AMERICAN UNIVERSITY OF BEIRUT

DISCOVERY OF NOVEL ANTIBACTERIAL AGENTS TARGETTING MRSA PATHOGENS FROM UNEXPLOITED ACTINOMYCETES

by
CHRISTELLE KAMAL YAMMINE

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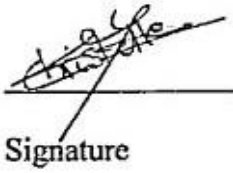

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AN ABSTRACT OF THE THESIS OF

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Title: Discovery of Novel Antibacterial Agents Targeting MRSA Pathogens from Unexploited Actinomycetes

Background: Antimicrobial resistance (AMR) has been present ever since the discovery of the first classes of antibiotics, where Sir Alexander Fleming warned about it in his Nobel Prize speech back in 1945. With the lack of new antibiotic classes and the continuous emergence of resistant bacteria, it is estimated that AMR will be killing 10 million people yearly by the year 2050 if no significant measures are taken. Discovering new antibacterial classes in addition to unexploited targets should be the main goal to overcome this problem. Natural resources, such as the soil, harbor microorganisms, *Actinomycetes* that can produce secondary metabolites that could have antibacterial activity, and could, therefore, be used in the search for new antibacterial classes. According to the World Health Organization (WHO) pathogen priority list, *Staphylococcus aureus* Methicillin-resistant (MRSA) was considered as a high priority pathogen in 2017 which makes it a relevant target for these prospective novel agents. This study aims to purify novel *Actinomycetes* from soil samples and screen them against a panel of pathogenic bacteria to discover new classes and targets of antibacterial agents.

Methods: Soil samples were collected from different regions in Lebanon and were dried, heated, and serially diluted before streaking on ISP3 and soil agar. The observed bacterial colonies were purified based on their morphology and stored in glycerol. The bacteria were then put under stress in 14 different production media and the secondary metabolites produced were extracted using Amberlite XAD-16N resin, methanol, and acetone and screened for antibacterial activity on *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* Newman, *Staphylococcus aureus* N315, *Enterococcus faecalis* ATCC 19433, *Klebsiella pneumoniae* DSM, *Acinetobacter baumannii* DSM 30008, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* MEXAB, and *Escherichia coli* ATCC 25922 using a broth microdilution assay. The medium that showed the highest number of wells with inhibition was then up-scaled and further separated into fractions. DNA was also extracted from bacteria of interest and 16S rRNA sequencing in addition to whole-genome sequencing was performed to identify novel *Actinomycetes* strains. NaCl and pH tolerance tests were also performed on the strain.

Results: The extract produced by strain ZK3 showed significant inhibition in medium RA3 on Gram-positive bacteria including *Staphylococcus aureus* N315, *and Enterococcus faecalis* ATCC 19433. Inhibition was also evident in the ethyl acetate and the chloroform fractions after separation of the upscale. The sequenced PCR product of 16S rRNA was blasted and the results show that the strain belongs to the *Streptomyces* species. Significant biosynthetic gene clusters were evident in the antiSMASH results of the WGS product. Testing also showed that 0% NaCl concentration and pH 7 were optimal conditions for growth.

Conclusion: In this study, we were able to isolate bacteria from soil, harboring inhibitory activity against MRSA, and other Gram-positive bacteria. The extract produced by this bacteria should be further purified and tested to discover the nature and the structure of the compound in addition to its mode of action.

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CHAPTER I

INTRODUCTION

Before Streptomycin, if a patient was diagnosed with *Mycobacterium tuberculosis,* the doctor's orders were ineffectively restricted to fresh air and rest [1]. Pneumonia once called "Captain of the Men of Death" by William Osler, used to kill about 40% of its victims [2]. Minor surgeries were fatal, epidemics and outbreaks were easily ignited, and microorganisms were widely feared. All of the above-mentioned examples were characteristics of a period known as the pre-antibiotic era. In 1928, Sir Alexander Fleming first noticed antibacterial activity on an uncovered petri dish left by the window and consequently, revolutionized medicine with this discovery [3]. A few years later, Prontosil was introduced in clinics after its discovery by biochemist Gerhard Domagk and the antibiotic era was initiated [4]. However, as dangerous as a time without antibacterial agents may seem, an even more dangerous era was about to unfold with the emergence of antimicrobial resistance (AMR), which threatened the antibiotic golden age and worried the medical community. Efforts were put into trying to understand why and how resistance was emerging and most importantly, what should be done to overcome resistance.

Now, over 70 years after the first observation of resistance to Prontosil, AMR imposes a great threat to human life. According to the Antibiotic Resistance Threats Report done by the Centers for Disease Control and Prevention (CDC), the number of deaths caused by resistant bacteria has increased by approximately 12,000 deaths per year from 2013 to 2019 in the United States of America (USA) [5] [6]. At this rate, it is

estimated that by the year 2050, AMR will be the number one cause of death worldwide, killing around 10 million people per year [7].

In order to address this issue, copious measures were and still are taken into consideration. These actions include: increasing awareness towards AMR, monitoring the use of common antibacterial agents, increasing and reshaping surveillance systems to get more accurate data in different countries, which all fall under the objectives of the Global Action Plan against Antimicrobial Resistance adopted by the World Health Organization (WHO) [8]. However, the biggest hope still lies in research dedicated to the discovery of new antibacterial agents. And although more than 40 new antibacterial agents by June 2019 have been involved in clinical trials, many of them share similar targets or belong to similar classes [9]. Therefore, patterns of resistance can be anticipated. That being said, for this discovery process to be effective, studies should focus on identifying new classes of antimicrobial drugs aimed against previously unexploited targets.

Going back to Sir Alexander Fleming, his discovery of Penicillin not only introduced the world to antibacterial activity but also to the fact that this activity can be harbored by a natural product. According to Newman and Craig, 42.5% of small-molecule antibacterial drugs were either natural products or derived from natural products while synthetic drugs made up 33% [10]. Natural products result from the secondary metabolism of diverse organisms residing in soil and the sea. The soil, in particular, serves as a habitat for a large phylum of Gram-positive, non-pathogenic, environmental bacteria known as *Actinomycetes*, which contains genus *Streptomyces* from which many of the current clinically used antibiotics originate, such as tetracyclines, macrolides, and aminoglycosides [11].

An additional fundamental aspect of research and discovery is knowing which pathogens to target. In 2017, the WHO published a pathogen priority list which aimed at shifting the focus of researchers towards pathogens that are imposing the most serious threats to human health in terms of resistance development. Methicillin-resistant *Staphylococcus aureus* (MRSA), according to this catalog, was considered a high priority pathogen, and therefore an important target for drug discovery [12]. Since the discovery of new antibacterials today is as important as it was back in the pre-antibiotic era, we aim at isolating and identifying novel *Actinomycetes* from soil samples and at screening their secondary metabolites against a panel of pathogenic bacteria including MRSA, to discover new classes of antibacterial agents with new targets.

CHAPTER II

LITERATURE REVIEW

A. Antimicrobial Resistance

1. Definitions and Origin

In 1945, Sir Alexander Fleming stated that "there is the danger that the ignorant man may easily under-dose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant" [13]. This statement was part of his Nobel Lecture on Penicillin and in it, the discoverer of Penicillin himself was able to predict the emergence of AMR.

The WHO defines AMR as being the consequence of constant exposure of microorganisms to antimicrobials. The modification that takes place due to this exposure causes the microbe to become resistant and renders the drug ineffective. AMR doesn't only refer to resistant bacteria, but also viruses, fungi, and parasites [14].

It is important to note that the emergence of resistance occurred in parallel to the discovery of the most famous classes of antibacterials that are still used in clinics today. For instance, both the introduction of Sulfonamides into healthcare and the emergence of resistance mechanisms against these antibiotics took place in the late 1930s, almost simultaneously. Another example would be the fact that penicillinase, which is an enzyme that inhibits the action of Penicillin, was discovered before Penicillin was even brought into the clinics [15]. This is shown in figure 2.1 below.

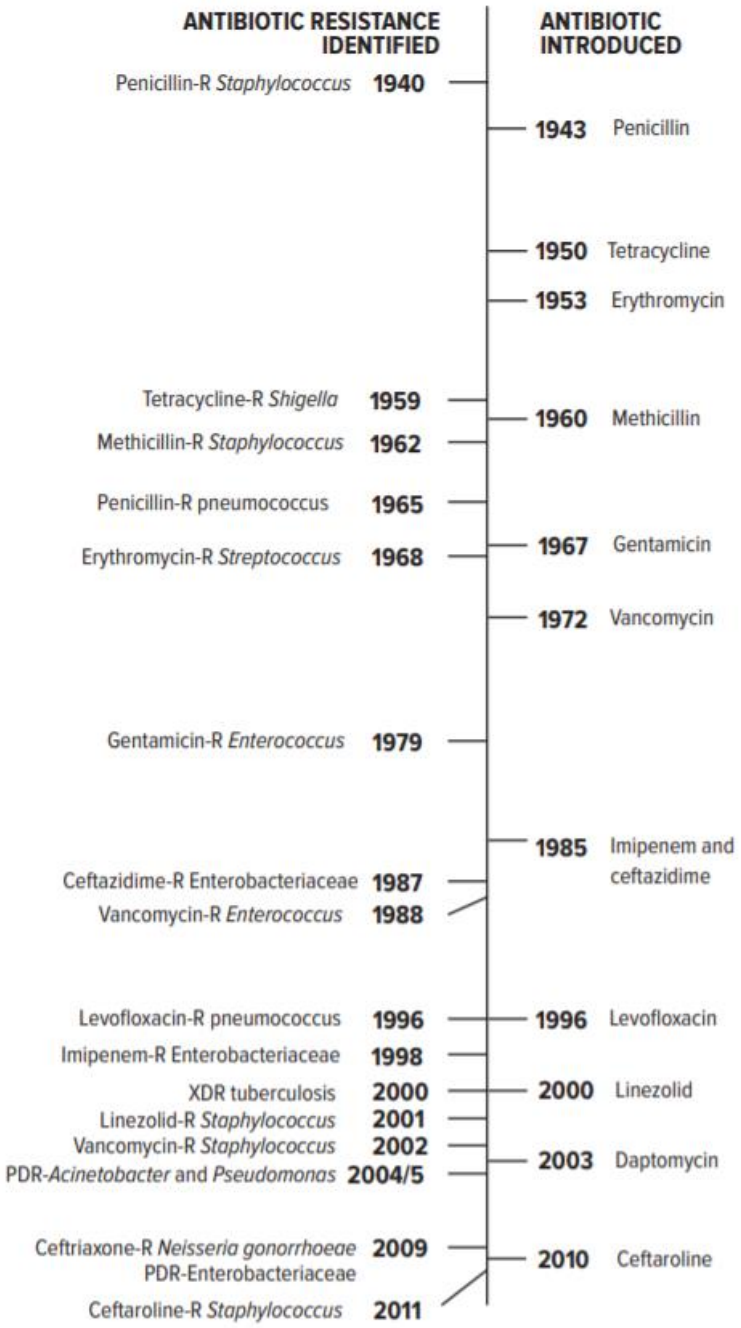


Figure 2. 1: Timeline showing the years in which antibiotics were introduced and the antibiotic resistance was identified. Adapted from [16].

Bacteria can be classified either based on the type of resistance it harbors or on the amount of the classes of antibacterials it can resist. To classify these pathogens under clearly defined terms, the categorization of antibacterials should be relevant to the

epidemiology of the bacterium. In other words, the antibacterials for which certain bacteria would be termed MDR, XDR, and PDR would essentially differ based on each bacterium. However, in general terms, and based on an article published in Clinical Microbiology and Infection [16], the following definitions are considered:

- MDR (multi-drug resistance) is defined as non-susceptibility to at least 1 antibacterial agent in 3 or more antimicrobial classes.
- XDR (extensively-drug resistance) is defined as non-susceptibility to at least 1 antibacterial agent in all except 2 or fewer antimicrobial classes.
- PDR (pan-drug resistance) is defined as non-susceptibility to all antibacterial agents in all antimicrobial classes.

The numbers attributed to MDR, XDR, and PDR bacterial strains today are alarming. Based on two studies done in two different tertiary care hospitals, bacterial isolates were tested. In the first study, which included Gram-positive and Gram-negative isolates, 37% of the total strains were found to be MDR and 13.8% were found to be XDR [17]. The second study included only Gram-negative isolates. 8.1% were found to be XDR while 0.9% were found to be PDR. This means that 11 out of 1,240 isolates were not susceptible to all antibacterial agents and 6 out of them were shown to be resistant to “last resort” antibiotics like colistin and tigecycline and therefore, leave no treatment options for physicians [18]. The numbers above might not seem significant but offer a glimpse of what should be expected if resistant strains keep emerging at this rate.

2. Causes

a. Microbial Behavior

Antibacterial resistance is essentially a survival mechanism employed by bacteria to be able to adapt to new environmental stresses. It is a form of natural selection. This takes place due to acquired bacterial resistance. Mutations, for example, will cause a change in the genome of the bacteria which can stop a certain protein from being expressed or express a different protein instead. [19]. If the mutation favors the resistance of the bacteria to a certain antibacterial, when this pathogen is exposed to this specific antimicrobial, the bacteria will survive and will pass on the mutation to its progeny [20].

Other forms of acquired resistance involve gene transfer to bacteria or what is known as Horizontal Gene Transfer (HGT). These include 3 main mechanisms [21]:

- Transformation occurs when specific bacteria in specific suitable conditions can pick up genetic material from the environment and integrate it either in its genome or in a plasmid.
- Conjugation takes place when there is a direct transfer of genetic material from bacteria to different bacteria through pili. Plasmids carrying resistance genes are commonly transferred through conjugation.
- Transduction involves bacteriophages that transmit genetic material to bacteria. Transferred sequences can either be chromosomal or plasmidic.

The acquired sequence could be crucial to the mechanism of resistance that would be employed by the bacteria. For instance, the newly expressed protein could work as an efflux pump, preventing the accumulation of the drug inside the cell. It could

also act as an enzyme that would modify the drug, deactivate it, or denature it. These are usually proteins coded by genes found in plasmids. The bacterial chromosome could code for some proteins that could even modify the target site of the drug making it unable to bind and therefore disabling the action of the antibiotic [22]. The different mechanisms of resistance explained above are depicted in Figure 2.2.

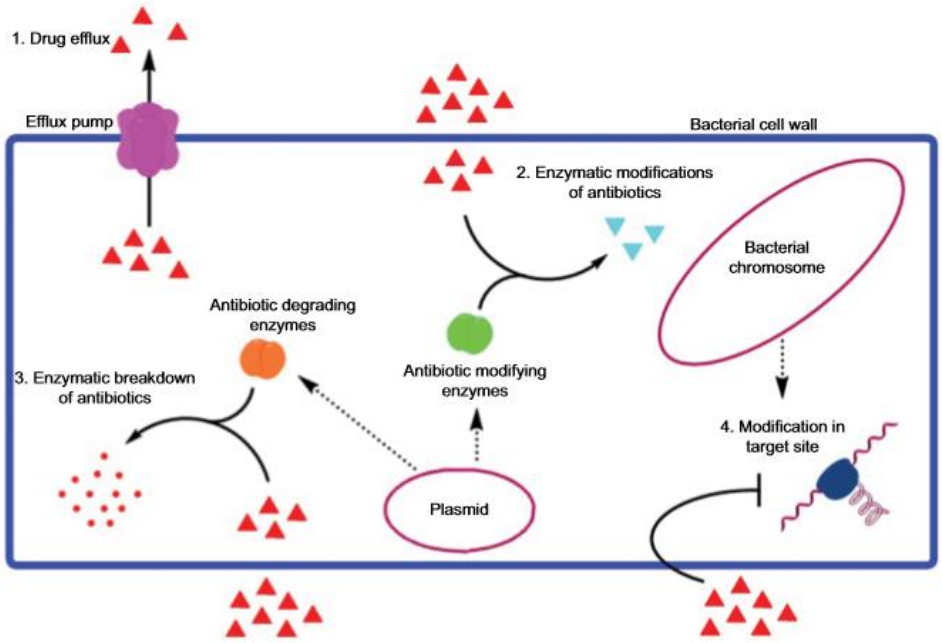


Figure 2. 2: Schematic representation of the main mechanisms employed by bacteria for antibiotic resistance. Retrieved from [24].

When a bacterial population is exposed to an antibacterial, the susceptible cells will die, leaving only those that acquired resistance to survive, thus enabling their replication without competition. Given the fast replication rate employed by most bacterial species, this leads to the formation of colonies with millions of bacteria that can resist the drug and survive in even harsher conditions than their predecessors. This is called selective evolution [19].

b. Human Behavior

Human behavior includes many actions done by both physicians and patients, as well as the use of antimicrobials outside the medical field. All of these events speed up the rate at which resistance is emerging and make the situation even more unanswerable. When patients visit their doctor's office suffering from symptoms that point to an infection, many physicians tend to prescribe antimicrobial drugs based on past experiences and assumptions. The absence of testing, either due to lack of time or available resources, usually leads the patients into taking excessive non-needed antibiotics [19]. According to the U.S Food and Drug Administration (FDA), out of 100 people who have sore throats and are usually prescribed antibiotics as treatment, only 15 suffer from a bacterial infection usually caused by *Streptococcus pyogenes* [23]. This is dangerous because it exposes the microbiota of the patient to unnecessary drugs that kill susceptible bacteria and spare bacteria that have become resistant [19].

The responsibility falls on the patient as well. The availability of antibiotics in the pharmacies as over-the-counter medicine has allowed people to bypass the physician and self-prescribe unnecessary antibiotics to treat themselves. In addition, people who self-medicate tend to stop the antibiotic course as soon as they start feeling better, thus accelerating more the spread of AMR [24]. This problem is not only restricted to pharmacies. The scope is widened due to the online availability of these drugs which makes it even easier for the patient to access them. Mainous et al. demonstrated in a study in the US in 2009 that 63.8% of online sellers would require from the patient to take an "online health history" before prescribing and selling a certain drug while 36.2% would provide the ordered antibiotic without any recommendation or physician's

prescription. The antibiotics that were available online were of different classes and some were even broad-spectrum antibiotics. The most commonly found were Penicillins and Macrolides [25].

Apart from taking or prescribing unneeded antibiotics for sick people, a more dangerous AMR-related concern imposes itself on the mass population, which manifests in the use of antibiotics in farm animals. Sometimes, antibiotics are given to treat animals for certain diseases or as a prophylactic measure to prevent specific illnesses. However, farmers tend to take advantage of the situation and provide antibiotics for farm animals to enhance their growth. This is usually done in large farms with high numbers of animals taking high doses of antibiotics simultaneously which creates a suitable environment for the emergence of resistant strains of bacteria in the animals. These strains subsequently end up in the human consumer [26]. Colistin (polymyxin E) is a very tangible example in this case. This is an antibiotic that is nephrotoxic and is usually considered as a “last resort” antibiotic for hospitalized patients. Colistin also happens to be one of the most commonly used antibiotics in poultry farms. A study done in 2015 in France showed that Colistin-resistant genes were present in the manure of chicken treated with Colistin. Findings also included that composting the manure did not get rid of the plasmids carrying the genes, which were still transferrable [27].

3. Global Burden

As mentioned earlier, AMR is a significant issue that troubled the world ever since the discovery of antimicrobials. The global work to overcome this problem began when the WHO tackled this issue for the first time in 1959 and decided that research

should be done on AMR [28]. Throughout the years, AMR shifted from being merely a concern to a global public health issue that threatens human lives as well as the international economy. Starting with the USA, the CDC estimated in 2013 in its report that AMR costs the USA yearly about 20 billion dollars as direct costs [5]. On average, 2.8 million Americans suffer from an infection that is antibiotic-resistant per year. 35,000 people die as a result [6]. In Europe, The European Commission published in 2017 a document pertaining to its action plan against AMR. In it, it is estimated that Europe loses around 1.5 billion euros per year as total costs due to AMR and around 25,000 human lives [29]. The reason behind these alarming numbers is the drastic impact that AMR leaves on healthcare systems and national economies. AMR costs hospitals more money due to the costly treatments and extended stays in hospitals and intensive care units. This leads to the utilization of more resources, whether human (nurses, physicians, healthcare workers…) or capital (hospital beds, equipment…) [30]. Based on the previously listed factors as well as many others, the extrapolations estimate that the world could lose up to 1 trillion dollars from now until 2050, in the absence of an effective approach to limit the spread of AMR [7].

B. Antimicrobials

1. Natural Antimicrobials

The use of natural products in medicine and for the treatment of illnesses was present long before the discovery of antimicrobials. Natural products can be produced from organisms living in natural resources like the soil or the sea via metabolic chemical reactions. The latter can belong to primary metabolism or secondary metabolism. Primary metabolism refers to the sum of the chemical reactions that

produce primary metabolites involved in basic survival. For example, amino acids, sugars, enzyme precursors, DNA, or RNA precursors, all fall under the category of primary metabolites [31]. Secondary metabolism, on the other hand, involves reactions that are not related to the direct survival of the organism but rather to its adaptability in a specific environment or defense mechanisms against other organisms. These could be reactions that have to do with the ability of the organism to acquire specific nutrients or kill its predators or its competitors for better survival conditions [32]. The significance of secondary metabolites lies in their ability to be used for therapeutic purposes.

The Journal of Natural Products (Newman and Cragg, 2020) published different reviews throughout the years showing the newest approved drugs and their sources. Based on these reviews, drugs were classified as being biological macromolecules (B), unaltered natural products (N), natural product derivatives (ND), and synthetics (S) among other classifications. According to the review published in 2020, from January 1981 until September 2019, a total of 401 anti-infective drugs including antibacterial, antifungal, antiviral, and antiparasitic drugs were approved. 107 of them were either natural products or derivatives of natural products [33]. This is depicted in Figure 2.3.

drug class	B	N	ND	S	S/NM	S*	S*/NM	V	total
antibacterial	4	11	78	36			1	32	162
antifungal	1		3	27	3				34
antiviral	17		6	19	9	26	21	87	185
antiparasitic		2	7	6		3		2	20
total	22	13	94	88	12	29	22	121	401
percent all (n = 401)	5.5	3.2	23.4	22.1	3	7.2	5.5	30.1	
percent small (n = 258)		5	36.3	34.4	4.6	11.2	8.5		

Figure 2. 3: Table showing the numbers of all anti-infective drugs based on their type and source from January 1, 1981, to September 30, 2019. Retrieved from [33].

The antibacterials alone made up 162 approved drugs out of the 401. 48% of those antibacterials were either natural products or derived from natural products. The exact numbers are depicted in the pie chart below [33].

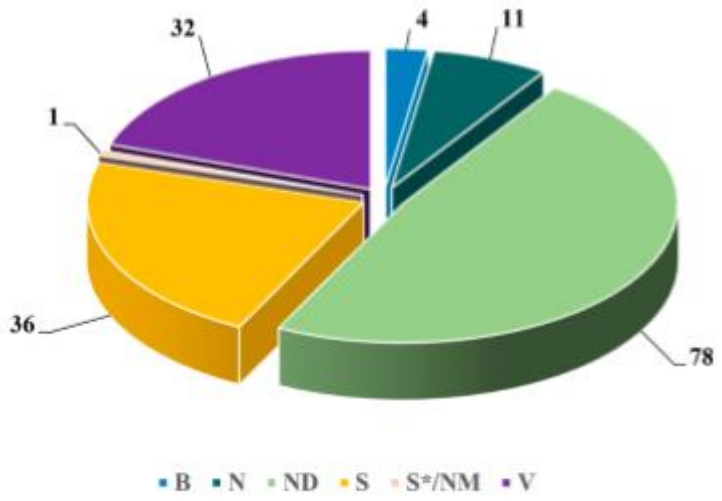


Figure 2. 4: Pie chart showing the percentages of antibacterial drugs based on their source from January 1, 1981, to September 30, 2019. Retrieved from [35].

a. Natural Products from Plants

There are a lot of chemical compounds of medicinal relevance extracted from plants that were studied throughout the years: alkaloids, phenolic compounds, and terpenoids being just a few. All of these compounds are byproducts of the secondary metabolism that plants undergo as explained previously. Alkaloids are nitrogen compounds that are also heterocyclic. An example of an alkaloid with antimicrobial activity would be berberine. Berberine is an alkaloid extracted from 3 plants; *Berberis vulgaris* commonly known as barberry, *Hydrastis canadensis* commonly known as goldenseal, and *Mahonia aquifolium* commonly known as the Oregon grape [34]. Berberine has been shown to have potential activity against some parasites like

trypanosomes [35] and some antifungal activity, acting against cell membranes of *Candida albicans* [36].

As for antibacterial activity, lots of plant products harbor such activity including cranberry, eucalyptus, and turmeric. Eucalyptus, scientifically known as *Eucalyptus globulus*, contains tannin which is also found in green tea and harbors in addition to its antibacterial activity, some antiviral activity as well [37]. Eucalyptus is commonly seen as an ingredient in sanitizers. A specific compound extracted from the essential oils of grapefruits and termed bergamottin epoxide was being tested against MRSA. Promising results were observed when this compound was used to sensitize MRSA to agents it was previously resistant to [38].

b. Natural Product from Fungi

Fungi are a very important source for tons of antimicrobials including some known antibiotics that were used since the beginning of the antibiotic era. A plethora of compounds was isolated from fungi over the years like the non-ribosomal peptides. One of the most commonly known natural products belonging to this group is Penicillin and the subsequent β-lactams Cephalosporins [39]. Cephalosporins were first isolated from *Acremonium* fungi by Italian pharmacologist, Giuseppe Brotzu [40]. β-lactams, in general, inhibit the synthesis of the bacterial cell wall which makes Cephalosporins active essentially against Gram-positive bacteria [41].

Another type of fungal natural products would be terpenoids. Terpenoids are not only natural products produced by plants but can also be produced by certain fungal species, especially those that live in the sea or have algae [42]. Terpenoids are made up

of isoprene units and are divided based on how many of these units they include in their structure [43]. Back in 2014, a study was performed on a specific *Aspergillus ochraceus* strain named as the Jcma1F17 strain. Compounds were extracted from this strain, one of which, the sesquiterpenoid insulicolide A57 showed antiviral activity against the H3N2 influenza virus and EV71 enterovirus [44].

c. Natural Product from Bacteria

Probably the most common source of natural products is bacteria. Bacteria are essentially found everywhere, but those that are of interest in the drug discovery field tend to be marine or soil-dwelling. Additionally, these bacteria tend to belong to a wide group called the *Actinomycetes* [45]. These are Gram-positive, mostly aerobic bacteria that can form hyphae and filaments. They are found abundantly on the surface of the soil and could be found at great depths as well. Since they are considered environmental bacteria, their optimal growth temperature would be around 28°C and they would grow usually at a neutral pH [46]. The Life cycle of *Actinomycetes* is depicted in the figure below.

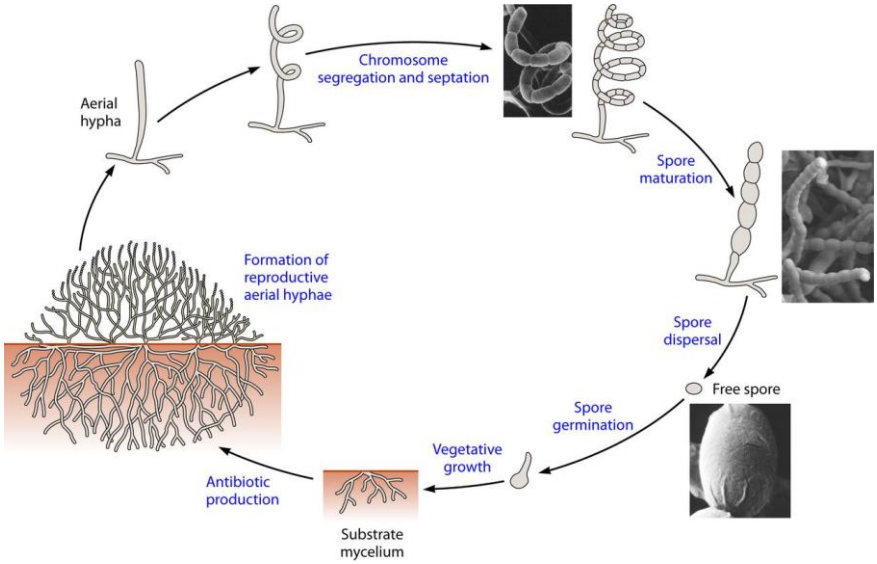


Figure 2. 5: Schematic representation of the life cycle of *Actinomycetes*. Retrieved from [48].

The most common genus of *Actinomycetes* that is found in the soil is *Streptomyces*. These are sporulating bacteria that form their spores on an aerial mycelium [45]. The life cycle of *Streptomyces* follows these steps: the germination of the spore, the formation of the primary mycelium which is also called the vegetative hyphae and is responsible for the absorption of nutrients, and the formation of the aerial mycelium where spores are produced [47]. *Streptomycetes* are mostly known for their production of secondary metabolites which serve as antimicrobials for infection treatment. When the bacteria undergo stress due to a deficiency in a specific nutrient, the primary mycelium will automatically degrade by a cell-death process which allows it to become a source of nutrients for the aerial mycelium [48]. The production of these nutrients will attract different microbes which pushes the bacterium to defend itself and its nutrients by producing secondary metabolites that would kill the invading organisms.

The appearance of *Streptomycetes* on agar is usually fluffy. This is attributed to the aerial hyphae. [46].

Daptomycin is an example of an antibiotic produced by *Streptomycetes* and is currently used in clinics. In the 1980s, scientists from Eli Lilly and Co, a pharmaceutical company based in the US, were able to isolate daptomycin from *Streptomyces roseosporus,* from soil located in Turkey in addition to a group of antibiotics referred to as A21987C [49]. Daptomycin is a lipopeptide, made up of 13 amino acids, a hydrophilic core, and a lipophilic tail. It works by attacking the plasma membrane of the bacterial cell. It does so by depolarizing the membrane via channels, eventually killing the cell, making Daptomycin bactericidal. The cell wall in gram-positive bacteria is not disrupted [50]. Concerning mechanisms of resistance to Daptomycin, it is yet not very clear how mechanisms of resistance work or whether resistance has been witnessed. This could be attributed to the mechanism of action of Daptomycin which doesn't involve DNA or proteins and therefore, has a lower chance of mutational resistance [51].

Also, as mentioned earlier, *Actinomycetes* and more precisely *Streptomycetes* can reside in the sea. Desotamide B is a cyclic peptide that was isolated from *Streptomyces scopuliridis* and which showed activity against *Staphylococcus aureus* and *Streptococcus pneumoniae* [52]. Another example would be Marfomycins A, B, E which were isolated from *Streptomyces drozdowiczii* and showed activity against *Micrococcus luteus* which is a Gram-positive coccus [53].

Other than *Streptomycetes*, *Actinomycetes* include different genera that are found in the environment, and that are also sources of many medically significant

natural products. For example, Gentamycin is a broad spectrum aminoglycoside that is isolated from *Micromonospora* genus [54]. Nocardicin A is a β-lactam that works against Gram-negative bacteria and is produced by the *Nocardia* genus [55].

C. Methicillin-Resistant *Staphylococcus aureus*

1. Origin

Staphylococcus aureus is a Gram-positive coccus. It is catalase-positive and is usually observed under the microscope as clusters. It is considered part of the normal flora of the mouth and the nares. It causes infection when it has access through the skin or into the bloodstream [56]. Soon enough after the clinical use of Penicillin, which was initially used to treat *S. aureus*, Penicillin-resistant *S. aureus* strains started emerging. These strains were producing β-lactamase which breaks down the β-lactam ring present in Penicillin, rendering the antibiotic inactive [57]. Physicians resorted to the use of Methicillin for treatment instead. Methicillin is a semisynthetic Penicillin that works against the same types of pathogens as Penicillin does, but is resistant to the action of β-lactamase which made it an appropriate treatment for Penicillin-resistant *S. aureus* [58]. The appearance of Methicillin-resistant strains of *S. aureus* (MRSA) was observed in 1961, only 2 years after the introduction of Methicillin and was mostly found in hospitals and nursing homes which characterized MRSA at the beginning as being a nosocomial infection before it was found in the community [59] [60].

2. Clinical Manifestations and Risk Groups

Initially, an *S. aureus* infection could be mistaken for an insect bite. At the site of infection, the typical signs of inflammation show; redness, swelling, pain… The infection could be presented as a boil, a lesion, or impetigo. Under certain circumstances, the simple skin infection could progress into cellulitis or purulent abscesses that might need to be drained surgically. If the patient is immune-compromised, and the infection is left untreated, it could lead to several illnesses ranging from pneumonia and deep tissue infection to endocarditis and meningitis and eventually sepsis [61]. The severity of the infection usually depends on whether the patient acquired it in the community (CA-MRSA), like for example in a gym, or whether he/she acquired it while being hospitalized (HA-MRSA), maybe due to a surgery or an in-dwelling catheter. CA-MRSA is typically associated with skin and soft tissue infections (SSTIs) [62]. People who contract the bacteria are mostly previously healthy, however, sequencing has shown that some of these strains carry the gene that codes for the Panton-Valentine toxin which is the causative agent for tissue necrosis [63]. Other illnesses related to progressive infection of CA-MRSA are osteomyelitis and Waterhouse-Friderichsen syndrome which causes bleeding in the adrenal glands. People at risk include soldiers, people with lacerations and open skin wounds, homosexuals… HA-MRSA is usually associated with hospitalized patients that stay in the hospital for a long time. These patients are more at risk of acquiring a severe MRSA infection especially patients who had just undergone surgery or are maintaining respiration on a ventilator, or have any type of indwelling catheter. Older individuals, previous colonization or infection with MRSA, or past intake of multiple antibiotics are risk

factors. The most common severe infections related to HA-MRSA include pneumonia, bacteremia, and arthritis [62].

3. Mechanism of Resistance to Methicillin

Methicillin's mode of action involves the inhibition of the cell wall synthesis of Gram-positive bacteria. It can bind to the Penicillin-binding proteins (PBPs) also known as the transpeptidases that are present on the surface of the bacterial cell wall. These proteins are responsible for the cross-linking in the peptidoglycan which makes up the bacterial cell wall of Gram-positive bacteria. Once Methicillin binds to them, the crosslinking stops and the cell wall is unable to form [64].

Methicillin-resistant bacteria differ from Methicillin-sensitive bacteria by possessing a specific genetic element in their genome termed as the Mec element which is 40 to 60 kb long. Within this element, the mecA gene is responsible for the expression of a specific type of PBP termed as PBP2a. This type of transpeptidase can perform the crosslinking job of the regular PBPs but is unable to bind Methicillin, making Methicillin ineffective in bacterial strains possessing such a gene [65].

4. Treatment

For patients who do not require hospitalization, skin infection is usually treated topically or drained. For patients with severe MRSA infection, intravenous Vancomycin is usually the drug of choice [66]. Vancomycin, isolated in the 1950s from *Streptomyces orientalis*, is a glycopeptide that works on cell wall synthesis, similar to Methicillin. However, it is a sterical inhibitor of peptidoglycan formation and does not bind to the

PBPs as Methicillin does, which makes it active against MRSA [67]. At the beginning of the 2000s, studies have shown that Vancomycin-resistant strains might be starting to emerge (VRSA) [68]. This was shown to happen due to MRSA strains acquiring the vanA operon. This operon works on expressing proteins that breakdown the Vancomycin target on the peptidoglycan and forms different peptidoglycan precursors that do not bind Vancomycin but are still able to form the bacterial cell wall [69].

Daptomycin could be an effective choice for people with MRSA infections because administering it at high doses was shown to be relatively safe [70]. When Vancomycin is not an option, Daptomycin could be an alternative. However, it has been shown that decreased susceptibility to Vancomycin might be correlated with decreased susceptibility to Daptomycin, which makes Daptomycin not a very good solution in VRSA strains [71]. In addition, a clinical trial was held between the years 2002 and 2005 that aimed at finding alternative treatment to endocarditis and bacteremia caused by *S. aureus*. Daptomycin was given to patients instead of standard treatment but the results showed non-inferiority which means that no significant difference was noted between the standard treatment, and the new one, which in this case is Daptomycin [72]. All of these facts highlight the urgency of discovering new treatment options for MRSA and VRSA.

5. Global Burden

Hospitals today face a serious issue with MRSA infections. In certain hospitals, MRSA infections make up more than half of all *S. aureus* infections [73]. In 2019, MRSA cost the US 1.7 billion dollars. 323,700 cases of MRSA positive cultures were

identified out of which 10,600 died. Those numbers only included hospitalized patients regardless of whether the infection was acquired in the hospital or the community. Non-hospitalized patients were not even counted. Even though the numbers have been decreasing since 2005, the rate of the decrease has slowed down in recent years specifically for hospital-acquired MRSA infections [6].

Figure 2.6 depicts the different criteria evaluated and followed by the WHO that led MRSA to be considered a high priority pathogen. Based on the studies done, MRSA was found to have the following characteristics among others [12]:

- High mortality, falling in the 21% – 40% range.
- Very high health care burden which means patients are usually hospitalized and are commonly admitted to the intensive care unit (ICU).
- High community burden which means that outside hospital settings, resistance to this pathogen is encountered frequently and can cause a systemic infection.

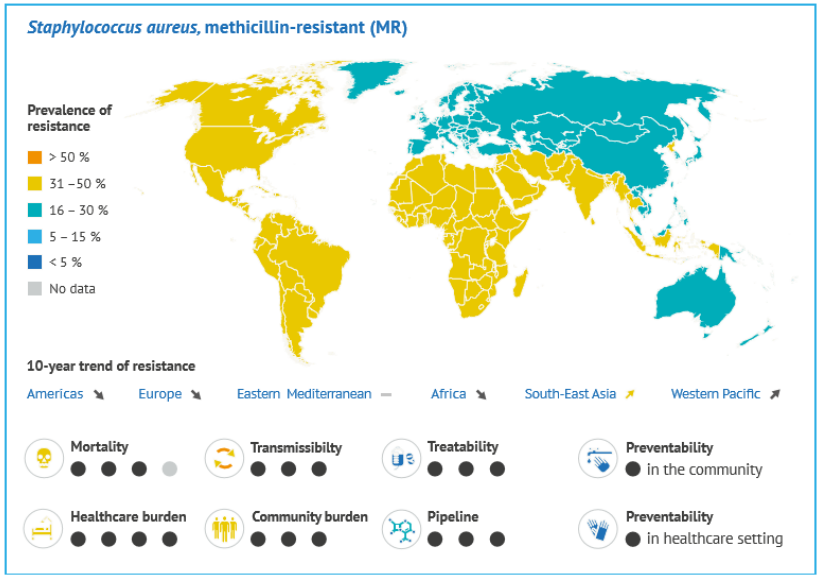


Figure 2. 6: Map showing the distribution of the prevalence of resistance and the criteria for prioritization by the WHO for MRSA. Retrieved from [12].

As AMR keeps ringing alarm bells, opportunities for discovery are always available. This research aims at attempting to resolve the resistance issue, not just in MRSA strains but also in different clinically relevant pathogens by taking advantage of the resources available, especially soil bacteria, isolating and screening novel and already existing strains, and eventually discovering new compounds.

CHAPTER III

MATERIALS AND METHODS

A. Soil Preparation and Bacteria Isolation

1. Serial Dilutions

Soil samples were collected from the Zekrit region in the Maten area, Mount Lebanon. Two soil samples were taken from the surface and two were taken from a depth of 10 cm below the surface. The specimens were dried at 37ºC for 7 days. 3 grams of each sample was heated with 100 mL of water at 55ºC for 30 minutes. The samples were serially diluted with autoclaved distilled water to obtain a final volume of 1 mL. The measures were as follows:

- 1/5: 200 µL non-diluted mixture + 800 µL distilled water
- 1/10: 500 µL of the 1/5 mixture + 500 µL distilled water
- 1/100: 100 µL of the 1/10 mixture + 900 µL distilled water
- 1/1000: 100 µL of the 1/100 mixture + 900 µL distilled water

2. Preparation and Streaking on ISP3 plates

From each dilution solution, 30 µL was streaked on both a soil agar plate and an ISP3 agar plate (International *Streptomyces* Project 3 Medium) [74]. The soil agar was prepared from the same soil as the streaked sample with the addition of soluble starch and bacteriological agar. ISP3, a medium used to isolate *Streptomyces* species, was prepared by mixing 20 grams of commercial oats, 18 grams of bacteriological agar,

and 2.5 mL of ISP3 trace elements ($FeSO_4$, $MnSO_4$, $ZnSO_4$) with 1 liter of distilled water. The plates were left for at least 1 week in the incubator at 28ºC.

3. Isolation

The isolation of strains was performed by taking a single colony from the soil plates using a loop or an autoclaved toothpick and streaking it on an ISP3 plate. The colonies were distinguished through color, morphology, and production of droplets, among a variety of other characteristics. This process was done for several different colonies and was repeated multiple times until the strain was completely pure. The plates were always covered with parafilm and kept in the incubator at 28ºC until spores were formed.

4. Storage in Glycerol

The strains, once purified, were stored in glycerol stocks at -80ºC. This was done by adding 2 mL of autoclaved Milli-Q water on the plate containing the pure strain. Using an autoclaved swab, the bacteria or spores were scraped off the plate and pipetted along with the water into an autoclaved Eppendorf tube. The tube was centrifuged at 4000 rpm for 10 minutes at 4ºC. The supernatant was discarded and the pellet was placed in a 50% glycerol solution to be used later on.

B. Metabolite Extraction

1. First and Second Seed Preparation

Prior to use, the pellet stored in 50% glycerol solution was thawed on ice. Then, 35 µL of the glycerol stock was inoculated in 5 mL of ISP3 broth (similar to the ISP3 agar but excluding the bacteriological agar). This was referred to as the first seed and was kept for 2 days in a shaker-incubator at 28ºC. 1 mL of the first seed was inoculated in 10 mL of ISP3 broth and kept again for 2 days in a shaker-incubator at 28ºC.

2. Production Media Preparation

14 production media that served as stress-inducing environments for the bacteria were prepared. The media recipes were as follows:

- Media V: Soluble Starch (24 g/L), Dextrose (1 g/L), Meat Extract (3 g/L), Yeast Extract (5 g/L), Tryptic Soy Broth (5 g/L), pH: 7.2
- Media Vegetative: Peptone (5 g/L), Soluble Starch (20 g/L), Meat Extract (2 g/L), Yeast Extract (3 g/L), Tryptic Soy Broth (2 g/L), $CaCO_3$ (1 g/L), pH: 7
- Media A: Peptone (4 g/L), Meat Extract (4 g/L), Yeast Extract (2 g/L), Tryptic Soy Broth (2 g/L), Maltose (20 g/L), Dextrose (10 g/L), pH: 7
- Media B: $CaCO_3$ (0.1 g/L), Glycerol (20 g/L), Glycine (2.5 g/L), NaCl (1 g/L), KH_2PO_4 (1 g/L), $FeSO_4$ (0.1 g/L), $MgSO_4.7H_2O$ (0.1 g/L), pH: 7
- Media C: $CaCO_3$ (0.1 g/L), Glycine (2.5 g/L), NaCl (1 g/L), KH_2PO_4 (1 g/L), $FeSO_4$ (0.1 g/L), $MgSO_4.7H_2O$ (0.1 g/L), Tween 60 (20 g/L), pH: 7
- Media INA: $CaCO_3$ (5 g/L), Glycerol (30 g/L), NaCl (2 g/L), pH: 7.3

- Media RA3: Peptone (2 g/L), Yeast Extract (4 g/L), Malt Extract (10 g/L), Glucose (10 g/L), Glycerol (5 g/L), $MgCl_2.6H_2O$ (2 g/L), pH: 7.4
- Media GPMY: Potato Starch (20 g/L), Yeast Extract (5 g/L), Malt Extract (5 g/L), Glycerol (20 g/L), pH: 7.02
- Media V6: Peptone (5 g/L), Meat Extract (5 g/L), Yeast Extract (5 g/L), Glucose (20 g/L), Hydrolyzed Casein (3 g/L), NaCl (1.5 g/L), pH: 7.05
- Media AF/MS: Dextrose (20 g/L), Yeast Extract (2 g/L), Tryptic Soy Broth (6 g/L), $CaCO_3$ (4 g/L), NaCl (1 g/L), pH: 7.3
- Media GYM: Yeast Extract (4 g/L), Malt Extract (10 g/L), Glucose (4 g/L), pH: 7
- Media M8: Soluble Starch (20 g/L), Meat Extract (2 g/L), Yeast Extract (2 g/L), Glucose (10 g/L), $CaCO_3$ (3 g/L), Glycine (4 g/L), pH: 7
- Media COM: Glucose (7.5 g/L), $CaCO_3$ (0.6 g/L), NaCl (0.6 g/L), KH_2PO_4 (0.045 g/L), Soy Flour (7.5 g/L), Dried Beer Yeast (0.9 g/L), Ammonium Sulfate (0.6 g/L), Soybean Oil (0.9 g/L), pH: 8.4
- Media NL2: Soluble Starch (9 g/L), Yeast Extract (0.75 g/L), $CaCO_3$ (3 g/L), Molasses (6 g/L), Soy Flour (4.5 g/L), pH: 7.8

3. Inoculation and Production Conditions

1 mL of the second seed was inoculated in 50 mL of each of these media and kept for 7 days in a shaker-incubator at 28ºC. On day number 7, 1 mL of Amberlite XAD 16N resin (Sigma-Aldrich) was added to each of the media to absorb the metabolites, and the mixtures were put in the same shaker-incubator at 28ºC. Three hours later, the flasks were removed from the incubator and the mixtures were

centrifuged at 4000 rpm for 20 minutes at a temperature of 4ºC. The supernatant was discarded and the pellet (containing cells and resins) was transferred to a new flask. Then, 30 mL of acetone and 10 mL of methanol were added to the pellet to extract the metabolites from the resins, and the mixture was put on a shaker at room temperature for 2 hours. Subsequently, the resins were filtered using glass wool and the liquid phase was left to dry, either for a couple of days in the chemical hood or directly via a rotary evaporator.

4. Storage of Crude Extract in DMSO

The dried extracts were then dissolved in 1 mL of DMSO, sonicated using the ultrasonic cleaner (DAIHAN Scientific), and stored in Eppendorf tubes at -20ºC.

C. Screening Against Pathogenic Bacteria

1. Broth Microdilution Assay

The screening process was performed using the broth microdilution (BMD) technique and done on a panel of pathogenic bacteria which includes; *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* Newman, *Staphylococcus aureus* N315, *Enterococcus faecalis* ATCC 19433, *Klebsiella pneumoniae* DSM, *Acinetobacter baumannii* DSM 30008, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* MEXAB, and *Escherichia coli* ATCC 25922. The staphylococcal species were streaked on Tryptic Soy Agar (TSA) (Lab M, NEOGEN), the *Enterococcus faecalis* was streaked on Brain and Heart Infusion Agar (BHI) (Lab M, NEOGEN) and the rest were streaked on Luria-Bertani Agar (LB) (Lab M, NEOGEN). All the media

were prepared as per instructions of the manufacturer and the plates were kept for 24 hours in an incubator at 37ºC. 195 µL of cation-adjusted Mueller Hinton broth (MHCAB) (BD Phoenix^{TM}), also prepared as per instructions of the manufacturer, was added in the first column of the 96 well-plate and 100 µL of the same broth was added in the remaining columns (2 to 12). 5 µL of the crude extract was added to the first column resulting in a total volume of 200 µL in each well of the first column. Using a multichannel pipette, 100 µL was taken from each well of the first column and pipetted in the adjacent column resulting in serial dilutions following a decreasing concentration gradient extending from column number 1 to column number 11. Column number 12 was left without extract and was considered a positive control. An additional row containing 100 µL of MHCAB only was added and was considered a negative control. No bacteria were added in the wells of this row.

Bacterial inoculum was then prepared using the following method. Approximately 2 mL of MHCAB was transferred into a sterile tube. The tube was put in a densitometer and optical density was adjusted to reach a difference of 0.5 McFarland between the clean broth and the bacterial suspension. The colonies used came from the overnight solid culture. 750 µL of the suspension in the tube was added to 14.25 mL of MHCAB resulting in a suspension with a concentration of $5x10^6$ CFU/mL. 10 µL of this suspension was added to each column excluding the row acting as a negative control, leading to a final bacterial concentration of $5x10^5$ CFU/mL in each well. The plates were kept on the shaker in a 37ºC incubator for 24 hours. The reading was based on the observation of inhibition in the wells. Growth was observed in column number 12 to validate the viability of the bacteria and no growth was observed in the row acting as a negative control to confirm that the used broth was clean.

D. Upscale and Bio-guided Fractionation

1. Upscale Fermentation

An upscale (12.5 L) of the media with the extract that showed the highest number of inhibited wells was prepared. First and second seeds similar in concept to those prepared for the extraction of metabolites were also prepared. An additional third seed was also done. It contained 10 mL of ISP3 broth and 1 mL of the second seed and was kept for 2 days in a shaker-incubator at 28ºC. 20 mL of the third seed was inoculated for each 1 liter of prepared media and the bacterial suspensions were kept for 10 days in a shaker-incubator at 28ºC.

On day 10, autoclaved Amberlite XAD 16N resin was added (25 mL/L media) to the media and the upscale was kept for 2 days in a shaker-incubator at 28ºC. On day 3, the media were removed from the incubator and left for 2 hours on the bench for the resin to sediment at the bottom of the flasks. The liquid phase was then decanted keeping the solid pellet in the flask. Using commercial gaze swabs and distilled water, the resins were further separated from any liquid residues and the extraction was done using acetone and methanol following the same proportions as for the small scale. 4 liters of acetone and 1 liter of methanol were added to the resins. The mixture was kept on a shaker at room temperature. The extraction this time was performed 3 times instead of only once to ensure a maximum product yield. The extract was stored in DMSO at -20ºC.

2. Liquid-Liquid Separation

This method was performed to separate the components of the crude extract into fractions, allowing thus to more easily purify the active compound. The stored vial was put on a lyophilizer until all the DMSO was removed. 180 mL of methanol was then added to the extract along with 20 mL of water. After making sure that the stopper was closed, the mixture was placed in a separatory funnel and 200 mL of hexane was added. The funnel was taken out of the ring stand, its top opening was closed, and the funnel was shaken well. Before placing it back on the stand, the stopper was opened to allow for the escape of accumulated gas and the release of pressure formed by the solution. This was repeated a few times. The funnel was then put on the ring stand while making sure that the stopper was closed. The liquids in the funnel separated into two phases, one containing the methanol and the water (at the bottom) since both are polar solvent and one containing the hexane (on top) which is a non-polar solvent. The stopper was slowly opened and the methanol-water combination was drained in a beaker. Once all the methanol-water was drained, the hexane was put in a separate beaker. The methanol-water was put again in the separatory funnel and 150 mL of hexane were added. The methanol-water and hexane fractions were collected again following the same procedure. The drained hexane was added to the hexane drained from the first round. This was repeated twice.

The methanol-water fraction was put on a rotary evaporator. The methanol was evaporated and the water left, was approximately 20 mL. More water was added until the volume became 200 mL. The 200 mL aqueous solution was put in the funnel. This time, 200 mL of chloroform was added. Chloroform was drained, put in a beaker, and

the water was again put in the funnel and mixed with 150 mL chloroform and drained again, twice. The chloroform collected from the 3 rounds were joined together.

The water solution collected was added to the funnel and 200 mL of ethyl acetate was also added and mixed in the same way described above. If no clear separation was observed, HCl was added. The water which was at the bottom was drained and the ethyl acetate (on top) was collected and the four fractions obtained were hexane, chloroform, ethyl acetate, and water.

3. Fraction Screening

After the separation into fractions, the broth microdilution assay was performed again on 3 of these fractions (hexane, ethyl acetate, and chloroform) in a way similar to the one performed on the small scale crude extracts described earlier. However, screening was done against only one bacteria of interest *S.aureus* N315. In addition to testing the 3 fractions against N315, the crude extract from the small scale was tested again as a control, and 2 dilutions for the small scale crude extract were prepared and tested as well. The first one included 25 µL DMSO and 25 µL extract (1/2 dilution of the extract, 2.5 mg/mL). The second included 45 µL DMSO and 5 µL extract (1/10 dilution of the extract, 0.5 mg/mL).

E. Phenotypic and Biochemical Characterization of Strain

1. pH and NaCl Percentage Growth

To determine the optimal growth conditions of the isolated environmental strains, biochemical tests were performed. For the sodium chloride tolerance test, Basal

Medium 5339 [74] was prepared. This medium included 10 g/L casein peptone, 5 g/L yeast extract, and 20 g/L bacteriological agar in addition to NaCl such that we get 5 different sodium chloride concentrations; 0%, 2.5%, 5%, 7.5%, and 10%. The pH was adjusted at 7 before sterilization in the autoclave. For the pH tolerance test, ISP2 Medium 5265 [74] was prepared. This medium included 10 g/L malt extract, 4 g/L yeast extract, 4 g/L glucose, and 15 g/L bacteriological agar. The pH was adjusted such that we get 9 different media each with a pH of 2, 3, 4, 5, 6, 7, 8, 9, and 10 before sterilization in the autoclave. The plates were then streaked fully by the strain of interest and kept for at least 14 days in an incubator at 28ºC.

F. Molecular Characterization of Strain

1. DNA Extraction Protocol

For DNA extraction, the QIAamp DNA Mini kit (50) from QIAGEN was used. After thawing the glycerol stock solution on ice, 50 µL was inoculated in 10 mL of Tryptic Soy Broth (TSB), prepared as per instructions of the manufacturer. This was done in duplicates for each sample and the falcon tubes were kept for 2 days or more in a shaker-incubator at 28ºC until sufficient bacterial growth is observed. On the day of the extraction, the duplicate bacterial solutions were merged and centrifuged at 4000 rpm for 10 minutes at 4ºC. After the centrifugation, the supernatant was discarded from each tube and the pellet was washed with a 10X phosphate-buffered saline solution (PBS) (LONZA) to remove debris and media residues. The PBS solution containing the bacterial pellet was transferred to an Eppendorf tube and centrifuged at room temperature and 4000 rpm for 5 minutes. The supernatant was discarded and the pellet was then suspended in 180 µL of a prepared lysozyme solution (20 mg Sigma-Aldrich

lysozyme/mL TE). The content of the Eppendorf tube was vortexed and incubated at 37ºC for approximately 1 hour. During this time, the lysozymes break down the bacterial cell wall. 20 µL of 20 mg/mL proteinase K solution was then added and the tube was put at 56ºC for at least 2 hours. Vortexing the content every 20 minutes was essential to ensure the complete lysis of the cells. The proteinase breaks down proteins that contaminate the DNA in addition to nucleases that might break down the genetic material.

After incubation, 200 µL of lysis AL buffer was added. The tube was vortexed and then kept for 10 minutes at 70ºC, then for 15 minutes at 95ºC. The droplets on both the wall and the lid of the tube were spun down after each incubation. The tube was then centrifuged at room temperature and 4000 rpm for 5 minutes and the pellet containing lysis debris was discarded. The supernatant was pipetted into another Eppendorf tube and 200 µL of 100% ethanol was added to the new tube to precipitate the DNA. The tube was vortexed and spun down. The contents of the tube were transferred to a spin column provided by the kit, and the column was centrifuged at room temperature and 8000 rpm for 1 minute. The column now contained the aggregated DNA only so the collection tube was discarded and the column was placed in another collection tube. The first wash was done by adding 500 µL of AW1 buffer followed by centrifugation at room temperature and 8000 rpm for 1 minute. The second wash was done by adding 500 µL of AW2 buffer followed by centrifugation at room temperature and 14,000 rpm for 3 minutes. Following each centrifugation, the collection tube was discarded and switched with a new one. An additional centrifugation step was performed at 14,000 rpm for 1 minute before transferring the column into an Eppendorf tube and adding 50 µL of the elution buffer (AE) and incubating at room temperature for 1 minute. Finally,

the column was centrifuged at 8,000 rpm for 1 minute and the flow-through in the collection tube containing the extracted DNA. Proteinase K and the buffers including the elution buffer were provided by the kit (QIAGEN).

Quantification of the DNA was performed using the NanoDrop (DeNovix) spectrophotometer. The machine was initially cleaned with distilled water using lint-free tissue paper and 2 µL of the AE buffer that was used for elution of the DNA was used here as well as a blank. After blanking, 2 µL of the extracted DNA was placed in the NanoDrop for checking both yield and purity. After quantification, the DNA extracts were kept at -20ºC.

2. 16S rRNA PCR

Polymerase chain reaction (PCR) was performed on the 16S rRNA gene of the extracted DNA. First, the reverse and the forward primers were diluted: For each 1 µL of primer, 9 µL of distilled autoclaved water was added. The PCR experiment was performed twice, each time for a different set of primers with the following sequences (both from Macrogen):

- Set 1 : SSU-bact-27F: 5'-AGAGTTTGATCMTGGCTCAG-3'
  SSU-bact-519R: 5'-GWATTACCGCGGCKGCTG-3'
- Set 2: 8F: 5'-AGAGTTTGATCCTGGCTCA-3'
  1492R: 5'-TACGGYTACCTTACGACTT-3'

The second set provides wider coverage for the 16s rRNA gene. The master mix was then prepared by adding 0.75 µL of the diluted forward primer, 0.75 µL of the diluted reverse primer, 6 µL of the FIREPol Master Mix (Solis Biodyne), and 19.5 µL

of autoclaved distilled water. In each well, 3 µL of the DNA was added to the 27 µL of the master mix, resulting in a 30 µL total volume. The PCR machine used was the BIO-RAD T100 Thermal Cycler. The protocols followed for each set of primers are depicted in figures 3.1 and 3.2 below.

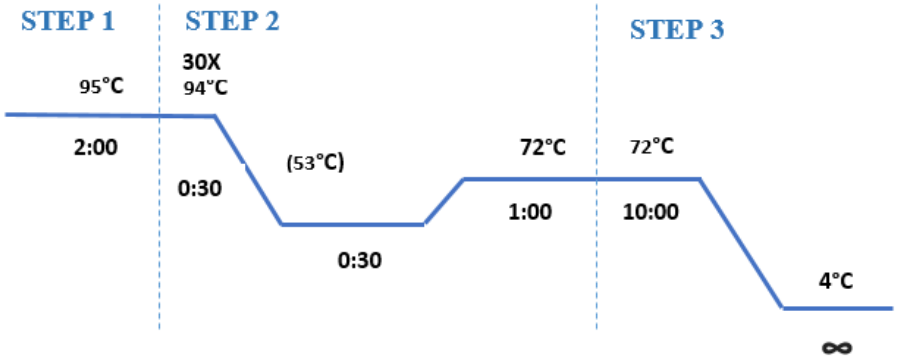


Figure 3. 1: Graph showing the PCR protocol for 16S SSU primer.

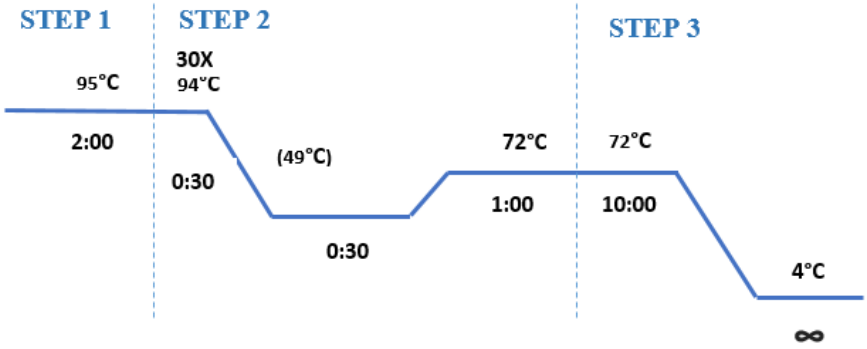


Figure 3. 2: Graph showing the PCR protocol for 16S 8 primer.

3. Gel Electrophoresis

After the run was finished, gel electrophoresis was performed. 100 mL of 10X Tris Borate EDTA buffer (TBE) (AMRESCO) was mixed with 900 mL of distilled water to obtain a 1X TBE solution. 100 mL of the prepared solution was used to completely dissolve 1 gram of LE agarose (VWR Life Science). The flask was heated in the microwave until the solution became clear and then waiting for the solution to cool down. 6 µL of Ethidium Bromide (EtBr) (BIO-RAD) was then added and the solution was mixed by manual swirling. The addition of EtBr is to allow the visualization of the DNA under the UV light. The warm gel was then added to the tray placed on a horizontal surface, straight, to ensure uniformity in the gel thickness and distribution. The comb was then attached to the gel to form wells for the DNA. Once the gel solidified, the comb was removed and the gel was placed in the electrophoresis apparatus where the electric current passes allowing for the migration of the DNA fragments. The initially prepared 1X TBE solution was added to the apparatus as a running buffer. The gel was now ready to be loaded. 5 µL of the 100 bp DNA ladder (Solis Biodyne) was loaded in the first well. Then, 1 µL of the 5X loading dye (Gel Pilot) was mixed with 5 µL of each PCR product by pipetting up and down on a piece of parafilm, and all 6 µL was loaded in the well. When the loading was done, the required voltage of 80 mV was set and the gel was run allowing for the migration of the DNA fragments. At the end of the run, the gel was removed from the apparatus and was put in a UV illuminator to observe the migration of the fragments.

4. Sequencing

The PCR samples were sent for sequencing in Macrogen. The results were aligned using the Molecular Evolutionary Genetics Analysis program (MEGA) and using the National Center for Biotechnology Information (NCBI)'s Basic Local Alignment Search Tool (BLAST), the strains were identified or presumed to be novel strains depending on the percentage of homology with already registered strains. Whole-genome sequencing was also performed and the results were entered on antiSMASH (Antibiotic and Secondary Metabolite Analysis Shell [75] version 5.1.2) to check for the presence of biosynthetic gene clusters.

Whole genome sequencing was also performed. Genomic DNA was extracted and NexteraXT libraries were prepared following the manufacturer's protocols (Illumina, San Diego, CA). The sequencing was done on an Illumina Miniseq High Output Kit (300 cycle).

G. Table of Materials

Material Used	**Brand Name**
Potato Starch	Sigma-Aldrich
Peptone	DIFCO Laboratories
Soluble Starch	Merck
Dextrose	DIFCO™ Laboratories
Meat Extract	CONDA
Yeast Extract	USB
Malt Extract	Lab M, NEOGEN

Glucose	Sigma-Aldrich
Maltose	Sigma-Aldrich
$CaCO_3$	Baker
Glycerol	Sigma-Aldrich
Glycine	Fisher
Hydrolyzed Casein	Fluka® Analytical
NaCl	Merck
KH_2PO_4	Merck
$FeSO_4$	Sigma-Aldrich
Tween 60	Sigma-Aldrich
Soy Bean Flour	Commercial
Dried Beer Yeast	Commercial
$(NH_4)_2SO_4$	Fisher
Casein Peptone	Sigma-Aldrich
Bacteriological Agar	Lab M, NEOGEN
TSB	BD Phoenix™
96-Well Plates	Corning®
PCR Plates	BIO-RAD
Incubator (37ºC)	Thermo Scientific
Incubator (28ºC)	Amerex Instruments
Centrifuge	Thermo Scientific
Rotary Evaporator	Heidolph

Table 3. 1: Table showing the brand names that were not mentioned in the text, of the materials used.

CHAPTER IV

RESULTS

A. Screening Against Pathogenic Bacteria

The screening for antibacterial activity of crude extracts produced by different environmental strains in our 14 distinct production media was performed on a panel of ESKAPE pathogens. These include *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* Newman, *Staphylococcus aureus* N315, *Enterococcus faecalis* ATCC 19433, *Klebsiella pneumoniae* DSM, *Acinetobacter baumannii* DSM 30008, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* MEXAB, and *Escherichia coli* ATCC 25922. Inhibition in the wells was demonstrated by lack of turbidity comparable to what is seen in the negative control wells, while growth in the wells was confirmed by turbidity or the presence of a bacterial pellet at the bottom of the well that was comparable to that of the positive control wells (column 12).

1. ZK3

The extracts produced by the strain ZK3 (figure 4.1) showed the most activity. As demonstrated in table 4.1 and figures 4.2, 4.3, 4.4, and 4.5 below, the secondary metabolites of strain ZK3 showed inhibition against Gram-positive bacteria, specifically *Staphylococcus aureus*. Starting with *S. aureus* ATCC 29213, 9 wells with inhibition were observed for medium RA3, 8 wells with inhibition were observed for media C, AF/MS, and GPMY, 5 wells with inhibition were observed for medium V, 3 wells were

observed for medium B, 2 wells were observed for media INA, GYM, and NL2, 1 well was observed in medium COM, and 0 wells with inhibition were recorded for media A, V6, M8, and Vegetative (Veg).

For *S. aureus* Newman, 10 wells with inhibition were observed for medium RA3, 9 wells with inhibition were observed for medium GPMY, 8 wells were observed for media C and AF/MS, 5 wells were observed for medium V, 4 wells were observed for medium B, 3 wells were observed for media INA, GYM, and NL2, 1 well was observed for the Vegetative medium, and 0 wells with inhibition were recorded for media A, V6, M8, and COM.

For *S. aureus* N315, 11 wells with inhibition were observed for medium RA3, 9 wells with inhibition were observed for medium GPMY, 8 wells were observed for medium C, 5 wells were observed for medium V, 3 wells were observed for media INA and NL2, 2 wells were observed for medium GYM, 1 well was observed for media A, B, COM, and the Vegetative medium, and 0 wells were observed for media V6 and M8.

Some activity against *E. feacalis* ATCC 19433 was also recorded. 7 wells with inhibition were observed for medium RA3, 6 wells with inhibition were observed for media GPMY and AF/MS, 1 well was observed for media INA and NL2, and 0 wells were observed for media V, A, B, C, V6, GYM, M8, COM, and the Vegetative medium. 0 wells with inhibition were recorded against any other bacteria from the panel. The negative control in all plates was clean and showed no growth indicating the absence of contamination. The positive control in all plates showed adequate growth.

ZK3		Media												
Bacteria	**V**	**Veg**	**A**	**B**	**C**	**INA**	**RA3**	**GPMY**	**V6**	**AF/MS**	**GYM**	**M8**	**COM**	**NL2**
***S. aureus* ATCC 29213**	5	0	0	3	8	2	9	8	0	8	2	0	1	2
***S. aureus* Newman**	5	1	0	4	8	3	10	9	0	8	3	0	0	3
***S. aureus* N315**	5	1	1	1	8	3	11	9	0	8	2	0	1	3
***E. feacalis* ATCC 19433**	0	0	0	0	0	1	7	6	0	6	0	0	0	1
K. pneumonaie DSM	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A.baumannii* DSM 30008**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***K. pneumonaie* ATCC 13883**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***P. aeruginosa* mexAB**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***E. coli* ATCC 25922**	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4. 1: Table depicting the number of wells with inhibition for crude extracts produced by strain ZK3 in 14 different media against a panel of pathogenic bacteria.

Figure 4. 1: ZK3 colonies on ISP3 agar.

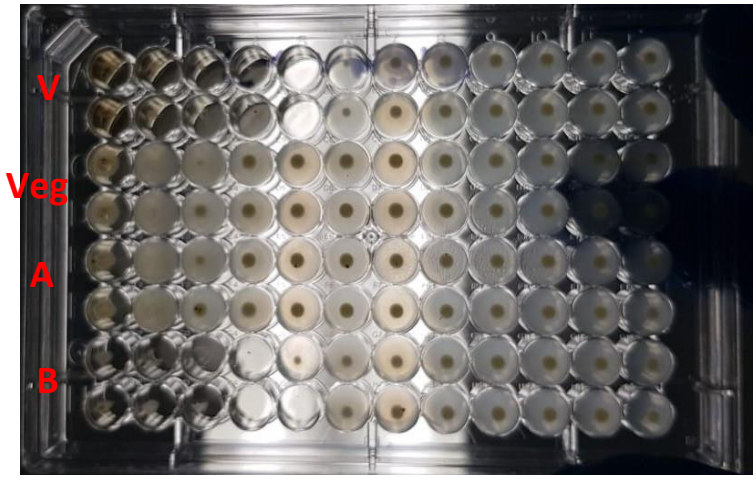


Figure 4. 2: Broth microdilution assay plate showing inhibition of growth of *S. aureus* N315 by crude extracts produced from strain ZK3 in media V, Vegetative, A, and B against.

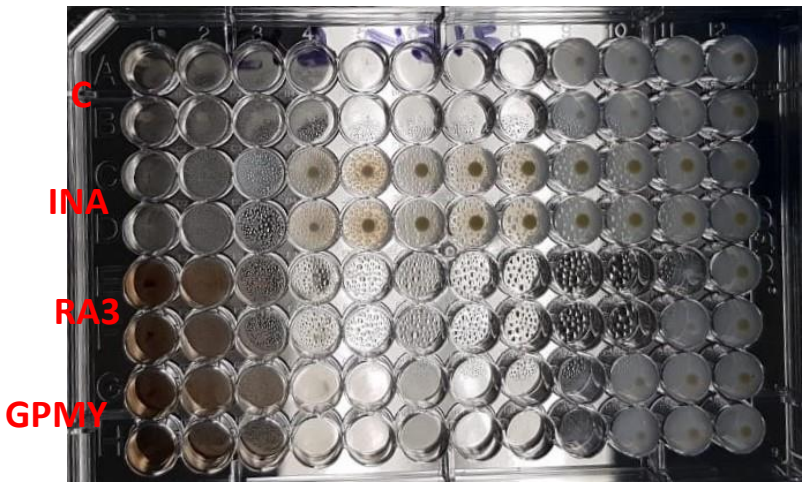


Figure 4. 3: Broth microdilution assay plate showing inhibition of growth of *S. aureus* N315 by crude extracts produced from strain ZK3 in media C, INA, RA3, and GPMY.

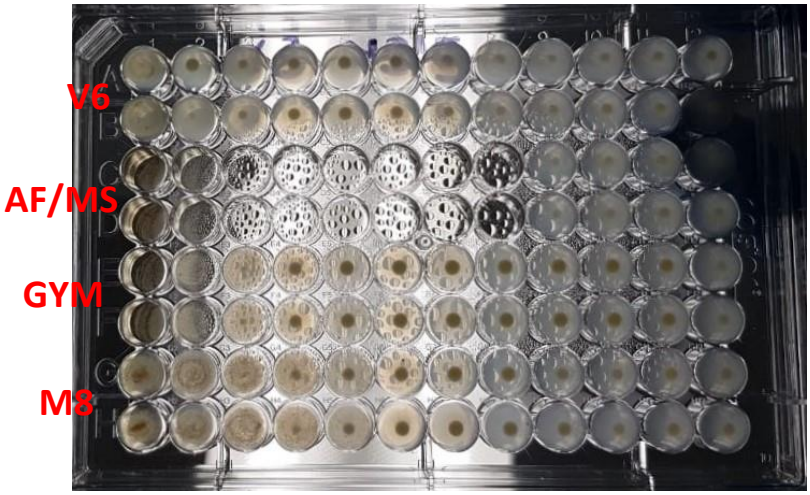


Figure 4. 4: Broth microdilution assay plate showing inhibition of growth of *S. aureus* N315 by crude extracts produced from strain ZK3 in media V6, AF/MS, GYM, and M8.

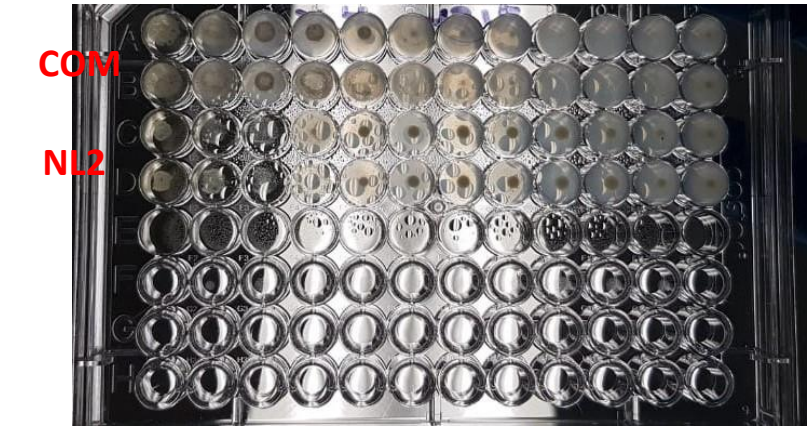


Figure 4. 5: Broth microdilution assay plate showing inhibition of growth of *S. aureus* N315 by crude extracts produced from strain ZK3 in media COM and NL2.

2. ZK4

Extracts produced by strain ZK4 (figure 4.6) showed inhibition on Gram-positive bacteria as well but this inhibition was mostly observed for medium NL2. For

S. aureus N315, 9 wells were observed in medium NL2. Activity against this bacteria was also recorded for medium V with 5 wells, medium B with 3 wells, and the Vegetative medium with 2 wells. For *S. aureus* ATCC 29213, 6 wells with inhibition were observed for the NL2 medium. Also for NL2 medium, 5 wells were observed for *S. aureus* Newman, and 4 wells were observed for *E. feacalis* ATCC 1943. Some Gram-negative activity was recorded also in media NL2 against *K. pneumonia* DSM. 2 wells were observed against this bacteria. Extracts produced in all other media showed 0 wells of inhibition against all other pathogenic bacteria. These results are depicted in table 4.2 below. The negative control in all plates was clean and showed no growth. The positive control in all plates showed adequate growth.

ZK4		Media												
Bacteria	**V**	**Veg**	**A**	**B**	**C**	**INA**	**RA3**	**GPMY**	**V6**	**AF/MS**	**GYM**	**M8**	**COM**	**NL2**
***S. aureus* ATCC 29213**	0	0	0	0	0	0	0	0	0	0	0	0	0	6
***S. aureus* Newman**	0	0	0	0	0	0	0	0	0	0	0	0	0	5
***S. aureus* N315**	5	2	0	3	0	0	0	0	0	0	0	0	0	9
***E. feacalis* ATCC 19433**	0	0	0	0	0	0	0	0	0	0	0	0	0	4
K. pneumonaie DSM	0	0	0	0	0	0	0	0	0	0	0	0	0	2
***A.baumannii* DSM 30008**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***K. pneumonaie* ATCC 13883**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***P. aeruginosa* mexAB**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***E. coli* ATCC 25922**	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4. 2: Table depicting the number of wells with inhibition for crude extracts produced by strain ZK4 in 14 different media against a panel of pathogenic bacteria.

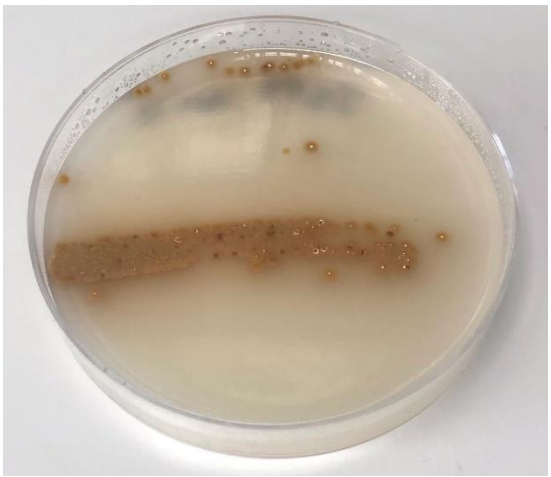


Figure 4. 6: ZK4 colonies on ISP3 agar.

3. ZK1, ZK2, ZK6, ZK8

Extracts from other strains were tested as well, all of which showed no inhibition against any of the tested bacteria and in any one of the 14 media. Below are tables 4.3, 4.4, 4.5, and 4.6 depicting these results for 4 strains; ZK1, ZK2, ZK6, and ZK8 respectively. The negative control in all plates was clean and showed no growth. The positive control in all plates showed adequate growth.

ZK1		**Media**												
Bacteria	**V**	**Veg**	**A**	**B**	**C**	**INA**	**RA3**	**GPMY**	**V6**	**AF/MS**	**GYM**	**M8**	**COM**	**NL2**
***S. aureus* ATCC 29213**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***S. aureus* Newman**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***S. aureus* N315**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***E. feacalis* ATCC 19433**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K. pneumonaie DSM	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A.baumannii* DSM 30008**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***K. pneumonaie* ATCC 13883**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***P. aeruginosa* mexAB**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***E. coli* ATCC 25922**	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4. 3: Table depicting the number of wells with inhibition for crude extracts produced by strain ZK1 in 14 different media against a panel of pathogenic bacteria.

ZK2		Media												
Bacteria	**V**	**Veg**	**A**	**B**	**C**	**INA**	**RA3**	**GPMY**	**V6**	**AF/MS**	**GYM**	**M8**	**COM**	**NL2**
***S. aureus* ATCC 29213**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***S. aureus* Newman**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***S. aureus* N315**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***E. feacalis* ATCC 19433**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K. pneumonaie DSM	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A.baumannii* DSM 30008**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***K. pneumonaie* ATCC 13883**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***P. aeruginosa* mexAB**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***E. coli* ATCC 25922**	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4. 4: Table depicting the number of wells with inhibition for crude extracts produced by strain ZK2 in 14 different media against a panel of pathogenic bacteria.

ZK6		Media												
Bacteria	**V**	**Veg**	**A**	**B**	**C**	**INA**	**RA3**	**GPMY**	**V6**	**AF/MS**	**GYM**	**M8**	**COM**	**NL2**
***S. aureus* ATCC 29213**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***S. aureus* Newman**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***S. aureus* N315**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***E. feacalis* ATCC 19433**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K. pneumonaie DSM	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A.baumannii* DSM 30008**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***K. pneumonaie* ATCC 13883**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***P. aeruginosa* mexAB**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***E. coli* ATCC 25922**	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4. 5: Table depicting the number of wells with inhibition for crude extracts produced by strain ZK6 in 14 different media against a panel of pathogenic bacteria.

ZK8		Media												
Bacteria	**V**	**Veg**	**A**	**B**	**C**	**INA**	**RA3**	**GPMY**	**V6**	**AF/MS**	**GYM**	**M8**	**COM**	**NL2**
***S. aureus* ATCC 29213**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***S. aureus* Newman**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***S. aureus* N315**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***E. feacalis* ATCC 19433**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K. pneumonaie DSM	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***A.baumannii* DSM 30008**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***K. pneumonaie* ATCC 13883**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***P. aeruginosa* mexAB**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***E. coli* ATCC 25922**	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4. 6: Table depicting the number of wells with inhibition for crude extracts produced by strain ZK8 in 14 different media against a panel of pathogenic bacteria.

B. Upscale and Bio-guided Fractionation

The medium RA3 for strain ZK3 was chosen to be upscaled considering it was the medium corresponding to the extract producing the highest number of wells showing inhibition against *Staphylococcus aureus* N315. The up-scale extract was separated into fractions as described earlier. Broth microdilution assay was performed on 3 of these fractions (ethyl acetate, hexane, and chloroform) against *Staphylococcus*

aureus N315. As shown in Table 4.7 below, the ethyl acetate fraction demonstrated 4 wells with inhibition, chloroform fraction had 1 well with inhibition, while 0 wells with inhibition were seen for the hexane fraction.

Besides, 9 wells with inhibition were observed for the 1/2 dilution of the small scale extract and 6 wells were observed for the 1/10 dilution of the small scale extract. It is noted that the non-diluted small scale crude extract was also screened as a control and it showed 11 wells. These data prove the potency of the extract produced by ZK3 in medium RA3 against *S.aureus* N315. The negative control in all plates was clean and showed no growth. The positive control in all plates showed adequate growth.

Fraction	Ethyl Acetate	Hexane	Chloroform	1/2	1/10
S. aureus N315	4	0	1	9	6

Table 4. 7: Table showing the number of wells with inhibition for each fraction of the upscaled extract produced by ZK3 in medium RA3 against *S. aureus* N315 in addition to the results of the 2 dilution samples done on the small scale crude extract.

A second upscale was performed, in the same way, but a bigger volume was prepared. The reason for doing so was to increase the amount of extract produced in hopes of getting better results. The liquid-liquid separation was performed again for the second upscale following the same procedure described above, but without the addition of the hexane. The screening was done only on *S.aureus* N315 and the ethyl acetate and the chloroform fractions both demonstrated 11 wells with inhibition. 3 dilutions for the fractions were prepared. The first one included 5 µL DMSO and 5 µL extract (1/2

dilution of the extract, 2.5 mg/mL). The second included 20 µL DMSO and 5 µL extract (1/5 dilution of the extract, 1 mg/mL). The third one included 45 µL DMSO and 5 µL extract (1/10 dilution of the extract, 0.5 mg/mL). For ethyl acetate, 11 wells with inhibition were evident for the 3 dilutions. For chloroform, 10, 9, and 9 wells with inhibition were evident for the ½, 1/5, and 1/10 dilutions respectively. The results are shown in the table below. Figures 4.7 and 4.8 show the plates done for ethyl acetate and chloroform.

Fraction	Number of wells with inhibition for the fraction	Number of wells with inhibition for ½ dilution	Number of wells with inhibition for 1/5 dilution	Number of wells with inhibition for 1/10 dilution
Ethyl Acetate	11	11	11	11
Chloroform	11	10	9	9

Table 4. 8: Table showing the number of wells with inhibition for each fraction of the second upscale produced by ZK3 in medium RA3 against *s. aureus* N315 in addition to the results of the 3 dilution samples done on the fractions.

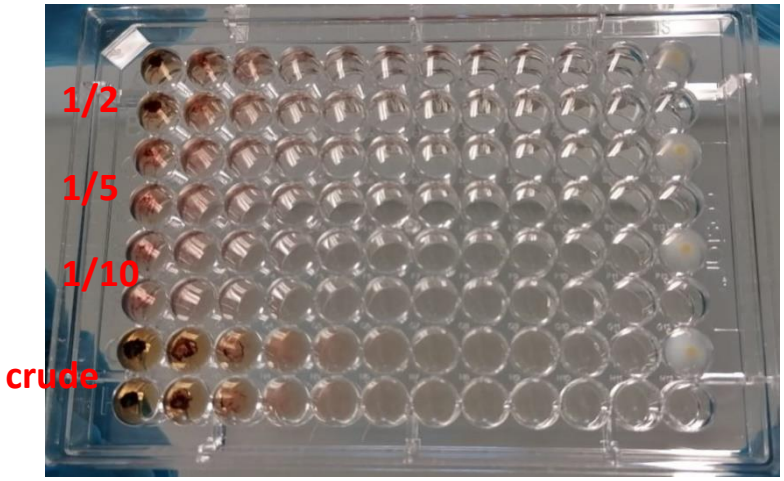


Figure 4. 7: Broth microdilution assay plate showing inhibition of growth of S. aureus N315 by the ethyl acetate fraction of the second upscale produced by ZK3 in medium RA3 in addition to the inhibition produced by the 3 dilution samples.

Figure 4. 8: Broth microdilution assay plate showing inhibition of growth of S. aureus N315 by the chloroform fraction of the second upscale produced by ZK3 in medium RA3 in addition to the inhibition produced by the 3 dilution samples.

C. pH and NaCl Percentage Growth

This experiment was performed on strain ZK3 considering it was the strain that showed the most significant activity on gram-positive bacteria and was, therefore, the only strain of interest.

For the NaCl percentage growth experiment, after 14 days in the incubator at 28°C, no bacterial growth was observed on the plates with 5%, 7.5%, and 10% NaCl whereas heavy growth was observed on the plates with 0% and 2.5% NaCl, with heavier growth on the 0% NaCl plate. The colonies appeared white, fluffy and produced a black pigment. The pictures of the plates are shown below (figure 4.9).

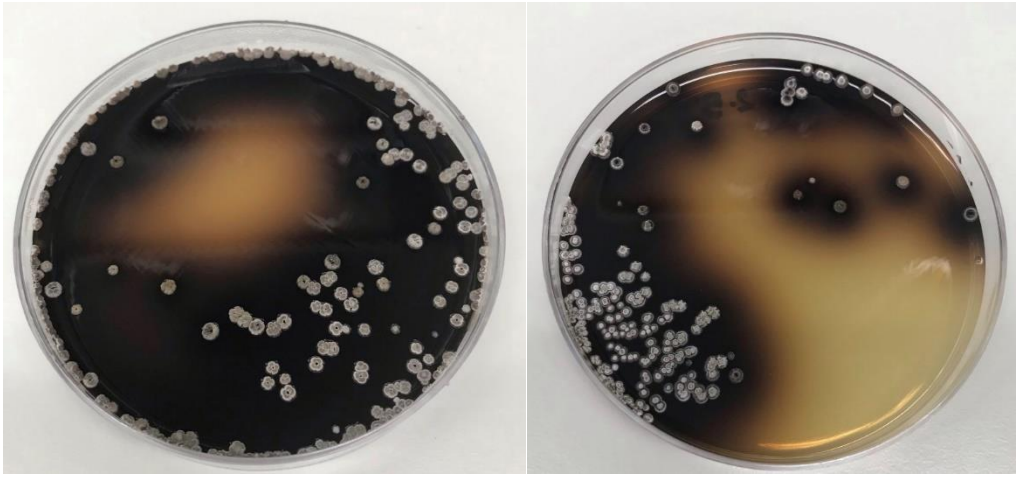


Figure 4. 9: ZK3 colonies on Basal Medium 5339 [76] agar with 0% NaCl and 2.5% NaCl.

For the pH growth experiment, after 14 days in the incubator at 28°C, no bacterial growth was observed on the plates with pHs 2, 3, 4, and 5. Red and pink colonies started to appear on the plate with pH 6 along with white spores appearing on some colonies (figure 4.10). On the plate with pH 7, growth was heavy and colonies were mostly white and sporulating with a few colonies remaining pink (figure 4.10). On the plate with pH 8 (figure 4.11), white colonies were observed with 1 darker colony, and the same was observed on a plate with pH 9 (figure 4.11). On the plate with pH 10 (figure 4.12), 4 colonies were observed but with a significantly different shape than what was observed before. They lacked the fluffy white appearance that was seen in the other plates. It is noted that on plates with pHs 6, 7, 8, and 9 when observed closely, droplets were seen on the top of the colonies (figure 4.13), which can correspond to the secretion of secondary metabolites by this strain.

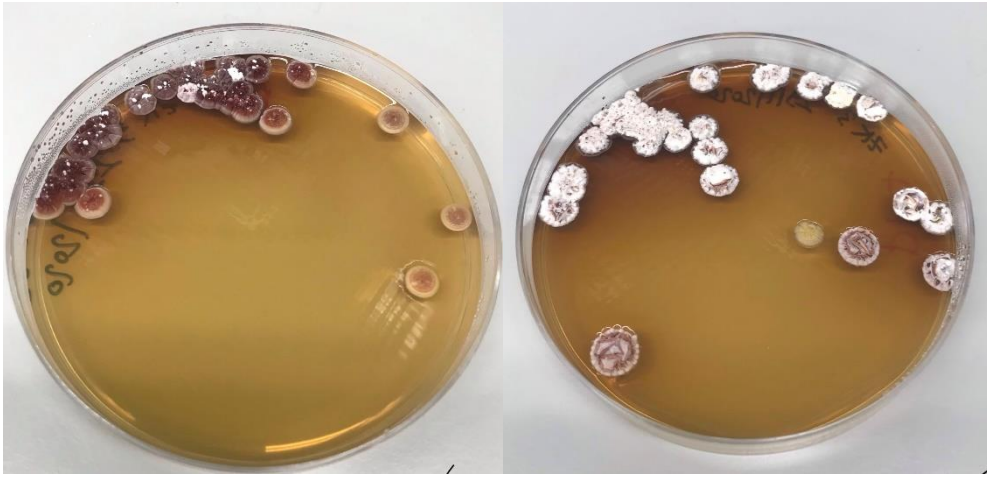


Figure 4. 10: ZK3 colonies on ISP2 Medium 5265 [76] agar with pH 6 and pH 7.

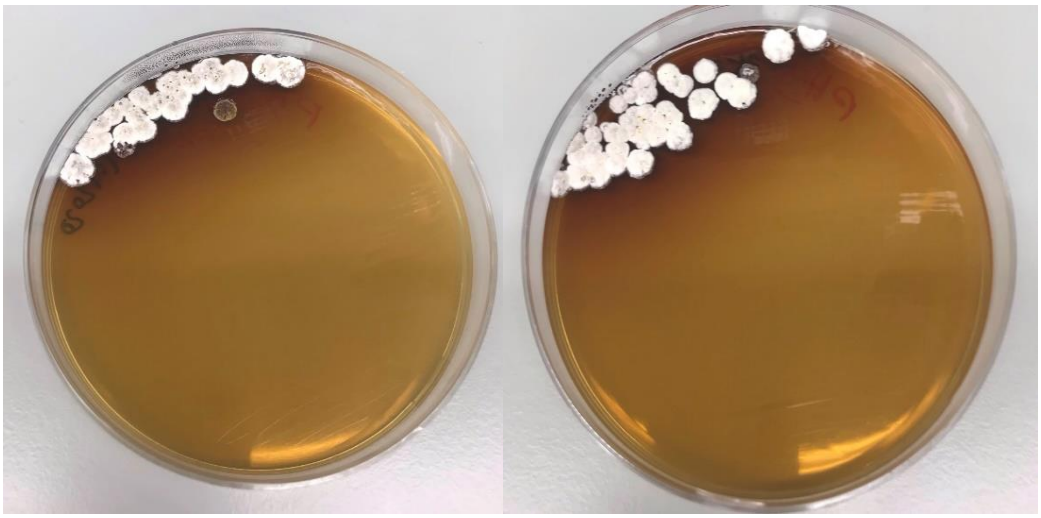


Figure 4. 11: ZK3 colonies on ISP2 Medium 5265 [76] agar with pH 8 and pH 9.

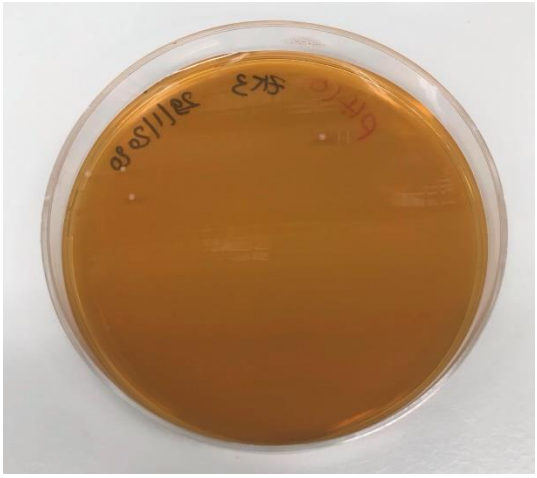


Figure 4. 12: ZK3 colonies on ISP2 Medium 5265 [76] agar with pH 10.

Figure 4. 13: Droplets seen on top of the ZK3 colonies grown on ISP2 Medium 5265 [76] agar with pH 7.

D. Molecular Characterization of Strain

1. DNA Extraction

DNA was extracted from most of the strains that were screened. Results related to the concentration and purity for all strains that were screened, including ZK3, are depicted in table 4.9.

Strain	Concentration (ng/µL)	A260/280	A260/230
ZK3	**291.653**	**2.01**	**2.36**
ZK4	**211.208**	**2.04**	**2.31**
ZK1	848.686	2.18	2.3
ZK2	313.734	1.96	2.45
ZK5	445.944	1.99	2.31
ZK6	181.158	2.03	2.34
ZK7	227.621	2.1	2.24
Zk8	516.709	2.07	2.35
ZK9	284.832	2.1	2.5
Zk11	1667.313	2.02	2.28
ZK12	691.038	2.17	2.43
ZK14	775.095	2.18	2.27
ZK15	841.185	2.16	2.34
ZK16	544.105	2.15	2.36
ZK17	276.56	1.95	2.4

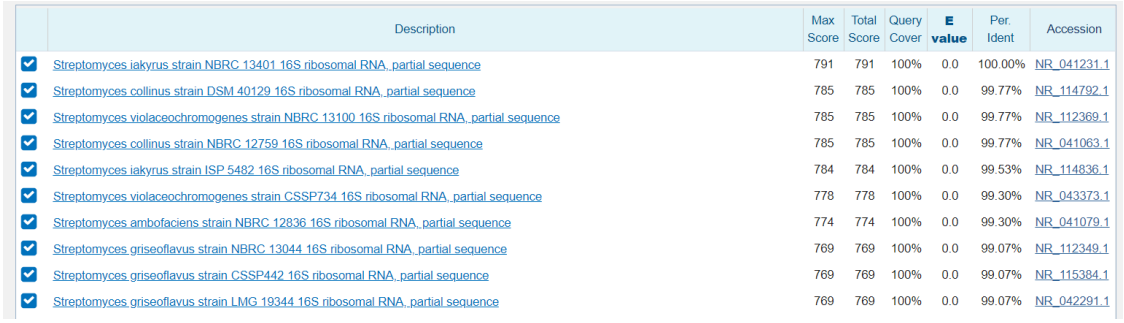
Table 4. 9: Table showing the DNA extraction results for different strains.

2. Sequencing and Blasting of the PCR Product

After alignment of the obtained forward and reverse sequenced PCR product for each strain, the alignment was blasted using the National Center for Biotechnology Information (NCBI)'s Basic Local Alignment Search Tool (BLAST) and the results for strains ZK3 and ZK4 in addition to ZK1, ZK2, ZK6, and ZK8 are shown below. The figures below show for each strain only the top ten results obtained after the blast of the aligned PCR products of the SSU primers. Based on BLAST glossary, the identity, shown in the figures below as a percentage, is defined as "the extent to which two (nucleotide or amino acid) sequences have the same residues at the same positions in an alignment" [76].

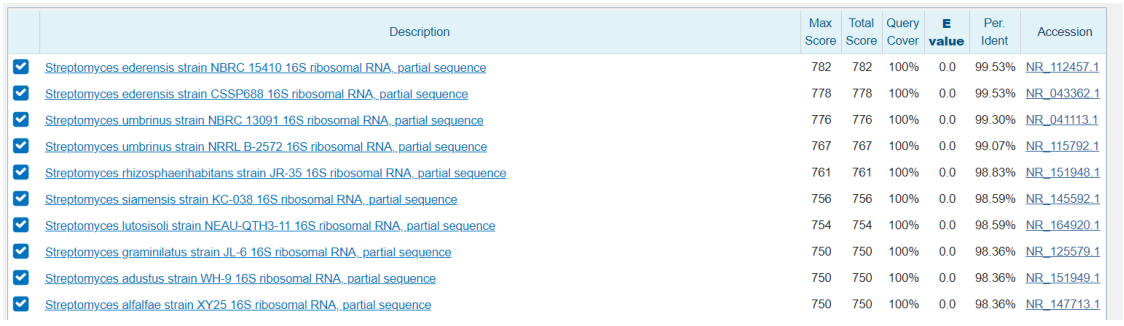
For ZK3, the identity percentage was 100% for the strain *Streptomyces Iakyrus*. This means that in the NCBI database, this strain, and ZK3 have 100% similarity

between the residues at the same positions in the alignment. This could possibly indicate that ZK3 is *Streptomyces Iakyrus*. For ZK1 and ZK8, more than 1 strain has 100% similarity which means that the difference in these sequences probably lies in different parts of the genome while the submitted alignment results match some strains in the database. For ZK4, ZK2, and ZK6, no result has a 100% identity match, but the top result for each of these shows at least a 99.25% identity match which means that the strain is most probably the one matched from the database.



Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Streptomyces iakyrus strain NBRC 13401 16S ribosomal RNA, partial sequence	791	791	100%	0.0	100.00%	NR_041231.1
Streptomyces collinus strain DSM 40129 16S ribosomal RNA, partial sequence	785	785	100%	0.0	99.77%	NR_114792.1
Streptomyces violaceochromogenes strain NBRC 13100 16S ribosomal RNA, partial sequence	785	785	100%	0.0	99.77%	NR_112369.1
Streptomyces collinus strain NBRC 12759 16S ribosomal RNA, partial sequence	785	785	100%	0.0	99.77%	NR_041063.1
Streptomyces iakyrus strain ISP 5482 16S ribosomal RNA, partial sequence	784	784	100%	0.0	99.53%	NR_114836.1
Streptomyces violaceochromogenes strain CSSP734 16S ribosomal RNA, partial sequence	778	778	100%	0.0	99.30%	NR_043373.1
Streptomyces ambofaciens strain NBRC 12836 16S ribosomal RNA, partial sequence	774	774	100%	0.0	99.30%	NR_041079.1
Streptomyces griseoflavus strain NBRC 13044 16S ribosomal RNA, partial sequence	769	769	100%	0.0	99.07%	NR_112349.1
Streptomyces griseoflavus strain CSSP442 16S ribosomal RNA, partial sequence	769	769	100%	0.0	99.07%	NR_115384.1
Streptomyces griseoflavus strain LMG 19344 16S ribosomal RNA, partial sequence	769	769	100%	0.0	99.07%	NR_042291.1

Figure 4. 14: BLAST results for ZK3.



Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Streptomyces ederensis strain NBRC 15410 16S ribosomal RNA, partial sequence	782	782	100%	0.0	99.53%	NR_112457.1
Streptomyces ederensis strain CSSP688 16S ribosomal RNA, partial sequence	778	778	100%	0.0	99.53%	NR_043362.1
Streptomyces umbrinus strain NBRC 13091 16S ribosomal RNA, partial sequence	776	776	100%	0.0	99.30%	NR_041113.1
Streptomyces umbrinus strain NRRL B-2572 16S ribosomal RNA, partial sequence	767	767	100%	0.0	99.07%	NR_115792.1
Streptomyces rhizosphaerihabitans strain JR-35 16S ribosomal RNA, partial sequence	761	761	100%	0.0	98.83%	NR_151948.1
Streptomyces siamensis strain KC-038 16S ribosomal RNA, partial sequence	756	756	100%	0.0	98.59%	NR_145592.1
Streptomyces lutosisoli strain NEAU-QTH3-11 16S ribosomal RNA, partial sequence	754	754	100%	0.0	98.59%	NR_164920.1
Streptomyces graminilatus strain JL-6 16S ribosomal RNA, partial sequence	750	750	100%	0.0	98.36%	NR_125579.1
Streptomyces adustus strain WH-9 16S ribosomal RNA, partial sequence	750	750	100%	0.0	98.36%	NR_151949.1
Streptomyces alfalfae strain XY25 16S ribosomal RNA, partial sequence	750	750	100%	0.0	98.36%	NR_147713.1

Figure 4. 15: BLAST results for ZK4.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Streptomyces albogriseolus strain NBRC 3709 16S ribosomal RNA, partial sequence	791	791	99%	0.0	100.00%	NR_112489.1
Streptomyces albogriseolus strain NBRC 3413 16S ribosomal RNA, partial sequence	791	791	99%	0.0	100.00%	NR_112487.1
Streptomyces viridodiastaticus strain NBRC 13106 16S ribosomal RNA, partial sequence	791	791	99%	0.0	100.00%	NR_112371.1
Streptomyces albogriseolus strain NBRC 12834 16S ribosomal RNA, partial sequence	791	791	99%	0.0	100.00%	NR_112297.1
Streptomyces griseorubens strain NBRC 12780 16S ribosomal RNA, partial sequence	791	791	99%	0.0	100.00%	NR_041066.1
Streptomyces viridodiastaticus strain CSSP719 16S ribosomal RNA, partial sequence	785	785	99%	0.0	99.77%	NR_043367.1
Streptomyces griseoflavus strain NBRC 13044 16S ribosomal RNA, partial sequence	780	780	99%	0.0	99.53%	NR_112349.1
Streptomyces griseoflavus strain CSSP442 16S ribosomal RNA, partial sequence	780	780	99%	0.0	99.53%	NR_115384.1
Streptomyces griseoflavus strain LMG 19344 16S ribosomal RNA, partial sequence	780	780	99%	0.0	99.53%	NR_042291.1
Streptomyces ambofaciens strain NBRC 12836 16S ribosomal RNA, partial sequence	774	774	99%	0.0	99.30%	NR_041079.1

Figure 4. 16: BLAST results for ZK1.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Streptomyces speibonae strain PK-Blue 16S ribosomal RNA, partial sequence	721	721	100%	0.0	99.25%	NR_025212.1
Streptomyces variabilis strain NRRL B-3984 16S ribosomal RNA, partial sequence	704	704	100%	0.0	98.50%	NR_043840.1
Streptomyces atrovirens strain NRRL B-16357 16S ribosomal RNA, partial sequence	702	702	100%	0.0	98.50%	NR_043508.1
Streptomyces griseoincarnatus strain LMG 19316 16S ribosomal RNA, partial sequence	697	697	98%	0.0	98.48%	NR_042290.1
Streptomyces variabilis strain NBRC 12825 16S ribosomal RNA, partial sequence	695	695	98%	0.0	98.48%	NR_112532.1
Streptomyces griseoincarnatus strain NBRC 12871 16S ribosomal RNA, partial sequence	695	695	98%	0.0	98.48%	NR_112312.1
Streptomyces griseoflavus strain LMG 19344 16S ribosomal RNA, partial sequence	695	695	98%	0.0	98.48%	NR_042291.1
Streptomyces albogriseolus strain NBRC 3709 16S ribosomal RNA, partial sequence	693	693	98%	0.0	98.48%	NR_112489.1
Streptomyces albogriseolus strain NBRC 3413 16S ribosomal RNA, partial sequence	693	693	98%	0.0	98.48%	NR_112487.1
Streptomyces atrovirens strain NBRC 15388 16S ribosomal RNA, partial sequence	693	693	98%	0.0	98.48%	NR_112449.1

Figure 4. 17: BLAST results for ZK2.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Streptomyces viridochromogenes strain NBRC 13347 16S ribosomal RNA, partial sequence	778	778	99%	0.0	99.30%	NR_112526.1
Streptomyces viridochromogenes strain NBRC 3113 16S ribosomal RNA, partial sequence	778	778	99%	0.0	99.30%	NR_112482.1
Streptomyces albogriseolus strain NBRC 3709 16S ribosomal RNA, partial sequence	776	776	99%	0.0	99.30%	NR_112489.1
Streptomyces albogriseolus strain NBRC 3413 16S ribosomal RNA, partial sequence	776	776	99%	0.0	99.30%	NR_112487.1
Streptomyces viridodiastaticus strain NBRC 13106 16S ribosomal RNA, partial sequence	776	776	99%	0.0	99.30%	NR_112371.1
Streptomyces albogriseolus strain NBRC 12834 16S ribosomal RNA, partial sequence	776	776	99%	0.0	99.30%	NR_112297.1
Streptomyces griseorubens strain NBRC 12780 16S ribosomal RNA, partial sequence	776	776	99%	0.0	99.30%	NR_041066.1
Streptomyces bellus strain ISP 5185 16S ribosomal RNA, partial sequence	771	771	99%	0.0	99.07%	NR_114828.1
Streptomyces coerulescens strain ISP 5146 16S ribosomal RNA, partial sequence	771	771	99%	0.0	99.07%	NR_114821.1
Streptomyces caelestis strain NBRC 12749 16S ribosomal RNA, partial sequence	771	771	99%	0.0	99.07%	NR_112512.1

Figure 4. 18: BLAST results for ZK6.

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☑	Bacillus proteolyticus strain MCCC 1A00365 16S ribosomal RNA, partial sequence	837	837	99%	0.0	100.00%	NR_157735.1
☑	Bacillus wiedmannii strain FSL W8-0169 16S ribosomal RNA, partial sequence	837	837	99%	0.0	100.00%	NR_152692.1
☑	Bacillus cereus strain IAM 12605 16S ribosomal RNA, partial sequence	832	832	99%	0.0	99.78%	NR_115526.1
☑	Bacillus tropicus strain MCCC 1A01406 16S ribosomal RNA, partial sequence	832	832	99%	0.0	99.78%	NR_157736.1
☑	Bacillus paramycoides strain MCCC 1A04098 16S ribosomal RNA, partial sequence	832	832	99%	0.0	99.78%	NR_157734.1
☑	Bacillus nitratireducens strain MCCC 1A00732 16S ribosomal RNA, partial sequence	832	832	99%	0.0	99.78%	NR_157732.1
☑	Bacillus luti strain MCCC 1A00359 16S ribosomal RNA, partial sequence	832	832	99%	0.0	99.78%	NR_157730.1
☑	Bacillus albus strain MCCC 1A02146 16S ribosomal RNA, partial sequence	832	832	99%	0.0	99.78%	NR_157729.1
☑	Bacillus cereus strain CCM 2010 16S ribosomal RNA, partial sequence	832	832	99%	0.0	99.78%	NR_115714.1
☑	Bacillus cereus strain NBRC 15305 16S ribosomal RNA, partial sequence	832	832	99%	0.0	99.78%	NR_112630.1

Figure 4.19: BLAST results for ZK8.

3. Whole Genome Sequencing Results

Whole-genome sequencing was performed on ZK3 and the antiSMASH results are shown in the figure below (figure 4.20).

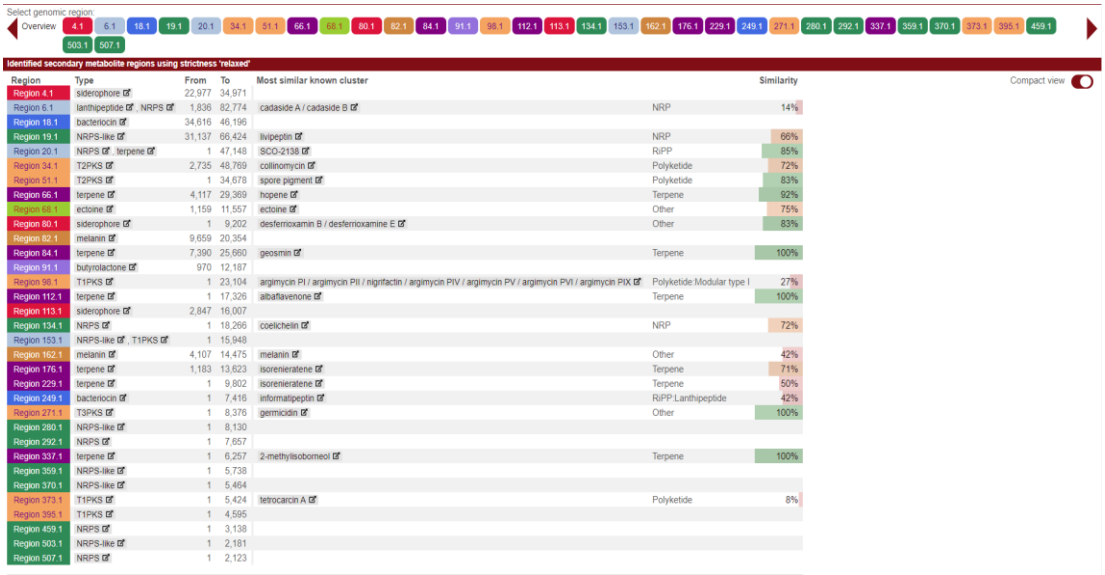


Figure 4. 100: AntiSMASH results for Zk3.

CHAPTER V

DISCUSSION

The aim of this project was to be able to isolate different *Actinomycetes* residing in soil and screen their secondary metabolites against pathogenic bacteria. A plethora of bacteria was purified from the soil, cultivated in production media, and their metabolites were screened for antibacterial activity. ZK4 did show some promising results against *Staphylococcus aureus* N315. However, the most significant results were those of a strain entitled ZK3. The extract produced by this strain showed significant inhibition on Gram-positive bacteria including *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* Newman, *Staphylococcus aureus* N315, *and Enterococcus faecalis* ATCC 19433. Out of these 3, *Staphylococcus aureus* N315, which is a Methicillin-resistant *Staphylococcus aureus* strain, was probably the most significant since MRSA is a very serious pathogen causing life-threatening nosocomial infections. Aside from its clinical manifestations that often lead to sepsis and death, it can develop resistance at a fast rate, and will probably acquire resistance on most, if not all of the drugs that are already in use to treat the infections it causes [60].

The broth microdilution assay (BMD) done against the N315 strain showed inhibition in 11 wells in the medium RA3. Usually, the presence of inhibition against Gram-positive bacteria is not taken into consideration if the number of wells is not high enough. In this case, it was considered to be significant because the number of wells was more than 5. It is important at this point to understand whether the extract present in ZK3 that is causing the inhibition is bacteriostatic, which means slowing the growth

of the bacteria, or bactericidal, which means killing the bacteria completely [77]. To do so, the plate that showed the 11 wells was kept for an additional 24 hours in the incubator at 37ºC. The first 4 wells of inhibition showed a clear broth and lack of turbidity in comparison to the other wells with inhibition at lower concentrations. 30 µL was taken out of each of these 4 wells and was streaked on Tryptic Soy Agar (TSA) plates. After 24 hours of incubation at 37ºC, no growth was observed on the plates. This proves that the extract, at high concentrations, is bactericidal.

The medium RA3 was chosen for the upscale considering it was the medium with the highest number of wells showing inhibition. After the upscale and the liquid-liquid separation, screening was performed on the obtained fractions. Inhibition was very significant in the ethyl acetate and chloroform fractions. The 11 wells in both fractions indicate that the compound responsible for the inhibition against MRSA probably resembles both ethyl acetate and chloroform in chemical characteristics and its polarity could be between the polarities both compounds. Part of the compound produced by ZK3 was fractioned with the ethyl acetate and part of it with the chloroform. However, it is more probable that the compound is leaning towards ethyl acetate in polarity because, at low concentrations, more inhibition was apparent for the ethyl acetate fraction than for the chloroform. On the polarity scale, ethyl acetate is found in the middle, so it is considered semi-polar [78]. This means that ethyl acetate can dissolve polar and non-polar compounds and the compound found in ZK3 could, therefore, be either.

The compound should be further purified using high-performance liquid chromatography (HPLC) and its molecular structure should be uncovered using nuclear magnetic resonance (NMR) to be able to understand better the significance of this

compound and how to extract it more efficiently. Additionally, this data could allow us to figure out if the ZK3-derived compound belongs to one of the known classes, or whether we are in the presence of a new class of antimicrobials.

The last experiment performed on the extract itself was the BMD on the diluted small scale crude extract. 9 wells for the 1/2 dilution and 6 wells for the 1/10 dilution were observed proving that even at low concentrations, the extract is still active and still showing inhibition with high numbers of wells. Further dilution was also done for the fractions after the second fractionation. The high number of wells shown in the results indicates a high level of production for a very potent antibiotic. In this project, minimum inhibitory concentration experiments (MIC) with known concentrations of the extract were not performed since the extract was not purified. However, the fact that the extract showed significant activity at low concentrations could be indicative of a low MIC, once performed, and therefore, to better therapeutic efficacy, in vitro at least [79].

On the ISP3 medium, ZK3 showed white colonies that produced a red pigment after prolonged incubation. It was a slow-growing strain that took more than 2 weeks to grow on the plate. The results obtained from the pH and NaCl growth experiments show that ZK3 grows optimally at a pH of 7 and with 0% salt concentration. The results also showed that as the pH changes, the morphology, structure, size, and color of the colonies on the plate change as well. The fluffy appearance most probably indicates the presence of the aerial mycelium [46]. The droplets on the surface of the colonies could be the secondary metabolite with the inhibitory activity or another secondary metabolite with inhibitory activity against another microorganism. One future perspective for this project could be growing ZK3 and other strains of interest on ISP plates, collecting the droplets and screening them against other bacteria or even viruses, fungi, and parasites.

Concerning the molecular aspect of the study, DNA extraction for ZK3 and ZK4 resulted in concentrations of 291.653 ng/µL and 211.08 ng/µL respectively. This is considered a good yield since it exceeded 200 ng/µL. As for the purity of the extracted DNA, and taking into consideration the absorbance ratio of 260 nm over 280 nm, the values were 2.01 for ZK3 and 2.04 for ZK4. Any number above 2 for DNA is considered pure [80]. Good concentration and purity of DNA make for a good PCR product which was sent for 16S rRNA sequencing at Macrogen. For further confirmation of the BLAST results, and since ZK3 was the main strain of interest, whole-genome sequencing was performed (WGS) on ZK3 and the FASTA file was also BLASTed on the NCBI database. No hits were found which probably means the strain is novel and not present in the database.

In addition, the WGS product was entered on antiSMASH. This software shows the different clusters present in the genome of ZK3 that would be responsible for the production of the secondary metabolites. Each cluster has a defined location on the genome and produces a type of metabolite with a specific percentage of similarity. If the similarity is 100%, the cluster is certainly producing this type of compound. The lower the percentage, the more probable it is that the cluster is producing a derivative or an analog of the compound rather than the compound itself [81]. Referring to figure 4.31, 33 biosynthetic gene clusters were observed, and those with 100% similarity include the geosmin cluster (region 84.1) [82], the albaflavenone cluster (region 112.1) [83], and the 2-methylisoborneol cluster (region 337.1) [84], all of which are producers of terpenes and responsible for the earthy smell produced by the strain. Another cluster with 100% similarity is a Type 3 polyketide synthase (T3PK) cluster known as the germicidin biosynthetic cluster (region 271.1) which is a polyketide producer and is

involved in the germination process of the *actinomycete* [85]. The clusters mentioned are not involved in the production of compounds that have anti-staphylococcal activity.

On region 98.1, a Type 1 polyketide synthase (T1PK) cluster that shares a 27% similarity with argimycin P1 biosynthetic gene cluster is found. This cluster is responsible for the production of argimycins which are alkaloid compounds that are involved in the growth of the strain and the development of its colonies [86].

One cluster of interest could be the bacteriocin cluster on region 249.1 which shares a 42% similarity with the informatipeptin biosynthetic gene cluster. This means that the cluster present in ZK3 could be producing informatipeptin derivatives or analogs. Informatipeptin belongs to the lanthipeptides class which is a class of ribosomally synthesized and post-translationally modified peptides (RiPPS) with activity against Gram-positive bacteria [87] [88]. Another cluster of interest is a non-ribosomal peptide synthetase cluster (NRPS-like) that showed 66% similarity with livipeptin biosynthetic gene cluster. Livipeptin (shown in figure 5.1) is a patented peptide aldehyde with anti-protease activity [89]. Proteases can be used by Gram-positive and Gram-negative bacteria to increase virulence and pathogenicity rendering them appropriate targets for antibacterial agents [90]. The derivatives or analogs of these 2 compounds produced by ZK3 could be responsible for the inhibitory activity evident in the results shown.

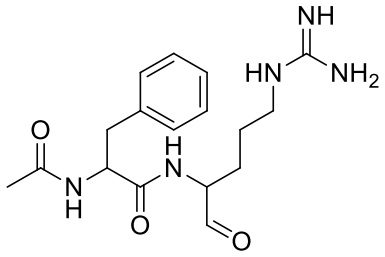


Figure 5. 1: Structure of livipeptin.

Further perspectives of the study would be to purify the extract at hand, using advanced techniques in chemistry such as those mentioned earlier and to test the purified compound again to assess whether the inhibitory activity is maintained, amplified, or diminished. Furthermore, induction of resistance experiments and WGS for the mutants obtained after induction could help in identifying the mode of action of the antibacterial agent and therefore its eventual medical significance. An important aspect of the project as well, was to isolate novel strains. Of the strains that were blasted using the NCBI database, none showed a significantly low percentage identity with a known strain. This doesn't necessarily mean that none of the strains are novel. WGS, such as that done for ZK3, should be performed on all the isolated strains to be able to discriminate between strains that are closely related or have identical 16s rRNA sequences.

Another de-replication method that should be done, is using LC HR-MS (liquid chromatography high-resolution mass spectrometry) to better understand the profile of the molecules produced by each strain. Using this technique would allow us to compare the measured masses to the database of the dictionary of natural products. Hence, we can understand which are known molecules and which are novel metabolites or derivatives.

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