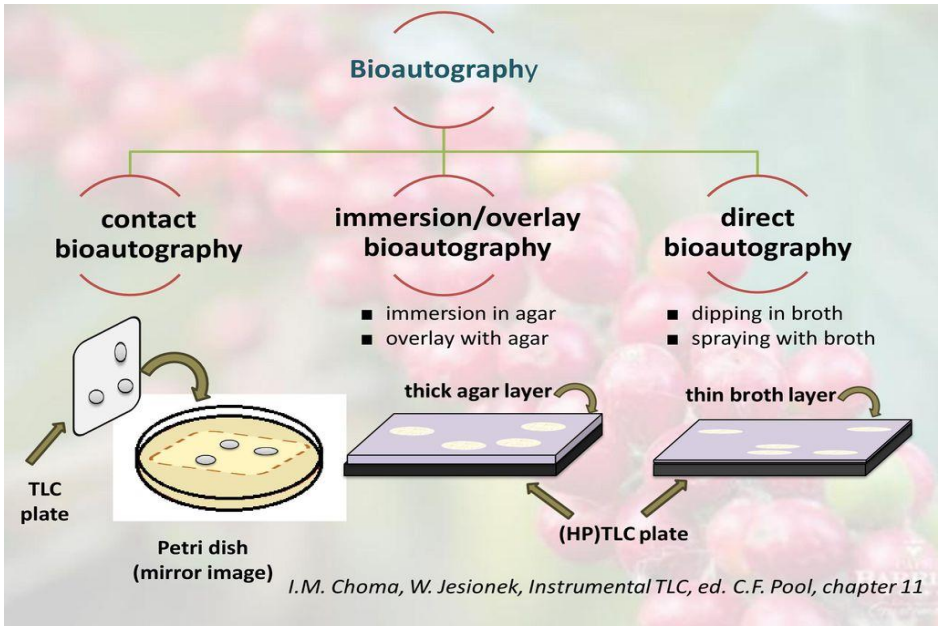

Figure 3.1: Bioautographic methods, namely contact bio-autography, overlay bio-autography and direct TLC bio-autography. These methods are used to localize antimicrobial compounds of a given extract (Choma & Jesionek, 2015).

The second method is fermentation and there two described processes of it. The first method is called submerged fermentation and is carried out in a liquid broth that has nutrients that are required for the producer's growth (Doriya et al., 2016). The solution would then be incubated for 24 hours or more and then centrifuged to remove all the cell debris. The second method is solid substrate fermentation that uses substrates like rice or wheat bran and are added to the medium. The solid substrate fermentation is the favoured one because it is easy to carry out, is cost effective and produces high yield of the desired compound (Sandhya et al., 2005).

There are several analytical methods that are used to determine the structure of an isolated pure compound. Nuclear Magnetic Resonance (NMR) spectroscopy is one of the tools mostly used in structure elucidation and can be used to study and search for new compounds produced by organisms. The technique plays an important role in most fields of science, especially chemistry and physics but has recently gained recognition in medicine and other disciplines. NMR-based metabolomics can be used in disease diagnosis and in drug development (Kayser & Quax, 2007; Emwas et al., 2015). With the diagnosis of some diseases there were several factors that were discovered to have major impacts on the use of NMR-based metabolomics like the age of the person, the type of food the person consumes, the sex of an individual, the way they live their lives and other related factors (Emwas et al., 2015). Metabolomics is defined as a technique used to study both the quality and quantity of all metabolites in a biological system (Kayser & Quax, 2007).

There are two main types of NMR; ^{1}H NMR and ^{13}C NMR. In ^{13}C NMR, each peak represents a carbon atom in a molecule and the higher the electro-negativity the atom that is next to the carbon atom, the higher the value of the chemical shift (ppm). This study only focuses on ^{1}H NMR. In ^{1}H NMR, each peak represents a hydrogen and if these hydrogen atoms are in the same environment they will be at the same chemical shift (ppm value) and the peak will be higher depending on how many hydrogen atoms are in the same chemical environment. Molecules which contain long alkane chains will have lower ppm values (chemical shifts) on the NMR spectrum, molecules with aromatic rings will have ppm values of about 7 to 9 ppm and those molecules that contain atoms that are high in electro-negativity (oxygen, nitrogen, bromine, etc.), will have even higher ppm values. Another method used in the identification of compounds, is Gas Chromatography-Mass Spectrometry (GC-MS). GC-MS is an analytical method that is used in different fields of science to identify and characterize volatile compounds and their masses in chemical and biological samples (Sneddon et al., 2007). The

identity of the compounds can be based on the size and fragmentation pattern of the compound and the area of the peak (Usha, 2011).

The aim of this chapter was to purify and identify the compound(s) that was antagonistic against four of the tested bacteria of which *Kocuria turfanensis* strain HO-9042 and *Microbacterium* sp. Clone 1c05, isolated from Namibian desert soil, was identified. The technique that was chosen to extract the compound, TLC bio-autography, identified bioactive regions which were scrapped off from the plates and then analysed with ^{1}H NMR and GC-MS. This method was chosen because it's usually more efficient to obtain pure compound(s) from the plates in small quantities for NMR and GC-MS analyses.

3.2. Materials and methods

3.2.1. Extraction of secondary metabolites

The Petri dish plates for this experiment were prepared as follows: 38 g of Mueller Hinton agar (MHA) was added to a 1 L glass bottle containing 1 000 ml of distilled water. The solution was mixed and autoclaved at 121 °C for 1 hour. The agar was let to cool off and poured into 30 Petri dishes to solidify. The Petri dish plates with *Streptomyces parvulus* only were incubated at 25 °C for 7 days. After 7 days, the test bacteria (*K. turfanensis* and *Microbacterium* sp.) were streaked perpendicular to the *S. purvulus* culture and incubated at 37 °C for 24 hours. Clear zones from 30 Petri dishes (*K. turfanensis* and *S. parvulus*) were cut out and used to extract possible secondary inhibiting compounds. An amount of 200 ml of ethyl acetate was added into a 1 L conical flask containing the cut agar from 30 MHA plates, placed on a magnetic stirrer shaker at room temperature for 2 hours and was re-extracted 3 times to get all the metabolites from the agar. The same procedure was conducted with Petri dishes of control plates with no bacterial cultures. The total volume of each sample after extraction was about 600 ml and was filtered to remove unwanted material.

3.2.2. TLC Bio-autography

TLC separation of extracted compounds was conducted following the method of Jesionek et al. (2015) with modifications. The ethyl acetate extracts were applied manually in 5 mm bands on silica TLC plates (Merck) of 20 cm X 20 cm, and the TLC plates were developed with a

mobile phase mixture of ethyl acetate: hexane (9:1) to an 8 cm distance in a glass chamber. All TLC separations were performed at room temperature (25 °C). After chromatographic separation, the adsorbent layers were dried at room temperature overnight to remove the mobile phase completely. This procedure was done with the *Microbacterium* sp. and the *K. turfanensis* grown in the presence of *S. parvulus* as well as negative control plates without bacterial extracts. Separated compounds were visualized with both UV light and vanillin by spraying it only along the edges of the plates, with the middle sections being covered with aluminium foil.

For the bio-autography, Mueller Hinton broth was prepared by adding 38 g into a 1L glass bottle containing 1 000 ml of distilled water. The solution was mixed and autoclaved at 121 °C for 1 hour. Bacterial colonies of *K. turfanensis* and *Microbacterium* sp. were inoculated in 100 ml MHB and incubated in a shaker incubator at 37 °C for 24 hours. After incubation, the bacterial liquid culture was centrifuged at 3 000 rpm for 10 minutes. A volume of 100 ml of freshly prepared MHB was added into a flask containing the bacterial pellet and stirred on a shaker for 5 minutes. The developed TLC plates were dried and then sprayed with the freshly prepared bacterial cell suspensions and then placed into a humid plastic box and incubated at 37 °C for 24 hours. After incubation, the plates were sprayed with INT (p-Iodonitrotetrazolium violet) solution (2 mg /10 mL) along a 1 cm edge of the plates and further incubated at 37 °C for 24 hours. The visualization of the inhibition zones of the separated compounds was based on dehydrogenase activity of metabolically active bacteria. This enzyme converts the yellow tetrazolium salt, MTT, into purple formazan. Thus, the inhibition zones were visible as clear bands against a purple background. The inhibition band was scraped off from 8 TLC plates at the same R_f value, then placed in a flask containing 200 ml of ethyl acetate. The flask was incubated on a shaker at room temperature for 2 days. The sample was then filtered, dried and weighed to be used later for chemical analysis.

3.2.3. NMR analysis

The ^{1}H NMR was conducted on a Varian 200 MHz spectrometer (University of Pretoria, Department of Plant and Soil Sciences). The samples were completely dried for 3 hours using a geneVec machine (GenVec® EZ-2 ^{PLUS}). The samples were weighed (5.2 mg for extract and 3.5 mg for control) and dissolved in 700 ul of deuterated acetone and dissolved by ultrasonic treatment for 1 minute and transferred into a 5 mm NMR tubes for processing. For each spectrum, 1 000 scans were recorded. The temperature was kept constant for each run at 25 °C and all the spectra were referred to their respective internal standards.

3.2.4. GC-MS analysis

Extracts of 1 mg extract/ml were prepared in distilled acetone (Merck) and analysed on a Shimadzu GC-MS-QP2010 with an electrical current of 70 eV. The compounds were separated using a Rtx – 5MS column (29.3 m x 250 µm x 0.25 µm i.d.; 0.25 µm df) with helium as carrier gas. Splitless injections of 1 µl were performed, with both the injector and detector temperature set at 300 °C. The oven temperature program was set at an initial temperature of 50 °C and held for 2 minutes, thereafter the temperature was increased to 300 °C at a rate of 10 °C per minute and held at 300 °C for 5 minutes, bringing the total run time to 30 minutes. The respective spectra were analysed using the Shimadzu GC-MS Lab Solutions software version 4.2.

3.3. Results and discussion

3.3.1. Extraction of secondary metabolites and visualizing of the compounds using TLC

The TLC technique was conducted to separate the extracts' constituents of *Streptomyces parvulus* (Jesionek et al., 2015; Valle et al., 2016) and the agar control. The compounds on the developed TLC plates were visualized with the use both UV light (short 254 nm and long 365 nm wavelengths) and vanillin (Figure 3.2).

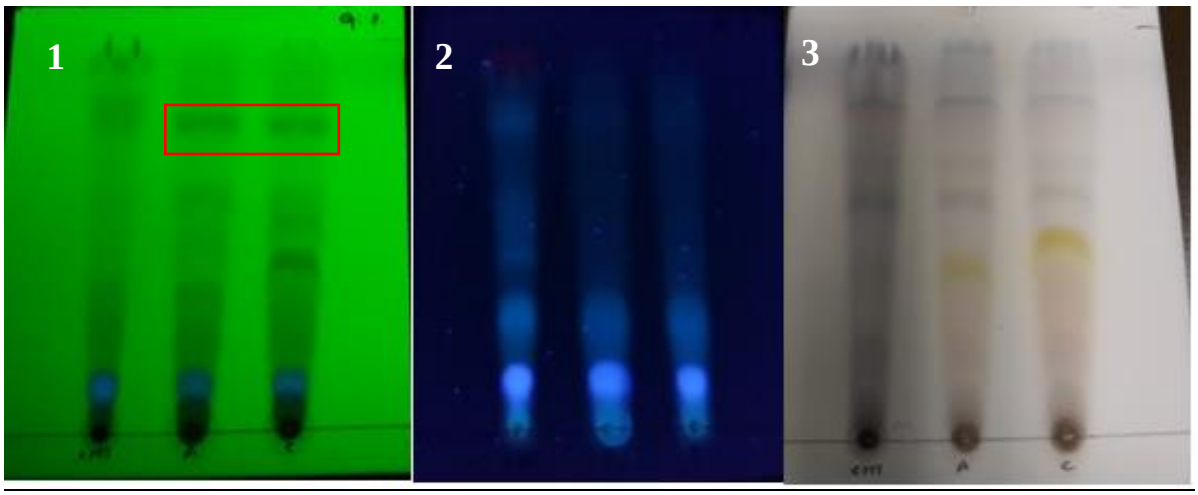

Figure 3.2: TLC plates of agar extractions of the control (no bacteria present) and agar from plates containing *Streptomyces parvulus* as well as other bacteria. The first band is the control, the second is sample A with *K. turfanensis* grown with *S. parvulus* and the third band is sample C with *Microbacterium* sp. grown with *S. parvulus*. 1: Samples visualized under short wavelength; 2: Samples viewed under long wavelength; 3: TLC plates sprayed with vanillin. The highlighted area in 1 represents the bands that absorb short wave UV light and only found in sample A and C.

Sherma & Fried (2003) mentioned that compounds that can absorb UV light are compounds that are aromatic, conjugated and unsaturated. As it can be seen in Figure 3.2, the compound(s) of interest (highlighted in red) absorbed UV under the short wavelength but only limited under long wavelength.

3.3.2. Antibacterial activity using direct TLC bio-autography

Soil microorganisms are known for their production of bioactive compounds and have been studied for many years. These bioactive compounds have been found to be antibacterial, antifungal and antitumoral (Rios et al., 1988; Berdy, 2005; Choma & Grzelak, 2010; Grzelak et al., 2016). In the direct-TLC bio-autography method that was conducted for our study, the ethyl acetate extract of a *S. parvulus* strain R5-344 that was isolated had shown to be active against one of the selected test bacteria, *Microbacterium* sp. and it can be seen by a clear white band against the purple background illustrated by a schematic diagram in Figure 3.3. Usha (2011) had conducted a similar study on a newly isolated *Streptomyces parvulus* KUAP106 and observed that extracts of ethyl acetate contained high antibiotic activity compared to the extractions with n-hexane, benzene and xylene. Another similar study by Ghadin et al. (2008) have reported that the ethyl acetate extract of endophytic *Streptomyces* sp. was active against pathogenic bacteria such as *Pseudomonas aeruginosa* and *Bacillus cereus* compared to xylene and n-hexane extracts. A study done by Parthasarathi et al. (2010) also used ethyl acetate as one of their extraction solvents and had shown that the ethyl acetate extracts of *Streptomyces hygroscopicus* produced better activity than against their other test pathogenic bacteria. Deepa et al. (2011) similarly found that the ethyl acetate extract of an isolated *Streptomyces* sp. is active against a *Microsporum* species.

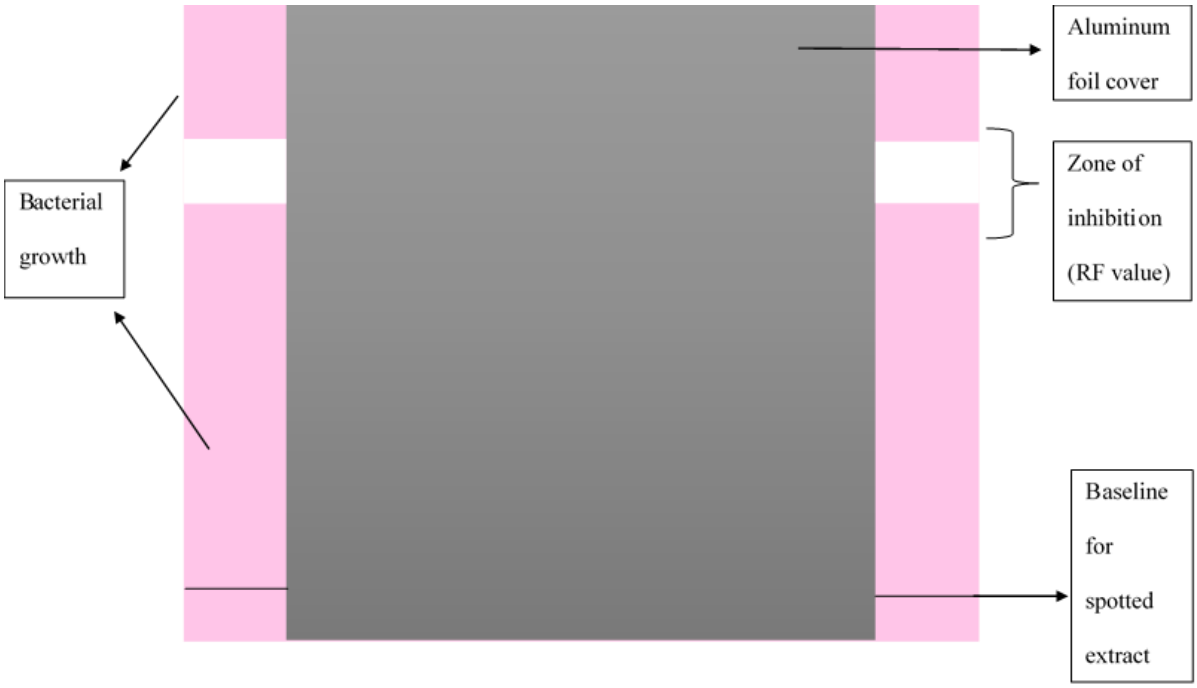

Figure 3.3: A schematic diagram of TLC bio-autography method showing a white area indicating the inhibition of the tested sprayed bacteria by the ethyl acetate extract of the isolated *Streptomyces parvulus* strain R5-344 used in the study.

The use of an appropriate solvent system is very important in TLC bio-autography. The solvents should be highly volatile and have a low boiling point which makes them easily removable by air-drying overnight. The reason behind this is that some solvents don't evaporate completely and therefore inhibit the growth of the sprayed microorganisms and interfere with the results (Islam et al., 2003; Grzelak et al., 2016). In this study ethyl acetate and hexane were used which evaporate easily when left overnight. Another important factor was to determine the R_f value of the compound of interest and the value was 0.76. The R_f value is measured as the distance travelled by the compound divided by the distance travelled by the solvent, so the higher the R_f value the higher the affinity for the solvent (Bele & Khale, 2011;

Pavia et al., 2015). Compounds with larger R_f value are said to adhere less to the stationary phase (which is polar) and therefore move faster on the TLC plate (Bele & Khale, 2011). From the given explanations we can conclude that the antibacterial compound is non-polar.

3.3.3. Analysis of the extract using Nuclear Magnetic Resonance and Gas Chromatography-Mass Spectrometry

After testing the extract for antibacterial activity, the extract was subjected to NMR. The mass of the control extract was 3.5 mg and for the *S. parvulus* strain R5-344 extract was 5.2 mg. The agar control was done to compare with the *S. parvulus* strain R5-344 extract. The two spectra shown in Figure 3.4 represent the control extract (in blue) and the bacterial extract (in red). There are several similar peaks between the two spectra, but also a few differences. The area in the red box showed a peak at a chemical shift of 4.73 ppm that was only present in the extract and not in the control. The region between chemical shifts of 4.15 ppm and 4.35 ppm of the extract spectrum (Figure 3.4 – blue box), had different peaks when compared to that of the control. This region is known to contain ester molecules, and this supported the results that were found in GC-MS, in which two of the identified compounds contained ester functional groups.

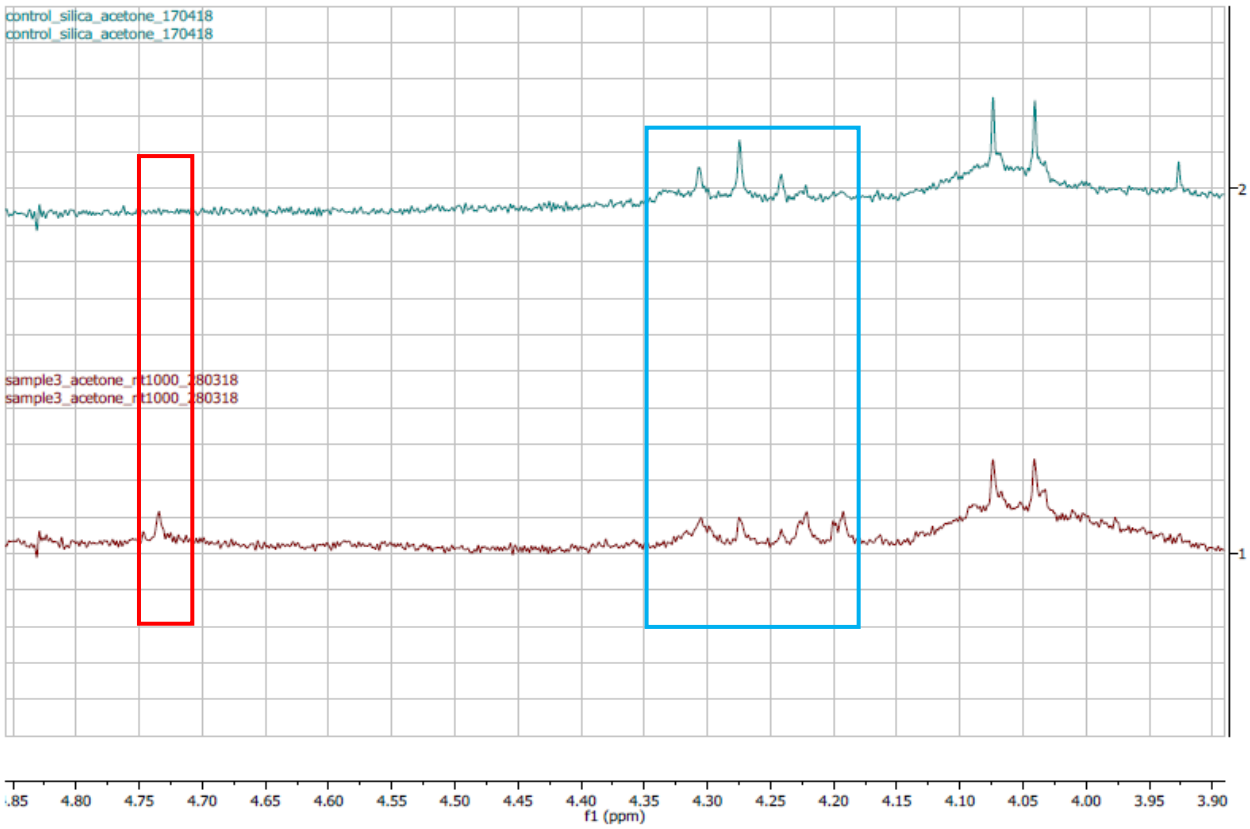

Figure 3.4: ^{1}H NMR spectrum of agar control (blue) stacked over the ^{1}H NMR spectrum of the *S. parvulus* extract (red). Unique peaks on the extract spectrum are seen at chemical shift of 4.73 ppm (red box) and region between 4.15 ppm and 4.35 ppm (blue box).

Another difference was spotted at a chemical shift of 0.90 ppm (Figure 3.5) which was only found in the inhibition band and not the control. The peaks in that area represent compounds that are long chain molecules of terpenoids, fatty acids or alkanes. Terpenes in bacteria, particularly actinomycetes, are not widespread as in plants and fungi (Rabe et al., 2013; Yamada et al., 2015).

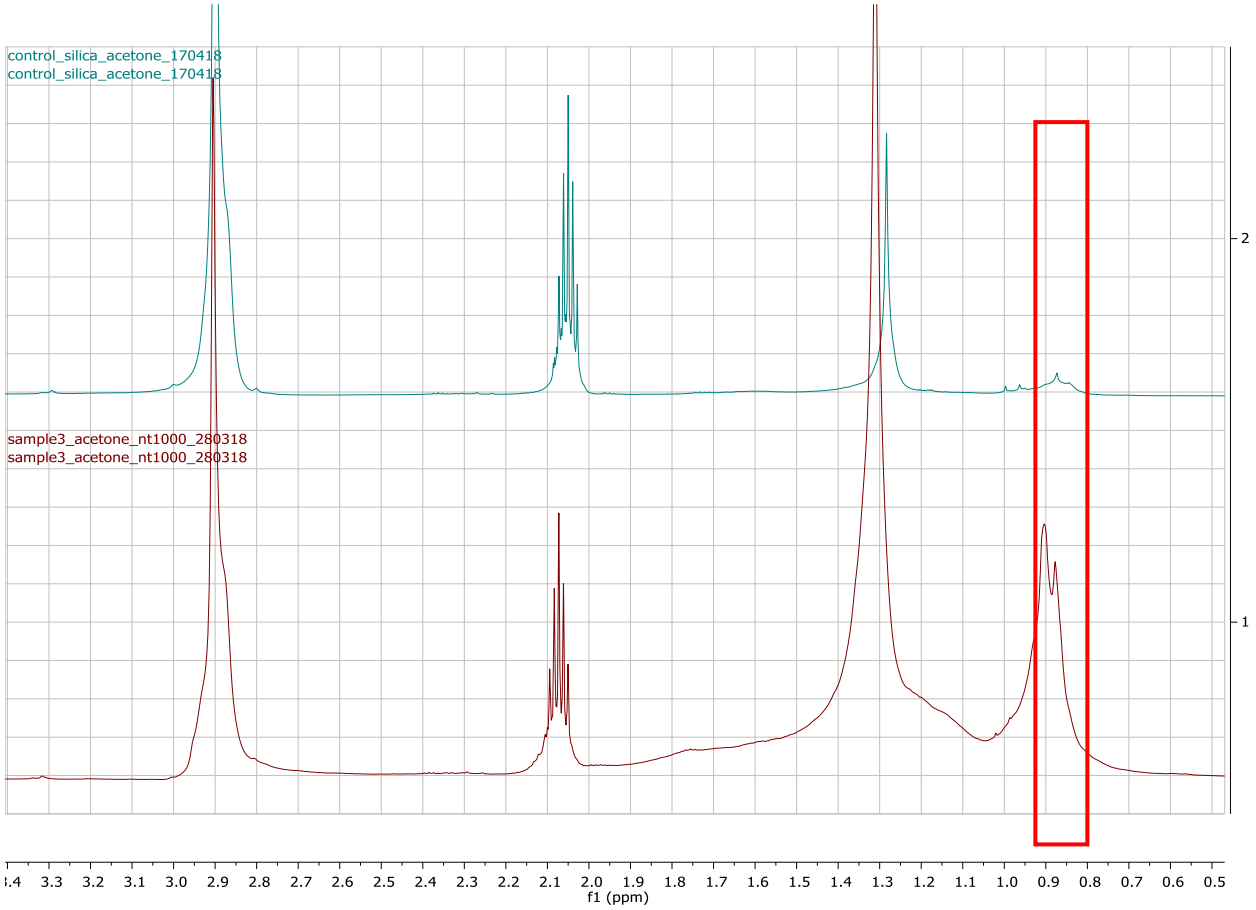

Figure 3.5: ^{1}H NMR spectrum of control (containing silica and solvents only) (green) stacked over the ^{1}H NMR spectrum of the inhibition zone's extract (red). A new doublet peak on the extract spectrum is seen at chemical shift of 0.90 ppm.

The two major groups of terpenes isolated from bacteria are hydrocarbon and alcohol terpenes and it was also mentioned that these terpenes (e.g. Geosmin) are responsible for the "earthly" smell of the soil (Scholler et al., 2002; Yamada et al., 2012; Yamada et al., 2015). Terpenes are known to play a role as antimicrobial and antitumor agents, pigments and hormones (Rabe et al., 2013).

GC-MS was conducted to identify the volatile compounds present in the ethyl acetate extract of *S. parvulus* strain R5-344. The GC-MS spectrum of the purified compound was compared to the

GC-MS spectrum of the control (silica) (Appendix, Figure 5.4 and 5.5) and from that the peaks were only visible on the spectrum of the antibacterial compound(s). Volatile metabolites that are widespread in organisms include alcohols, terpenes, hydrocarbons, esters, and many more (Rowan, 2011; Audrain et al., 2015). Volatile metabolites are produced as mode of interactions in bacteria in different environments (such as biofilms) and play an important role in food and cosmetics industries (Audrian et al., 2015).

Based on the compounds that were identified in this study, two contained an ester functional group, typical of volatile metabolites. At a retention time of 16.425 minutes, one of the compounds was found to be 92 % similar to a compound 2-methyloctacosane (Figure 3.6). A similar compound was identified by Nandhini et al. (2015), that was present in *Streptomyces cacaoi* and was found to be active against bacteria such as *Pseudomonas aeruginosa* and *Salmonella typhi*. The compound belongs to the class of alkanes that contains a long chain of C-H bonds making it very hydrophobic.

Another compound that was identified at a retention time of 22.185 minutes with a similarity index of 87 % to octacosyl acetate. The structure of octacosyl acetate contains a functional ester group accompanied by a long chain of C-H bonds. Octacosyl acetate is an ester molecule with several hydrocarbons and was found in the wax produced by *Melipona bicolor* (Koedam et al., 2002). No information about the compound being produced by bacteria has been found.

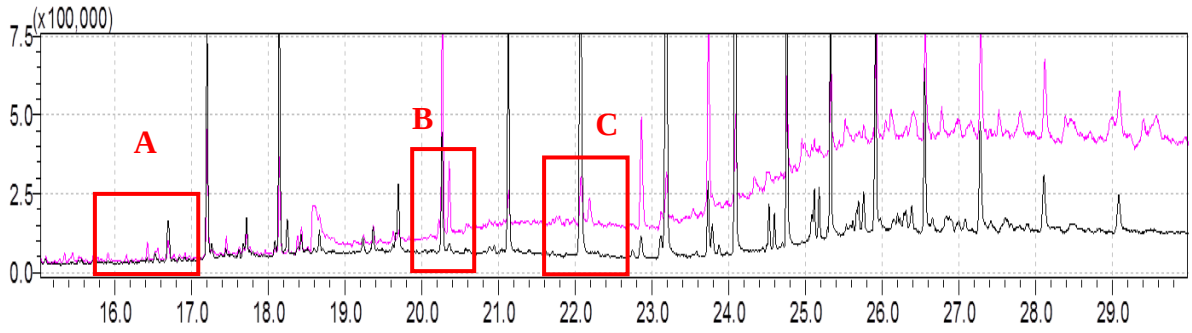

Figure 3.6: GC-MS chromatogram of the control (black) vs bacterial inhibition zone's extract (pink) chromatogram showing three major compounds only present in the *Streptomyces* bacterial extract's chromatogram identified at retention times of 16.420 (A), 20.355 (B) and 22.185 (C) minutes.

The third compound was identified at 20.355 minutes and had a 92 % similarity index to acetic acid n-octadecyl ester. The structure of acetic acid n-octadecyl ester is similar to octacosyl acetate but differ in the number of C-H bonds (Table 3.1). There has not been any evidence that this compound has been identified in bacteria, however it has been found in several plant and fungal species. Sivagnanam & Vialliammai (2016) identified acetic acid n-octadecyl ester as one of the compounds found in the plant chloroform extract of *Croton bonplandianusby* and Siddiquee et al. (2012) also found it in hexane extract of *Trichoderma harzianum*. Karthi et al. (2015) mentioned that acetic acid n-octadecyl ester had been reported to have antimicrobial activity.

Table 3.1: Ethyl acetate extract of *Streptomyces parvulus* was analysed with GC-MS and three compounds were identified.

Compound name and structure	**Retention Time (minutes)**	**Percentage similarity (%)**
Octacosyl acetate 	22.185	87
Acetic acid n-octadecyl ester 	20.355	92
2-Methyloctacosane 	16.420	92

The discovery of new drugs that are active against some of the most lethal bacteria-related infections has decreased over the years and therefore the need for new antibiotics is of a crucial matter. In 2000 and 2003, the FDA had introduced the newly approved antibacterial compounds, linezolid and daptomycin (Table 3.2) with novel modes of action (Spellberg et al., 2004). Linezolid is a synthetic drug that belong to the oxazolidinones that prevents the process of protein synthesis (Livermore, 2003) and daptomycin is a lipopeptide antibiotic produced by *Streptomyces roseosporus.* Daptomycin is used to treat Gram-positive bacteria-related infections and it does so by disrupting their cell membranes (Steenbergen et al., 2005).

Table 3.2: Two new antibacterial compounds with novel mode of actions (indicated in red box) approved by the FDA of USA (Spellberg et al., 2004).

Drug	Year approved	Novel mechanism
Rifapentine	1998	No
Quinupristin/dalfopristin	1999	No[a]
Moxifloxacin	1999	No
Gatifloxacin	1999	No
Linezolid	2000	Yes
Cefditoren pivoxil	2001	No
Ertapenem	2001	No
Gemifloxacin	2003	No
Daptomycin	2003	Yes

[a] The mechanism of the streptogramins (quinupristin and dalfopristin) is closely related to that of the macrolide/lincosamide families [63].

3.4. Conclusion

The ethyl acetate extract of *Streptomyces parvulus* strain R5-344 had good antibacterial activity against the *Microbacterium* sp. and the R_f value of the compound(s) was 0.76 making it non-polar. The extract was further analysed using ^{1}H NMR and peaks were found at 4.73 ppm, 4.35 ppm and 0.90 ppm, which were not present in the agar control. To identify this compound(s), GC-MS was conducted, and three main compounds were found and were tentatively identified to be similar to 2-methyloctacosane with a 92 % similarity, acetic acid n-octadecyl aster with a 92 % similarity and octacosyl acetate with a 87 % similarity at retention times of 16.425, 20.355 and 22.185 minutes, respectively. Two of the compound's structures have an ester functional group with a long chain of C-H bonds and the other one only contains a long chain of C-H bonds with one methyl group attached.

3.5. References

1. Asha-Devi, N. K., Rajendran, R., & Karthik-Sundaram, S. (2011). Isolation and characterization of bioactive compounds from marine bacteria. Indian Journal of Natural Products and Resources, 2 (1): 59-64.
2. Audrain, B., Farag, M. A., Ryu, C. M., & Ghigo, J. M. (2015). Role of bacterial volatile compounds in bacterial biology. FEMS Microbiology Reviews, 39 (2): 222–233.
3. Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). "Methods for in vitro evaluating antimicrobial activity: A review." Journal of Pharmaceutical Analysis, 6 (2): 71-79.

4. Bele, A. A., & Khale, A. (2011). An overview on Thin Layer Chromatography. International Journal of Pharmaceutical Sciences and Research, 2 (2): 256-267.
5. Berdy, J. (2005). Bioactive microbial metabolites: a personal view. Journal of Antibiotics, 58 (1): 1-26.
6. Choma, I. M., & Grzelak, E. M. (2010). Bioautography detection in Thin-Layer Chromatography. Journal of Chromatography A, 1-8.
7. Deepa, L. S., Gowthami, K., & Kumar, K. S. (2011). In vivo Screening of Antimicrobial Activity of Soil Actinomycetes against Human Pathogens. World Journal of Agricultural Sciences, 7 (5): 624-628.
8. Dewanjeea, S., Gangopadhyayb, M., Bhattacharyaa, N., Khanraa, R., & Dua, T. K. (2014). Bioautography and its scope in the field of natural product chemistry. Journal of Pharmaceutical Analysis, 5 (2): 75-84.
9. Doriya, K., Jose, N., Gowda, M., & Kumar, D. S. (2016). Chapter Six - Solid-State Fermentation VS Submerged Fermentation for the Production of l-Asparaginase. Advances in Food and Nutrition Research. S.-K. Kim and F. Toldrá, Academic Press, 78: 115-135.
10. Emwas, A. H. M., Merzaban, J. S., & Serrai, H. (2015). Theory and Applications of NMR-Based Metabolomics in Human Disease Diagnosis. Chapter 3. Rahman, A., & Choudhary, I. (Eds). Applications of NMR spectroscopy, 1: 93-130.
11. Ghadin, N., Zin, N. M., Sabaratnam, V., Badya, N., Basri, D. F., Lian, H. H., & Sidik, N. M. (2008). Isolation and characterization of a novel endophytic *Streptomyces* SUK 06 with antimicrobial activity from Malaysian plant. Asian Journal of Plant Sciences, 7: 189-194.
12. Grzelak, E. M., Huang, C., Cai, G., Nam, J. W., Choules, M. P., Gao, W., Lankin, D. C., McAlpine, J. B., Mulugeta, S. G., Napolitano, J. G., Suh, J. W., Yang, S. H., Cheng, J.,

Lee, H., Kim, J. Y., Cho, S. H., Pauli, G. F., Franzblau, S. G., & Jaki, B. U. (2016). Bioautography with TLC-MS/NMR for Rapid Discovery of Anti-Tuberculosis Lead Compounds from Natural Sources. ACS Infectious Disease, 2: 294-301.

13. Islam, N., Parveen, S. A., Nakazawa, N., Marston, A., & Hostettmann, K. (2003). Bioautography with the fungus *Valsa ceratosperma* in the search for antimycotic agents. Pharmaceutical Biology, 41: 637-640.
14. Jesionek, W., Moricz, A. M., Alberti, A., Ott, P. G., Kocsis, B., Horvath, G., & Choma, I. M. (2015). TLC-Direct Bioautography as a Bioassay Guided Method for Investigation of Antibacterial Compounds in *Hypericum perforatum* L. Journal of AOAC International, 98 (4): 1013-1020.
15. Karthi, S., Somanath, B., & Ali, A. J. H. (2015). Efficacy of Methanolic Extract of a Marine Ascidian, *Lissoclinum bistratum* for Antimicrobial Activity. Journal of Chemical, Biological and Physical Sciences, 5: 4119-4125.
16. Kayser, O., & W. J. Quax (2007). Medicinal plant biotechnology: from basic research to industrial applications, Wiley-VCH.
17. Koedam, D., Jungnickel, H., Tentschert, T., Jones, G. R., & Morgan, E. D. (2002). Production of wax by virgin queens of the stingless bee *Melipona bicolor* (Apidae, Meliponinae). Insectes Sociaux, 49: 229 – 233.
18. Livermore, D. M. (2003). Linezolid in vitro: mechanism and antibacterial spectrum. Journal of Antimicrobial Chemotherapy, 51 (2): ii9–ii16.
19. Nandhini, U. S., Sangareshwari, S., & Lata, K. (2015). Gas Chromatography-Mass Spectrometrey analysis of Bioactive Constituents from the Marine Streptomyces. Asian Journal of Pharmaceutical and Clinical Research, 8 (2): 244-246.
20. Parthasarathi, S., Kim, C. J., Kim, P. K., Sathya, S., Manikandan, M., Manikandan, T., & Karuppaiya, B. (2010). Taxonomic characterization and UV/VIS analysis of antagonistic

marine actinomycete isolated from South West Coast of South Korea. International Journal of Medicobiological Research, 1 (2): 99-105.

21. Pavia, D. L., Kriz, G. S., Lampman, G. M., & Engel, R. G. (2015). A Small Scale Approach to Organic Laboratory Techniques, Cengage Learning.
22. Rabe, P., Citron, C. A., & Dickschat, J. S. (2013). Volatile Terpenes from Actinomycetes: A Biosynthetic Study Correlating Chemical Analyses to Genome Data. ChemBioChem, 14: 2345-2354.
23. Rhee, I. K., Van de Meent, M., Ingkaninan, K., & Verpoorte, R. (2001). Screening for acetylcholinesterase inhibitors from Amaryllidaceae using silica gel thin-layer chromatography in combination with bioactivity staining. Journal of Chromatography A, 915 (1-2): 217-223.
24. Rios, J. L., Recio, M. C., & Villar, A. (1988). Screening methods for natural products with antimicrobial activity: A review of literature. Journal of Ethnopharmacology, 23: 127-149.
25. Rowan, D. D. (2011). Volatile Metabolites. Metabolites, 1 (1): 41–63.
26. Sandhya, C., Sumantha, A., Szakacs, G., & Pandey, A. (2005). Comparative evaluation of neutral protease production by *Aspergillus oryzae* in submerged and solid-state fermentation. Process Biochemistry, 40 (8): 2689-2694.
27. Scholler, E. C., Gurtler, H., Pedersen, R., Molin, S., & Wilkins, K. (2002). Volatile metabolites from actinomycetes. Journal of Agricultural and Food chemistry, 50: 2615−2621.
28. Sherma, J., & Fried, B. (2003). Handbook of Thin-Layer Chromatography, Taylor & Francis.
29. Siddiquee, S., Cheong, B. E., Taslima, K., Kausar, H., & Hasan, M. M. (2012). Separation and Identification of Volatile Compounds from Liquid Cultures of *Trichoderma*

harzianum by GC-MS using Three Different Capillary Columns. Journal of Chromatographic Science, 50: 358–367.

30. Sivagnanam, S., & Valliammai, R. (2016). Determination of Bioactive and Pharmaceutical Components of *Croton bonplandianus* by GC-MS Analysis. International Journal of Pharmaceutical Sciences and Nanotechnology, 9 (5): 3488-3493.
31. Sneddon, J., Masuram, S., & Richert, J. C. (2007). Gas Chromatography-Mass Spectrometry basic principles, instrumentation and selected applications for detection of organic compounds. Analytical letters, 40 (6): 1003-1012.
32. Spellberg, B., Powers, J. H., Brass, E. P., Miller, L. G., John E., & Edwards, J. E. (2004). Trends in Antimicrobial Drug Development: Implications for the Future.Clinical Infectious Diseases, 38 (9): 1279–1286.
33. Steenbergen, J. N., JAlder, J., Thorne, G. M., & Tally, F. P. (2005). Daptomycin: a lipopeptide antibiotic for the treatment of serious Gram-positive infections. Journal of Antimicrobial Chemotherapy, 55 (3): 283–288.
34. Suleiman, M. M., McGaw, L. J., Naidoo, V., & Eloff, J. N. (2010). Detection of antimicrobial compounds by bioautography of different extracts of leaves of selected South African tree species. African Journal of Traditional Complementary and Alternative Medicines, 7 (1): 64 – 78.
35. Usha, R. (2011). Antibiotic production, purification, characterization and biological activity of newly isolated *Streptomyces parvulus* KUAP106, PhD thesis.
36. Uzair, B., Ahmed, N., Ahmad, V. U., Mohammad, F. V., & Edwards, D. H. (2008). The isolation, purification and biological activity of a novel antibacterial compound produced by *Pseudomonas stutzeri*. FEMS Microbiology Letters, 279: 243-250
37. Valle Jr., D. L., Puzon, J. J. M., Cabrera, E. C., & Rivera, W. L. (2016). Thin Layer Chromatography-Bioautography and Gas Chromatography-Mass Spectrometry of

Antimicrobial Leaf Extracts from Philippine *Piper betle* L. against Multidrug-Resistant Bacteria. Evidence-Based Complementary and Alternative Medicine, 1-7.

38. Yamada, Y., Kuzuyama, T., Komatsu, M., Shin-Ya, K., Omura, S., Cane, D. E., & Ikeda, H. (2015). Terpene synthases are widely distributed in bacteria. Proceedings of the National Academy of Sciences, 112 (3): 857-862.
39. Yamada, Y., Cane, D. E., & Ikeda, H. (2012). Chapter Seven - Diversity and Analysis of Bacterial Terpene Synthases. Methods in Enzymology. D. A. Hopwood, Academic Press, 515: 123-162.

CHAPTER 4: GENERAL CONCLUSIONS AND FUTURE PROSPECTS

4.1. General Conclusion

Research on discovering and developing new antibiotics has been of great importance over the years and has shown that many bacteria in the soil have antagonistic activity against some of the most infectious bacterial species. Bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE) and *Pseudomonas* species as well as other multi-resistant bacteria have reduced the effectiveness and use of some of the most important antibiotics known. Treatment of infections caused by these multi-resistant bacteria had become expensive and less available to the public. The antibiotics that have lost their effectiveness have been manipulated structurally and by doing so; the previously resistant bacteria will no longer be susceptible to the antibiotic.

Three *Streptomyces* spp. were isolated from the soil using a serial dilution method and all showed good antagonistic activity against some of the selected test bacteria (also isolated from the same soil sample as the *Streptomyces* spp.). The cross-streak method on Mueller Hinton agar was used to achieve the results. From the three *Streptomyces* spp. isolates, only one with the best activity was chosen for further studies. The inhibition zones were not determined because the growth of the test bacteria were so reduced that it was not visible to measure. To determine whether the results were reliable, all the tested bacteria were grown individually as controls and observed that they grew very well, indicating that the medium used was not the reason behind the inhibition. The three isolated *Streptomyces* spp. were identified based on the colony morphology and 16S rRNA sequencing, which confirmed that all three belong to the genus *Streptomyces*. However in this dissertation only the sequencing results for one *Streptomyces* sp. was shown which was identified to be *S. parvulus* strain R5-344 (with 100 % identity). Two of the other soil isolated susceptible bacteria used to indicate antibacterial

activity were also identified and are most probably *Kocuria turfanensis* strain HO-9042 (with 100 % identity) and uncultured *Microbacterium* sp. clone 1c05 (with 99 % identity). Many bacteria with the potential to produce antibiotics were isolated from the Namibian soil and the majority belonged to actinomycetes. Amongst the actinomycetes, the genus *Streptomyces* is known to produce naturally occurring antibiotics (Velho-Pereira & Kamat, 2011; Hossain & Rahman, 2014).

MHA plates containing *S. parvulus* strain R5-344 tested on the *Microbacterium* sp. were used to extract the inhibitory compound, with ethyl acetate as the extraction solvent. The extract was then subjected to direct TLC bio-autography and applied to a TLC plate and the TLC plate then sprayed with the *Microbacterium* sp. suspension after 24 hours. It was observed that the extract exhibited good antibacterial activity against the *Microbacterium* sp. The compound had an R_f value of 0.76 and based on this information it was concluded that the antibacterial compound was non-polar. To identify the structure of the antibacterial compound, it was subjected to ^{1}H NMR and GC-MS analyses. Based on the ^{1}H NMR results, unique peaks were observed at chemical shifts of 4.73 ppm, 4.35 ppm and 0.90 ppm in the *S. parvulus* extract spectrum which were not found on the agar control spectrum. Furthermore, GC-MS analysis of the extract was conducted and three compounds were identified, namely, 2-methyloctacosane at retention time of 16.420 minutes, octacosyl acetate at retention time of 22.185 minutes and acetic acid n-octadecyl ester at retention time of 20.355 minutes. These compounds contained similar long chains of saturated C-H bonds and this support the non-polar polarity. A study by Nandhini et al. (2015), identified 2-methyloctacosane as one of the compounds produced by *Streptomyces cacaoi* and it was found to be active against bacteria such as *Pseudomonas aeruginosa* and *Salmonella typhi*.

The hypothesis was partially supported because the soil isolated *S. parvulus* does produce antibacterial compounds which are active against some Gram-positive bacteria and to fully support/reject the hypothesis, more analytical studies must be conducted.

4.2. Future prospects

The TLC-bioautography must be repeated to obtain higher quantities of the active compounds for NMR and GC-MS analyses. The minimum inhibition concentration (MIC) of the compounds on several pathogens must then be determined so that a quantitative conclusion about the antibacterial activity can finally be reached.

4.3. References

1. Hossain, N., & Rahman, M. (2014). Antagonistic activity of antibiotic producing *Streptomyces* sp. against fish and human pathogenic bacteria. Brazilian Archives of Biology and Technology, 57 (2): 233-237.
2. Nandhini, U. S., Sangareshwari, S., & Lata, K. (2015). Gas Chromatography-Mass Spectrometry analysis of Bioactive Constituents from the Marine *Streptomyces*. Asian Journal of Pharmaceutical and Clinical Research, 8 (2): 244-246.
3. Velho-Pereira, S., & Kamat, N. M. (2011). Antimicrobial Screening of Actinobacteria using a Modified Cross-Streak Method. Indian Journal of Pharmaceutical Sciences, 73 (2): 223–228.

CHAPTER 5: APPENDIXES

5.1. Cross-streak method used to test for antagonistic activity of *Streptomyces* (centre) against four known pathogens

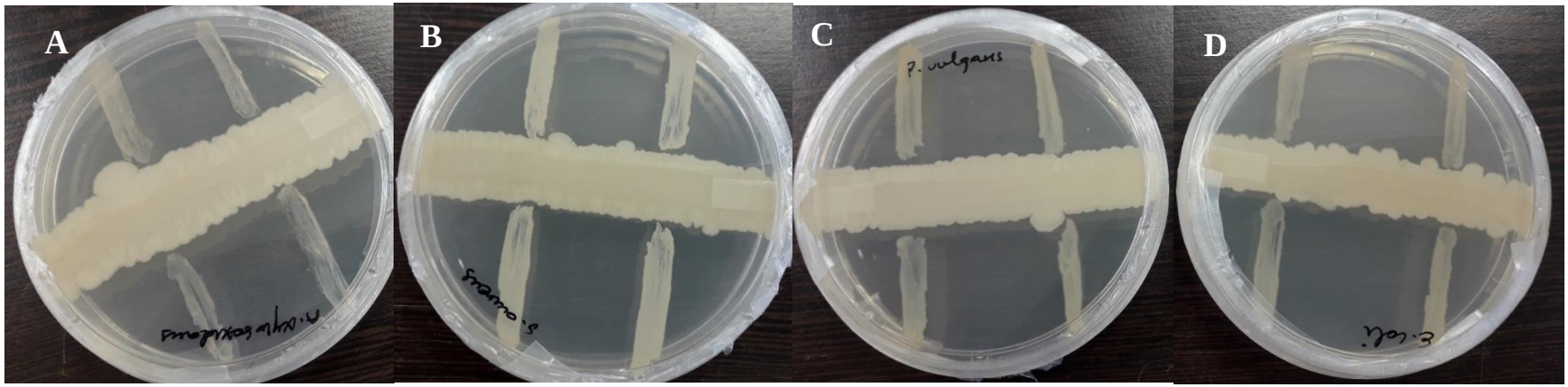

Figure 5.1: *Streptomyces purvulus* tested against the four known pathogens. A: *Achromobacter xylosoxidans*, B: *Staphylococcus aureus*, C: *Proteus vulgaris*, D: *Escherichia coli*.

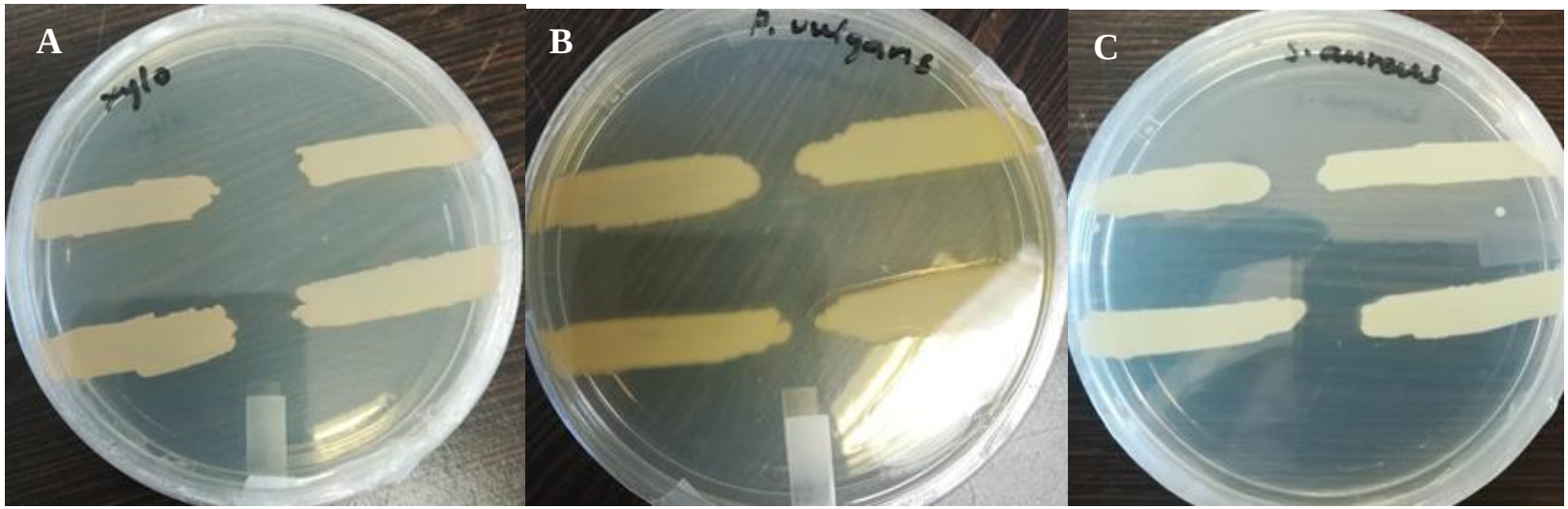

Figure 5.2: Negative controls of the tested pathogens. A: *A. xylosoxidans*, B: *P. vulgaris*, C: *S. aureus*.

5.2. Gel electrophoresis after DNA extraction and PCR

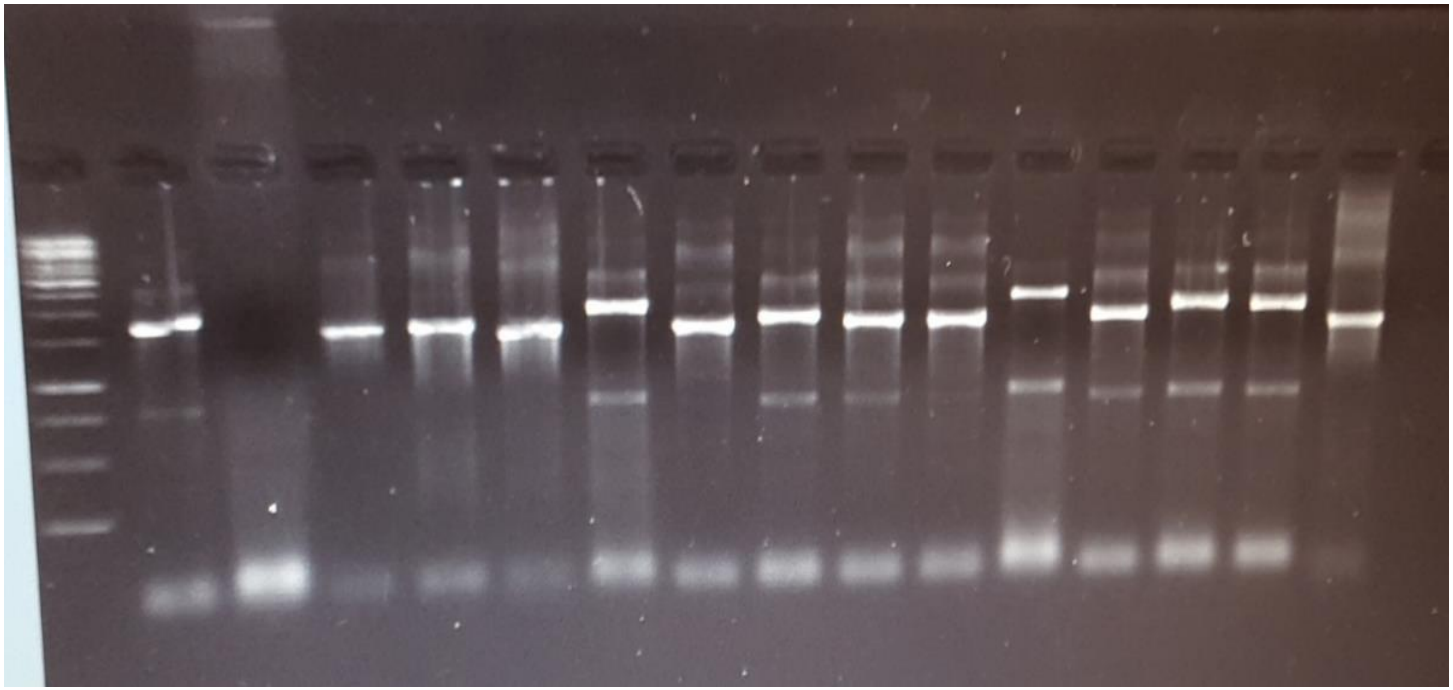

Figure 5.3: Gel electrophoresis was done after DNA extraction to show the presence of the extracted DNA in the sample. The brighter the band, the more DNA concentration meaning that is good enough to perform PCR. The other bands might be too faint, but they were still used for PCR.

5.3. GC-MS spectra of the controls

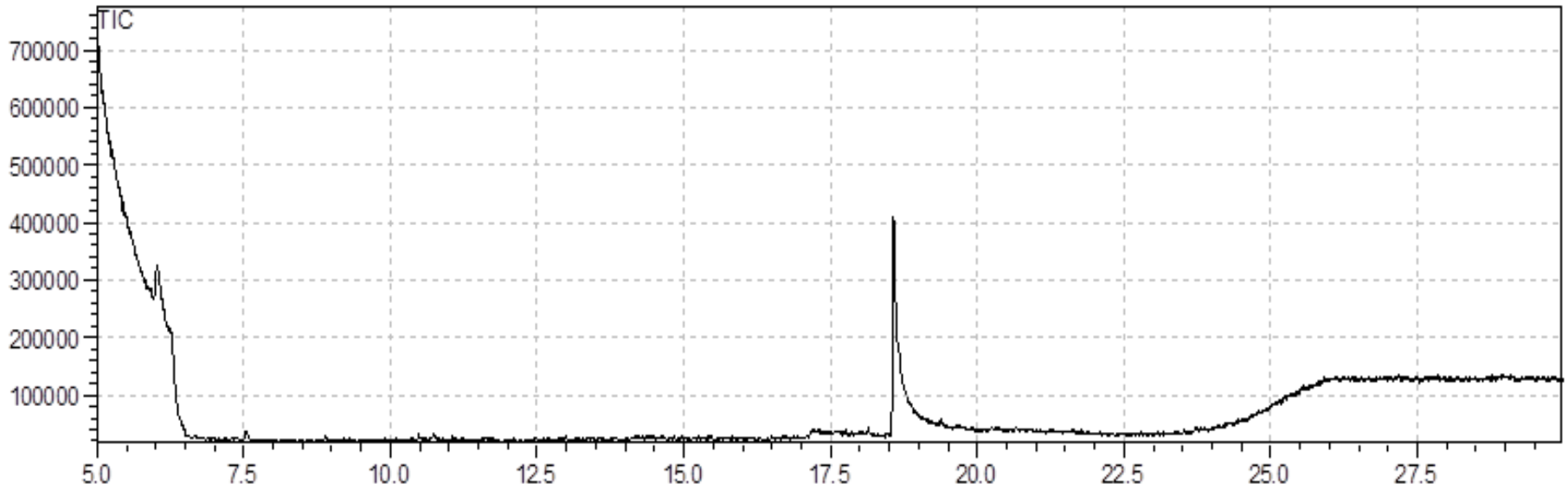

Figure 5.4: The full GC-MS spectrum of the solvent acetone without the extract or silica.

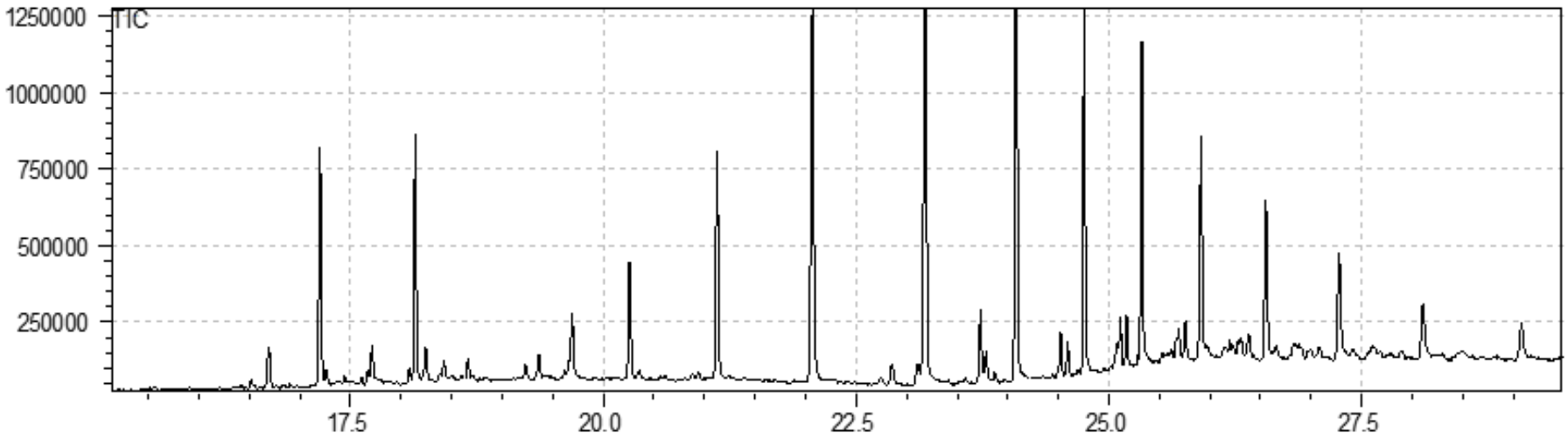

Figure 5.5: The full GC-MS spectrum of silica scrapped from TLC plate used as a negative control.