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Discovery of a Novel Antibiotic from a *Bacillus* Bacterium Cultivated from its Natural Environment

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I hereby reaffirm the Lawrence University Honor Code

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P. McMonagle

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Abstract

The current primary method of treating bacterial infections is using antibiotics. However, this continued treatment of these illnesses caused by pathogenic bacteria is causing the rate of evolution of these disease-inducing organisms to increase. Antibiotic resistance is forcing scientists to search for new forms of antibiotics to compete with these new 'super bugs.' I pursued the search for novel antibiotics through their natural source – antibiotic-producing microorganisms. As microbes have a direct advantage when producing antibiotics, it is my thought that they will be the best resource to discovering new and effective antibiotics. I conducted an exploratory search for antibiotic-producing microbes by sampling for microbes in the environment of the Lawrence University Campus. Once samples were cultured, I tested for the production of an antibiotic agent, and characterized the organisms. I then used a series of methods to extract, isolate, and identify the antibiotic agent. I extracted and identified an antibiotic agent from a microorganism in the *Bacillus* genus.

Acknowledgements

I would like to thank Ron Peck for his dedication on this project. Without his advising, this research would not have been as successful. I would also like to thank him for his strenuous critiquing of my paper. I would like to thank Beth De Stasio for editing my introduction and Nancy Wall for helping with final corrections to the paper. I thank JoAnn Stamm and Wayne Krueger for supplying equipment and materials as well as their helpful guidance with techniques and machines I did not understand.

Introduction

Defining Antibiotics

An antibiotic is any class of organic molecule that inhibits or kills microbes by specific interactions with bacterial targets (Davies 2010). Naturally-produced antibiotics are typically extracted from microorganisms but can also be produced by plants and mushrooms. Antibiotics may also be synthesized by altering the chemical structure of previously discovered antibiotic agents. Useful antibiotics are selectively toxic. Selective toxicity is the ability of an antibiotic agent to be toxic for a desired pathogen, without being toxic to host cells. Penicillin is an example of a selectively toxic antibiotic as it targets the cell wall of a pathogen. Since human cells do not have cell walls, it is selectively toxic against bacteria, but is harmless to human cells.

The Reason to Continue Searching for New Antibiotics

We continue to treat illnesses caused by pathogenic bacteria with antibiotics, causing the emergence of antibiotic-resistant strains to increase. As the antibiotics are killing off the antibiotic-sensitive variants, those that survive the assault are continuing to infect humans. There are very limited treatment options against these antibiotic-resistant variants. Antibiotic resistance is forcing scientists to search for new forms of antibiotics to compete with these new "superbugs." The term "superbugs" is defined as microbes with an enhanced mortality caused by multiple mutations giving the microbe a high level of resistance to antibiotic classes (Davies 2010). Fortunately, just as pathogenic bacteria are evolving increased resistance, other microbes continue to develop their own novel antibiotics in an on-going arms race. These antibiotic-producing bacteria are developing new killing agents to defend themselves and their environment from encroaching organisms. Thus, it is beneficial for microbiologists to search for these antibiotic-producing bacteria in hopes of finding new ways of killing pathogens.

Microorganisms create antibiotics for their own survival. By secreting a substance that kills organisms around them, it protects the organism from other microbes that may try to cause them harm. Releasing this substance into their environment also decreases the need to fight for space and resources within the environment. It protects the microbe from being out-competed in their niche. As a result, it is evolutionarily advantageous for the microbes to produce these agents in order to better survive and reproduce. Just as it is advantageous to create these antibiotic agents, it is also advantageous to gain resistance to these substances. Resistance to antibiotics occurs when a microbe with a specific characteristic that protects it from the antibiotic's effects survives in the presence of the antibiotic and reproduces.

Most antibiotic resistance develops through lateral gene transfer (Clardy 2009). Lateral gene transfer is the transfer of genetic material across species barriers. Lateral gene transfer allows individual genes, organelles, or fragments of genomes to move horizontally from one lineage to another (Freeman and Herron 2007). Lateral gene transfer typically occurs using plasmids; small loops of DNA that can self-replicate (Sadaca et al. 2011). The rate of this phenomenon increases under strong selective conditions of antibiotic medication in humans. Consequently, the antibiotic-resistant microbes will be the only ones to survive in the environment to continue to spread and exhibit pathogenic activities. Infrequent or improper use of antibiotics exacerbates the selection for resistant strains of bacteria, which are then harder to treat effectively. As a result, informed physicians have become more careful in prescribing antibiotics.

Currently, antibiotics are the main tools known to fight against bacterial infections. The World Health Organization (WHO) reported that about 440,000 new cases of multidrug-resistant tuberculosis (MDR-TB) emerge annually, causing at least 150,000 deaths. This creates a dire

need to decide how to remedy this phenomenon. While over 8000 antibiotics are known to exist and hundreds more are discovered yearly, few prove to be commercially useful (Al-Ajlani, MM 2006). Just as with past scientists, it is the duty of modern scientists to continue to experimentally derive new and innovative ways to discover novel antibiotics and methods to treat illnesses. It is thus my goal to assist in the search for novel antibiotics through exploratory research.

Antibiotics: A History

Selman Waksman first coined the term 'antibiotic' in 1941 and defined it as any small molecule made by a microbe that antagonizes the growth of other microbes (Clardy 2009). Presently, 'antibiotic' is defined as, "a chemical or protein, which kills bacteria by disrupting a particular biochemical process" (Sadaca 2011). The first to isolate an antibiotic was Alexander Fleming in September of 1928 when a mold contaminated a plate containing *Staphylococcus* colonies. A large colony of a contaminating mold caused the *Staphylococcus* colonies to become transparent and the colonies were determined to be undergoing lysis (Fleming 1929). This phenomenon spurred a curiosity to discover what was creating this inhibitory area. In 1929, Fleming published a paper titled, "On the Antibacterial Action of Cultures of a Penicillium, with Special Reference to their use in the Isolation of *B. influenzae*," in which he discusses his observation of the "bactericidal" and his experimentation to determine the characteristics of the antibiotic agent and its spectrum of effectiveness across the domain.

Penicillin was not immediately recognized to have clinical potential as Sulfa Drugs were widely used during that time. However, sulfa drugs were limited to treating gram-positive bacteria such as *Streptococcus* [Davies 2010]. Most other bacterial infections were uncontrollable. Gerhard Domagk first discovered sulfa drugs in the 1930s, creating the first synthetic antibiotic. The first synthetic antibiotic was named Prontosil. They resulted from a

screening of chemicals for activity against *streptococcal* infections in experimental animals. Sulfa drugs are a series of growth factor analogs, which inhibit the growth of bacteria. The most common sulfa drug is Sulfanilamide, an analogue of p-aminobenzoic acid. P-aminobenzoic acid is a part of the vitamin, folic acid – a nucleic acid precursor. Sulfanilamide is selectively toxic by blocking the synthesis of folic acid; thus inhibiting the synthesis of nucleic acid. Daniel Bovet of the Pasteur Institute later identified the active ingredient, Sulfanilamide.

Resistance occurs when the organism develops the ability to take up folic acid from its environment (Davies 2010). Therefore, while sulfa drugs were highly used, there was still a need for a more successful antibiotic. In 1929, Howard Florey and his colleagues developed a process for producing penicillin on a large scale. Penicillin was dramatically effective in controlling *staphylococcal* and *pneumococcal* infections in military personnel. It was also more effective at treating *staphylococcal* infections than sulfa drugs (Sykes 2001).

In 1945, penicillin became available for general use. Pharmaceutical companies began to look for, and develop, other antibiotics; leading to drugs that revolutionized the treatment of infectious diseases. These major classes of antibiotics include the tetracyclines, macrolides, aminoglycosides, cephalosporins, chloramphenicol, glycopeptides, and rifamycins (Madigan et al. 2012).

From the 1960s to the 1970s, antibiotic-resistant bacteria began to emerge in the hospital environment, creating the urgency and drive to search for new antibiotic compounds. "Most of the low-hanging antibacterial natural products fruit had already been picked from microbial fermentations" (Fernandes 2006). After the late 1960s, screening methods were only discovering known antibiotics. No longer able to find new antibacterial agents led to chemists developing semi-synthetic antibiotics. "Overall, these new semi-synthetic antibiotics were more potent, less

susceptible to inactivating enzymes that cause resistance, or simply tighter binders to their bacterial targets" (Fernandes 2006). By improving previously established, naturally-occurring antibiotics, selectivity and low toxicity were already presented within the "antibiotic backbone." Many of these semi-synthetic antibiotics were immediately successful and continue to be used highly in the medical field.

In the 1980s, two decades worth of developing analogues led to a dried-up well. There are a limited number of changes that can be made to the chemical structure of an antibiotic in order to counter resistance while still maintaining its effectiveness. Thus, once chemists had created all of the possible analogues from the known antibiotics, no new possibilities were left. Pharmaceutical companies were finding it impossible to find any new leads from old natural products (Devasahayam et al 2010). Concurrently, bacteria were continuing to develop resistance to antibiotics - as the antibiotics became an evolutionary selective pressure (Fernandes 2006). Moreover, no new antibiotic classes were being found and compounds that were discovered were so complex that they were difficult to synthesize and thus could not be modified.

A brief moment of relief came from the discovery of the fluoroquinolone class, which was developed by modifying nalidixic acid (Fernandes 2006). This discovery led to new quinolone programs in almost every pharmaceutical company (Fernandes 2006). Just as before, the boom of the fluoroquinolones and the analogues produced from them declined after two decades. Companies began to redirect their efforts to screening small-molecule libraries, a source already found to have successful results (Fernandes 2006). While this is the same method Domagk used when he discovered Prontosil, the spread of screening increased as well as the libraries being screened. In addition, the knowledge of what to screen and what to look for became more defined.

Recently, these libraries have not produced a significant amount of new antibiotic agents. Some companies have reverted back to analyzing old antibiotic compounds that did not originally meet optimal potency levels or target levels when they were first discovered. It is thought that analogues from known compounds will be less likely to fail because of toxicity than completely new classes of compounds (Fernandes 2006).

Antibiotic Classes and their Mechanisms of Action

In order to fully grasp the need for this research, it is necessary to first understand the different antibiotic classes, what they target in pathogenic organisms, and how resistance may occur. As there are so many classes of antibiotics and mechanisms of action, the search for new antibiotics could lead to a wide range of results. It is thus important to know what novel antibiotics might be similarly related to and how they will interact with pathogens in order to

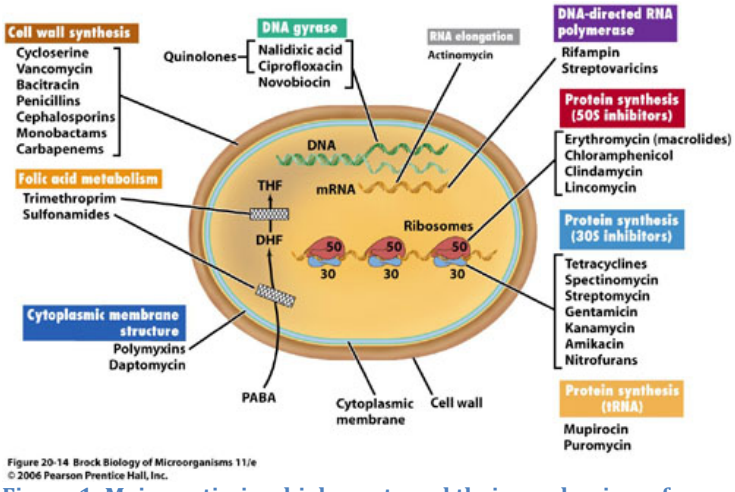

Figure 1: Major antimicrobial agents and their mechanism of action. It also displays the major targets exploited by antibiotics.

fully understand a newly discovered antibiotic agent.

Many antibiotics are derived from microorganisms. It appears that many bacteria and fungi produce these antibiotics for the sole purpose of inhibiting or killing other microorganisms. We now know thousands of different antibiotics that have been produced, but less than one percent of these come to be useful (Al-Ajlani 2009). The majority of the lack of clinical uses is

because they are not selectively toxic, and thus is severely toxic to hosts. They may also lack the ability for host cells to uptake them. As a result, several antibiotics are artificially modified in order to enhance their efficacy and decrease their toxicity. These antibiotics are classified as semi-synthetic.

There is a wide spectrum of antimicrobial activity due to the targets of the antibiotic. An antibiotic target is a part of the pathogenic organism that the antibiotic binds to causing cell death or preventing reproduction. Important targets include ribosomes, cell wall, cytoplasmic membrane, lipid biosynthesis, and DNA replication and transcription elements [Figure 1]. Antibacterial agents are classified according to their chemical structure, or their target within the bacterial cell. Each antibiotic affects a very limited and specific group of microorganisms. Some agents are so specific that they only affect the growth of microbes within a single genus. In fact, some of the most useful antibiotics are directed against unique structural features of bacteria.

Gram staining is a method used to determine the chemical and physical characteristics of a bacterial species' cell wall. Essentially, the stain detects a molecule called peptidoglycan, which is the major component in the cell wall of gram-positive bacteria. It thus divides bacteria into two large groups, gram-positive or gram-negative. The effectiveness of antibiotics that target the cell wall depends on whether the cell wall is gram-positive or gram-negative. Penicillin for example generally affects all gram-positive bacteria but gram-negative bacteria are naturally resistant (Madigan et al. 2012). Broad-spectrum antibiotics are typically effective against both groups. They have proven to be very successful for medical use and have a wider use than

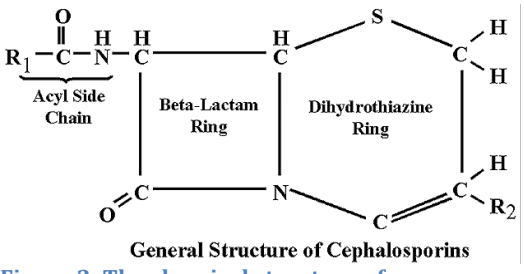

Figure 2: The chemical structure of Cephalosporins. Important sections of the structure include the Acyl side chain and the

narrow spectrum antibiotics. Examples of broad-spectrum antibiotics are ß-lactams and tetracyclines, which are described in further detail later in this section. Limited-spectrum antibiotics are effective against fewer pathogens, but are typically advantageous for controlling those that fail to respond to other antibiotics. For example, vancomycin is a narrow-spectrum glycopeptide antibiotic that is highly effective against gram-positive bacteria, which are resistant to penicillin - commonly from the genera of *Staphylococcus*, *Bacillus*, and *Clostridium* (Kahne et al. 2005).

Cell wall synthesis is a very common antibiotic target. Cell wall synthesis and its synthesis mechanisms are unique to bacteria and therefore inhibitors are likely to be selectively toxic. Penicillins, Cephalosporins, and Cephamycins are all ß-lactam antibiotics. ß-lactam antibiotics are one of the most important groups of antibiotics - both historically and medically. They account for over half of all antibiotics produced worldwide. ß-lactam antibiotics share a structural component called the ß-lactam ring [Figure 2] (Nussbaum 2012).

An important enzyme required for bacterial cell wall synthesis is transpeptidase. Transpeptidase enzymes bind to penicillin or other ß-lactam antibiotics. When transpeptidases bind to penicillin, they cannot catalyze the transpeptidase reaction but cell wall synthesis can be continued. As a result, the newly synthesized bacterial cell wall is no longer cross-linked and thus cannot maintain its strength. The antibiotic-transpeptidase complex also stimulates the release of autolysins – enzymes that digest the existing cell wall. This action results in a weakened and self-degrading cell wall. The osmotic pressure differences between the inside and the outside of the cell cause cell lysis (Nussbaum et al. 2012).

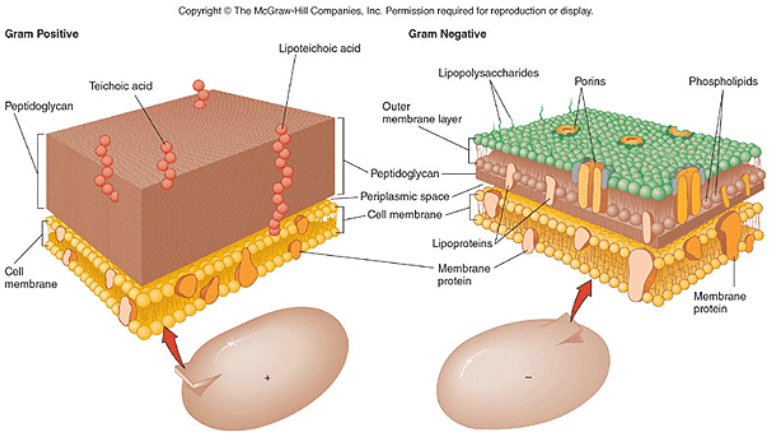

Figure 3: The cell wall structure of gram-positive (left) and gram-negative (right) bacteria. Notice the width of the peptidoglycan layer as it is the essential target for cell wall inhibition.

Penicillin is active against gram-positive bacteria. Gram-negative bacteria are impermeable to the antibiotic. Chemically modifying the structure can create significant changes to the properties. Many chemically modified, semisynthetic types of penicillin are effective against gram-negative bacteria. Structural differences in the n-acyl group of semisynthetic penicillins allow the antibiotic to be transported inside the gram-negative outer membrane in order to inhibit cell wall synthesis [Figure 3]. Penicillin is sensitive to ß-lactamase, an enzyme produced by a number of penicillin-resistant bacteria. To counteract this in patients, oxacillin and methicillin are two widely used semisynthetic penicillins that are resistant to ß-lactamase (Madigan et al. 2012). Vancomycin is another cell wall synthesis inhibitor, but does not bind to PBPs. Instead, it binds directly to the terminal D-alanyl-D-alamine peptide on the peptidoglycan precursors; thus blocking transpeptidoglycan synthesis [Figure 3].

Cephalosporins are a group of clinically important ß-lactam antibiotics; produced by the fungus, *Cephalosporium sp*. Cephalosporins are a semisynthetic antibiotic with a broader spectrum of activity than penicillins. They are more resistant to enzymes such as ß-lactamase that destroy ß-lactam rings because they differ structurally from penicillins. The structure is the ß-lactam ring connected to a 6-member dihydrothiazine ring [Figure 2], instead of a 5-member

thiazolidine ring. They maintain the same mechanism of action as penicillins - they bind irreversibly to PBPs and prevent the cross-linking of peptidoglycan.

Quinolones interfere with bacterial DNA gyrase therefore preventing the supercoiling of DNA in the bacterial cell. Since DNA gyrase is found in all bacteria, fluoroquinolones are effective for treating both gram-positive and gram-negative bacterial infections (Madigan 2012).

Inhibiting transcription, RNA synthesis is another successful target. Rifampin and the streptovaricins inhibit RNA synthesis by binding to the B-subunit of RNA polymerase. These antibiotics work specifically against RNA of bacteria, chloroplasts, and mitochondria. Actinomycin inhibits RNA synthesis by binding to DNA and blocking RNA elongation. Actinomycin binds strongest at guanine-cytosine base pairs by fitting into the major groove in the double strand where RNA is synthesized (Madigan 2012).

Protein synthesis is an important target for antibiotics as it causes inhibition of cell growth by interacting with the ribosome and disrupting translation. The interactions are specific and several involve binding to rRNA. Several antibiotics that inhibit protein synthesis are found to be medically useful. However, the mechanism of action is very different for different inhibitors of protein synthesis. For example, puromycin binds to the A site of the ribosome [Figure 4]. Growing peptide chains are transferred to the puromycin instead of the amino acyl-transfer RNA complex. The puromycin-peptide complex is then released from the ribosome. Thus halting elongation prematurely. In contrast, chloramphenicol inhibits elongation by blocking formation of the peptide bond (Madigan 2012).

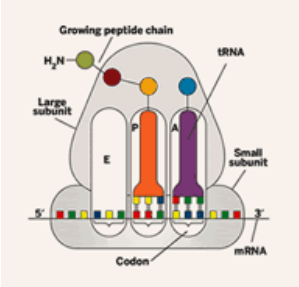

Figure 4: Diagram of a Ribosome and is function

Tetracyclines are protein synthesis inhibitors, which interfere with 30S ribosome subunit function. Tetracycline is produced by several species of *Streptomyces*, and has a vast medical

use including veterinarian medicine. They were some of the first broad-spectrum antibiotics capable of inhibiting both gram-positive and gram-negative bacteria. The active portion of their structure consists of a naphthalene ring system. Substitutions to the basic ring structure occur naturally and form new analogues. Semisynthetic analogues have also been developed. Tetracyclines and ß-lactam antibiotics are the two most important groups of antibiotics in the medical field (Madigan 2012).

Aminoglycosides contain amino sugars bonded by glycosidic linkage, which is clinically useful against gram-negative bacteria. They inhibit protein synthesis by targeting the 30S subunit of the ribosome. An example of an aminoglycoside is streptomycin, produced by *Streptomyces griseus*. Streptomycin was the first effective antibiotic used for the treatment of tuberculosis. Currently a synthetic antibiotic is the regular treatment. Aminoglycosides, such as neomycin, are found in most topical medications. Oral medications of aminoglycosides are rare as it can be toxic to several organs including kidneys and nerves. In addition, resistance develops quickly. Thus, its use is reserved for when other antibiotics fail (Madigan et al. 2012).

Many antibiotics inhibit ribosomes of organisms from only one phylogenetic domain. Chloramphenicol and streptomycin specifically target the ribosomes of bacteria whereas cycloheximide only affects the cytoplasmic ribosomes of eukarya.

Macrolides are broad-spectrum antibiotics, comprised of lactone rings bonded to sugars. Variations of both the ring and the sugars lead to a large number of macrolide antibiotics. Erythromycin, produced by *Streptomyces erythreus*, represents twenty percent of the total world production and use of antibiotics. Macrolides target the 50S subunit of bacterial ribosomes, thus inhibiting protein synthesis. Macrolides are typically used in patients who are allergic to penicillin or other ß-lactam antibiotic (Nussbaum 2012).

Daptomycin is also produced from the *Streptomyces* genus and has a unique mechanism of action. Its structure is comprised of a cyclic lipopeptide. It is used mainly to treat gram-positive infections. Daptomycin binds specifically to cytoplasmic membranes, forms a pore, and induces rapid depolarization of the membrane. The depolarized cell quickly loses its ability to synthesize macromolecules such as nucleic acids and proteins, resulting in cell death. Alterations in the cytoplasmic membrane structure accounts for very rare instances of resistance (Madigan 2012).

Platensimycin is a new antibiotic, produced from *Streptomyces platensis*. It selectively inhibits a bacterial enzyme, which is key to the biosynthesis of fatty acids, thus disrupting biosynthesis. It possesses a broad range of activity against gram-positive bacteria, including nearly untreatable infections caused by Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococci*. It has already been known to be effective in eradicating *S. aureus* in mice and shows no signs of toxicity (Madigan 2012).

Recent Approaches to Antibiotic Discovery

There have been a series of methods that have contributed to discovering and developing new antibiotics since the boom of antibiotic discovery. After resistance became a serious problem, companies began to search for new methods to discovering or creating new antibiotics.

Metagenomics became an important method in the search, beginning in the 1990s. Metagenomics is the study of genetic material recovered directly from environmental samples. The reasoning behind this approach was based on the notion that only a small sample of microorganisms in any environmental sample can be cultured by standard techniques (Peláez 2006). In other words, some microorganisms worth studying further, will either be out-competed by other microorganisms that have been sampled and studied before, or may not survive well in the synthetic environment created in a laboratory. As a result, a vast genetic pool is left

unexplored by these conventional methods. It was thought that environmental DNA might possess genes involved in secondary metabolite biosynthesis (Peláez 2006). Metagenomics has yet to offer expected leads that would be useful towards antibiotic development. One theory on this unfortunate occurrence is that there currently is not a good method for translating the "early proof-of-concept experiments into a technology suitable for drug discovery at the industrial scale" (Peláez 2006). Consequently, most of the companies that began these studies have since abandoned this strategy or have chosen a new route (Peláez 2006). As a result, it is unlikely that new useful antibiotics will come from this strategy.

Scientists are discovering useful antimicrobial peptides as a new group of natural products. These peptides vary in mass, below 25-30 kDa. They are composed mainly of cationic and hydrophobic amino acids (Peláez 2006). The structure is believed to be the cause of their ability to disrupt cell wall membranes. The peptides carry a positive charge, making them selective against bacterial cell membranes. It is still unclear how successful these peptides will be for therapeutic purposes.

Microorganisms regulate their metabolic processes to adapt to different environments so varying growth conditions may cause them to produce substantially different, yet potentially useful, compounds. Unfortunately, using a high number of conditions forces researchers to limit study to only a few strains due to feasibility. Concurrently, maximizing the number of strains is also a significant parameter as it increases the evolutionarily variability of the genes they possess. When sampling from the environment it also becomes difficult to decide which conditions should be given to the organism to optimize production of a potential antibiotic without previous knowledge of the organism (Peláez 2006). In addition, setting growth conditions at random will most likely result in redundancy of metabolites screened.

One recent approach to ease the difficulties of maximizing metabolic potential is to grow the microbes in a microtiter plate [Figure 5] which allows researchers to handle multiple strains at the same time, making it easier to experiment with multiple variables (Peláez 2006). This system has been piloted using *Streptomyces* with success (Peláez 2006).

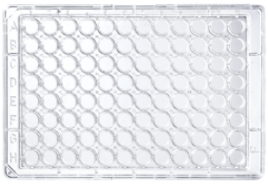

Figure 5: An example of a microtiter plate

Another recent approach is high-throughput screening (HTS). HTS studies have been initiated in the hopes of discovering bacterial proteins as potential targets for antibiotics. These targets have then been cloned to produce proteins for HTS in order to find inhibitors from previously established combinatorial libraries. Some promising enzyme inhibitors have been found through this method of screening (Fernandes 2006). Unfortunately these leads have yet to produce effective antibacterial agents. Thus, it is clear that a good enzyme inhibitor does not necessarily guarantee it to be a good antibacterial agent. In addition, the complexity and diversity of the molecules established from HTS do not carry the same degree of tight binding and multi-site inhibition, as do naturally occurring antibiotics (Peláez 2006).

Combinatorial biology is a growing field in small companies and labs, focusing on cloning and expressing genes, or clusters of genes, from antibiotic-producing bacteria to make "unnatural antibiotics." The bacteria being used to pilot these experiments are antibiotic-producing *Streptomycetes*. This approach to accelerate evolution has produced several new chemical entities. However, scientists face the same problem of, "how to obtain significant improvements in activity while maintaining safety [or preventing toxicity] and scale-up production" (Fernandes 2006).

Scientists have also begun to design new antibiotics based on natural host-defense

peptides isolated from high-level eukaryotes such as frog skin and human immune system cells. It is thought that this approach may produce antibiotics for superficial infections, but "systemic administration of membrane active agents" will not be as widely used except for multidrug-resistant infections because of the high chance for toxicity (Fernandes 2006).

Interestingly, theories and methods are developing around genetically modifying bacterial strains to either over-express or under-express essential genes, thus rendering them resistant or hypersensitive to antibiotics acting on the specific targets when compared to a wild-type strain. The modified strains can be used in experiments with wild-type strains to enable the detection of antibiotics acting specifically on a desired target (Peláez 2006). Another strategy would be to use empiric screening to look for activity against a specific target microbe, a common method perform in earlier antibiotic research (Peláez 2006). Thus, only the extracts acting on the desired targets would be prioritized in further studies.

My Approach

In my project, I pursued the search of novel antibiotics from natural sources. As microbes have a direct advantage in producing antibiotics, it was my thought that they will be the best resource for discovering new and effective antibiotics. Thus, it was my hypothesis that a novel antibiotic could be discovered and isolated from a microbe found in the environment.

Much like initial discoverers of antibiotics, I sampled the surrounding environment for antibiotic producing microbes. I then introduced the sample to growth media in order to encourage the growth of a microbe that out-competes the others. Hopefully, one of the reasons for their advantage in the media is their ability to produce antibiotics. Once grown, I tested each experimental organism against a gram-negative and a gram-positive model microorganism. If the microbe is able to create a zone of inhibition against one or both microbes, preventing the intrusion of the model organism, it can be assumed that the experimental microbe is producing

an antibiotic agent. Once I identified antibiotic-producing microbes, I isolated them and created pure cultures. I then isolated and extracted the antibiotic from the microbe and its environment. The ultimate goal was to identify the antibiotic agent's chemical structure.

Searching for novel antibiotics using previous methods is again a promising strategy because of our advances in technology and our increased understanding of the microbial world. Screening and isolation methods have greatly improved due to modern purification and analytical characterization tools. We now have the technology to rapidly identify known antibiotics with modern day chromatography and mass spectrometry (Fernandes 2006). Now mere traces of these "novel structures" can be identified and characterized. Thus, products can be isolated and analyzed fairly quickly and without the difficulties of attempting to mass-produce an antibiotic that may not even prove useful. In addition, new research, including and metagenomics, has shown that the microbial diversity is much greater than previously thought. This creates an opportunity to re-explore environments for potentially new antibiotic producers that were previously not known to exist.

With all of the trial and error of attempting new approaches to discovering new antibiotic agents, we know that nature has a history of successfully producing new structures and will continue on this trend. Microbes have coexisted with other microbes and fungi in the environment and it is thus thought four billion years of natural selection has likely resulted in a large diversity of antimicrobial compounds. Recently, new chemical leads for novel antibacterial agents have resulted from old, and previously underdeveloped antibiotics (Fernandes 2006). For example, hygromycin and pleuromutilin are two old antibiotics that have been used for several years in veterinary medicine have recently been modified to create new classes of antibiotics (Fernandes 2006). In addition, the protein crystal structure of these old antibiotics bound to the

bacterial targets of interest is being studied to learn new binding sites for potential analogues. "The complexity of the molecules and their multifaceted interactive sites make them more useful as starting points for designing new antibacterial agents" (Fernandes 2006).

Still more scientists continue the argument supporting this with other ideas including the following: the unparalleled structural diversity that can be found in nature, the fact that natural antibiotics have apparently been shaped by evolution to make them effective in killing microorganisms, and the suggestions that the field still unexplored is huge. The vast microbial diversity supports this argument, carrying the potential for researchers to trigger the expression of silent pathways by manipulating the conditions of cultivation (Peláez 2006). Moreover, the number of molecular targets still to be exploited for antibiotic therapy is unknown. This structural diversity has historically been crucial to the progress that has already been made in the discovery, research, and implementation of these antibiotic agents.

Overview

I conducted an exploratory search for antibiotic-producing microbes by sampling for microbes in the environment of the Lawrence University Campus. I then tested the samples to determine if any microbes secreted an antibacterial agent into their environment, which inhibited the growth of two known model microbes that I introduced to the synthetic environment. If the experimental microbe is able to create a "barrier" between itself and the competing microbe, then I can assume that the experimental microbe is producing and releasing an agent into the environment that is killing, or inhibiting the growth of, the other organism. Once the antibiotic-producing microbes were discovered, they were grown in a pure culture, identified, and characterized based upon their colony morphology and DNA sequence.

I then attempted several different methods to isolate and extract the antibiotic from the microbe and its environment. I discovered a successful method for extracting and isolating a

novel antibiotic from an experimental isolate. I was able to identify, extract, and isolate an antibiotic agent. The promising results from discovering and extracting the antibiotic agent could lead to further exploration, including discovering the agent's chemical makeup and determining its molecular target in bacteria.

Methods

Obtaining environmental isolates of microbes

Microbial isolates were obtained by swabbing surfaces with sterile cotton swabs. Thirty samples were collected across the Lawrence University Campus [Table 1]. Two samples were taken from each site using a sterile cotton swab, and stored in a sterile test tube for each sample.

Table 1: Recorded locations of each site where sampling occurred along with descriptions of the location when necessary. Temperature was also recorded.

Sampling 1 (9/19/11)			
Name	**Location**	**Comments**	**Temp (°C)**
A, A'	Compost pile of SLUG	Dirt, decomposing vegetables	63 °C
B, B'	Drain pipe of WCC	Murky water, dirty	63 °C
C, C'	Fox River water	Shallow was clear and deep was murky	18 °C
D, D'	Tree stump on River Walk		63 °C
E, E'	Mushroom on stump	Large, white, flat	63 °C
F, F'	Iron pin from railroad bridge	Rusty	63 °C
G, G'	Fallen tree in river	Base of tree	63 °C
H, H'	Mushroom on River Walk	Green/gray and spirally	63 °C
I, I'	Ashtray outside WCC	Muddy	63 °C
J, J'	Trash dumpster outside Phi Kappa Tau		63 °C
K, K'	Grill ashes outside Russell Sage Hall		63 °C
L, L'	Keyboard from second floor of WCC		63 °C
M, M'	WCC rock sign		63 °C
N, N'	Light post	Loaded with cob webs and dead river flies	63 °C
O, O'	Door handle of Steitz Hall (3rd floor)		63 °C
Sampling 2 (10/3/11)			
AM, AY	Compost pile of SLUG	Dirt, decomposing vegetables	75 °C
BM, BY	Drain pipe of WCC	Dusty	75 °C
CM, CY	Fox River water	Cloudy Water	16 °C
DM, DY	Tree stump on River Walk		75 °C
EM, EY	Mushroom on Stump	Large, white, flat	75 °C
FM, FY	Iron pin from railroad bridge	Rusty	75 °C
GM, GY	Fallen tree in river	Base of tree	75 °C
HM, HY	Mushroom on River Walk	Green/gray and spirally	75 °C
IM, IY	Ashtray outside WCC		75 °C
JM, JY	Trash dumpster outside Phi Kappa Tau		75 °C
KM, KY	Grill ashes (sage)		75 °C
LM, LY	Keyboard from second floor of WCC		75 °C
MM, MY	WCC rock sign		75 °C
NM, NY	Light post	Cob webs and dead river flies	75 °C
OM, OY	Door handle of Steitz Hall (3rd floor)		75 °C
PL, PM, PY	River Bed soil		17 °C

Unless otherwise noted, all items were sterilized using an autoclave. In the lab, each swab was broken in half, leaving the cotton end in its original test tube. Lysogeny Broth (LB) (10 mL) was added to each sample's test tube. LB is a nutrient-rich medium, which is comprised of tryptone, yeast extract, and NaCl – essential materials for bacterial growth. Therefore, the most efficient bacteria for this laboratory environment will be able to out compete other organisms and grow in the media, potentially by producing a chemical that inhibits the growth of other microbes. All samples were initially tested in LB to assure the sample sites contained microbes as it contains all essential materials a microbe will need to survive and reproduce.

After inoculation, the test tubes were placed in an incubator at 37˚C shaking at 250 rpm, and left to grow overnight. 37˚C is an optimal growth temperature for many bacteria. Shaking the samples during incubation allows a continual supply of oxygen to be added to the media, enabling the bacteria to grow rapidly.

One week later, a second sampling was performed from the same locations. As before, two samples were taken from each site. Two new media were added to these samples, in the hopes of growing different organisms than those originally cultivated, but found in the same locations. In this sampling, M9 and YPD media were used. M9 is a defined media, lacking amino acids necessary for cell growth. As a result, organisms unable to produce their own amino acids will be unable to grow. YPD is an optimal growth media for fungi. It has been previously observed that some fungi produce antibiotics, thus it is beneficial to attempt to grow such organisms.

Paper Disk Assay

Once the cultures were grown, the experimental bacteria were tested against two different model bacteria, *Staphylococcus aureus* and *Escherichia coli*. Lab strains of these organisms were chosen because each possesses different characteristics such as cell wall structure: *S. aureus* is gram-positive and *E. coli* is gram-negative. In addition, these lab strains are related to pathogenic organisms. Although they are not pathogenic themselves, they possess similar characteristics as their pathogenic relatives. The experimental bacteria were given identifying labels based on their order of sampling and their culture media. The bacteria were tested using a paper disk assay. This assay involved creating a lawn of the model organism across an LB agar plate. Two sets of plates were created, one for *S. aureus* and the other for *E. coli* for each sample. Immediately after inoculating the agar with the model organism, a filter paper disk was dipped into the experimental bacteria and applied directly on top of the freshly created lawn. Four different experimental bacteria were applied to each plate. The plates were incubated at 37˚C for approximately 24 hours. Once the bacteria were allowed to grow, zones of inhibition were measured in millimeters (mm). Zone of

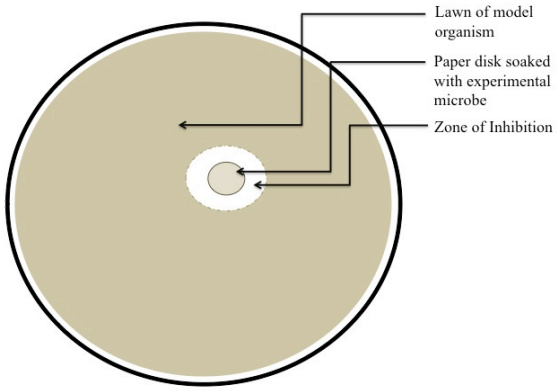

Figure 7: : Diagram of how to identify a zone of inhibition

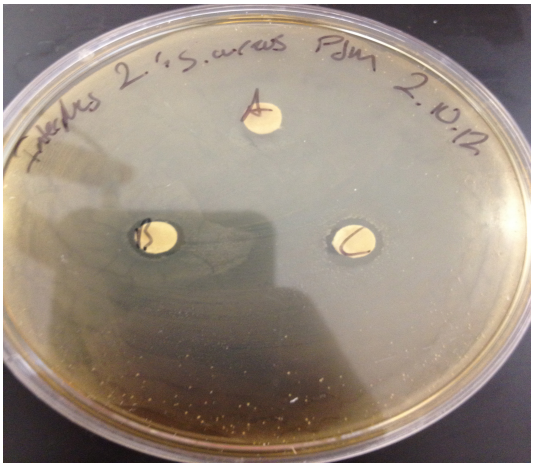

Figure 6: An example of an actual zone of inhibition. Zones can be seen on B and C.

inhibition is the location between the experimental bacteria and growth of the model bacteria [Figure 6]. If the model organism was placed across the entire plate, then growth should have been observed in these regions. The observation of this zone implies that there is an agent in that region that is killing off the model organism. This agent is assumed to have antibiotic properties. Observed antibiotic producing bacteria were measured and recorded.

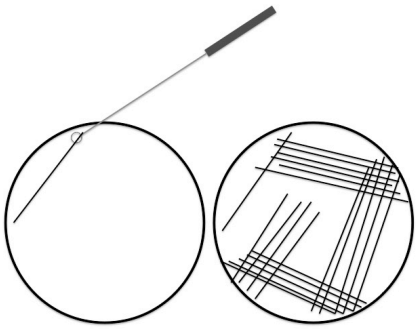

Figure 8: Diagram of Streaking.

Pure Culture

After measuring the zones of inhibition, those experimental bacteria in which a zone was observed were applied from the test plate and streaked onto a new LB agar plate in order to create a pure culture [Figure 8]. The plates were placed in an incubator set to 37˚C and left to grow overnight. These pure culture plates were used for further antibiotic testing and for growing liquid pure cultures.

Colony Morphology

Once the cultures were grown and a pure culture was observed their colony morphology was recorded. Colony Morphology is qualified using several defining characteristics (Smibert and Krieg 1994).

The diameter of each experimental colony was measured in milimeters. The pigmentation of the colonies were then described. The form, elevation, and margin was observed along with an analysis of whether the colonies were smooth (shiny glistening surface), rough, (dull, bumpy, granular, or matte surface), or mucoid (slimy or gummy appearance) [Figure 9]. Finally, the opacity of the colonies (transparent, translucent, or opaque) was recorded and the texture of each colony was tested using an inocculating need - defininging the texture as butyrous (buttery

texture), viscous (gummy), or dry (brttle or powdery).

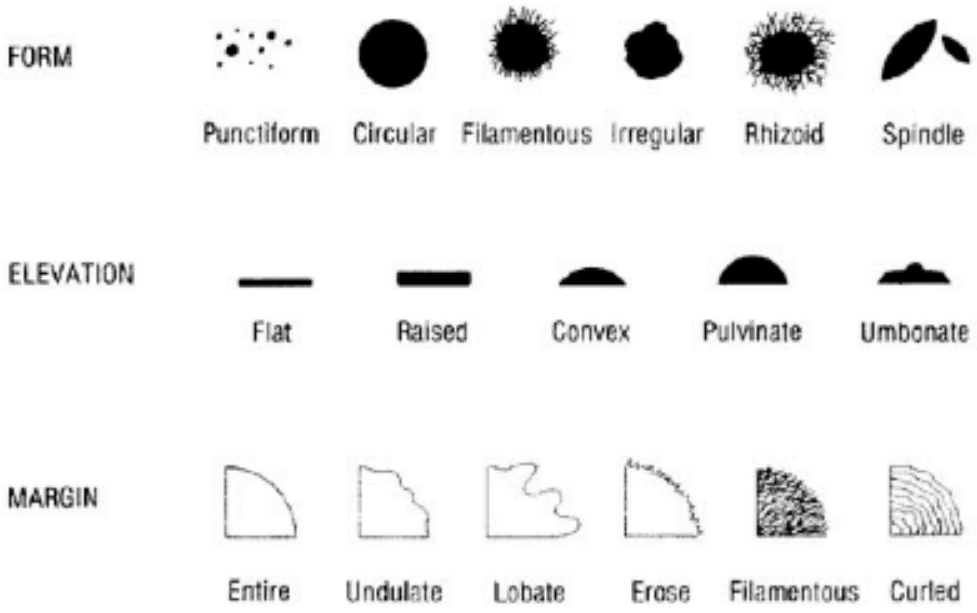

Figure 9: Diagram illustrating the descriptions for form, elevation, and margin

Storage and Freezer Stock

In order to preserve the experimental bacteria, freezer stocks were created from the pure cultures. 400 microliters (µL) of eighty percent glycerol were added to 1600 µL of each experimental bacteria liquid pure culture and placed into a cryovial. Two cryovials were generated for each experimental organism; one was placed in a liquid nitrogen (N_2) container and the other was placed in an -80˚C freezer.

DNA Extraction and Sequence Analysis

DNA was extracted from all desired microbes in order to perform a Polymerase Chain Reaction (PCR) to allow sequencing. DNA was extracted using the QIAquick DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) following the protocol for Gram$^-$ bacteria. 16S rDNA was amplified using PCR with the primers AGAGTTTGATCCTGGCTCAG and AAGGAGGTGATCCAGCC with the following temperature program (Wiesburg et al. 1991).

Recipe used for PCR:

35 µl genomic DNA
10 µl 5x reaction buffer
1 µl dNTP mix (10mM concentration of each dATP, dTTP, dGTP, dCTP)
0.25 µl 100 µM fD1 universal eubacterial 16S rDNA primer
0.25 µl 100 µM rD1 universal eubacterial 16S rDNA primer
0.5 µl Taq polymerase (Promega, Madison, WI)

Program used for PCR:

95˚C for 5 minutes
Then 25 Cycles of the following 3 steps:
95˚C for 2 minutes
42˚C for 30 seconds
72˚C for 4 minutes
Then a final elongation step:
72˚C for 20 minutes
Then a hold temperature to prevent DNA degradation
8˚C until placed in refrigerator
Primers and PCR program obtained from the article Weisburg et al. (1991)

The PCR product was then purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA to assure that only the desired DNA was present in the sample.

Gel Electrophoresis and Photography

Gel Electrophoresis was performed to test if the appropriate length of DNA was cloned and amplified from the DNA extraction and PCR, before purification. A gel was cast using 40 g agarose gel with 40 mL of 1x TBE buffer to make a 1% agarose gel. 2 µl of loading dye were added to 8 µl of the purified PCR product. 10 µl of DNA marker were added to the first slot in each row in order to measure the base pair (bp) length. The gel was run with TBE buffer at 100v for 45 minutes. After it was run, the gel was bathed in ethidium bromide for 10 minutes and then was laid in an ultraviolet lamp and photographed at three separate exposures (55, 60, and 65) in order to assure a decisive visual measurement. Ethidium bromide is used as a fluorescent tag for DNA. When the ethidium bromide is exposed to ultraviolet light, it will fluoresce with an orange

color.

DNA Analysis

4 µl of PCR product were added to 2 µl of primer and 6 µl of sterile, double-deionized water for each experimental bacterial DNA. Both primers were used for sequencing (forward and reverse) in case a difference is observed. Excess PCR purified product was placed in a freezer container for storage. Samples prepared for sequencing were sent to the DNA analysis facility on Science Hill at Yale University.

Antibiotic Extraction Procedures – Trial 1

Two antibiotic testing experimental procedures were designed in order to extract and test the antibiotic agent produced by the experimental bacteria. The first test was an attempt to extract the antibiotic through the agar, which the bacteria were grown on. The experimental bacteria were plated heavily on one-third of an LB agar plate. It was then incubated for four days at 27˚C, which is an optimal growth temperature for producing materials. *S. aureus* was then spread across the remaining two-thirds of the plate and incubated for two days at 37˚C, the optimal temperature for growth. The zones of inhibition were measured and then cut out using a sterile razor. The razor was sterilized using ethanol and then passed through a Bunsen burner to remove excess ethanol. The removed zone was placed in a sterile test tube. Approximately 3 mL of 50% aqueous solution acetone were added to the test tube and the mixture was ground using a tissue grinder until the agar was well lysed. The mixture was tested using the paper disk assay. The agar mixture was centrifuged at 8,000 rpm for two minutes. The supernatant was added to a new centrifuge tube and left in a hood overnight to evaporate the solvent.

The second test involved centrifuging a liquid culture of the experimental bacteria and testing the supernatant. A liquid culture of the experimental bacteria was incubated at 27˚C, shaking at 250 rpm for two days. 1 mL of the liquid pure culture was added to a sterile centrifuge

tube and centrifuged for 2 minutes at 14,000 rpm. 700 µl of the supernatant were filter sterilized and added to a new sterile centrifuge tube. The sterilized supernatant was then tested for antibiotic activity using the paper disk assay against both *E. coli* and *S. aureus*.

Re-activation of Antibiotic Production

After a pause in experimentation, a sample of the culture from the freezer stock was taken, incubated in 10mL of LB and re-tested for antibiotic production using the paper-disk assay. The freezer stocks of the experimental organisms had lost their antibiotic-producing characteristic. An experiment was designed in order to encourage the microbes to continue antibiotic production. 10 mL of LB were inoculated for each experimental microbe and slowly grown to approximately 50% of maximum turbidity based on visual observation of the media. The microbes were grown at room temperature without shaking in order to control the rate of growth. The liquid culture of experimental microbes was divided into two test tubes of 5 mL each. An additional 5mL of sterilized LB were then added to each tube. The test tubes were inoculated with 100 µL of *E. coli* or *S. aureus* saturated culture and incubated in a shaking incubator at 37˚C for 24hrs. Paper-disk assays were conducted to test antibiotic production against both *E. coli* and *S. aureus* for both test tubes.

Antibiotic Extraction Procedures – Trial 2

A second set of experiments was designed to attempt to extract the antibiotic agent. The first test involved separating the antibiotic agent using a solvent. The experimental bacteria were grown in LB (50 mL) in an Erlenmeyer flask and incubated at 27˚C, shaking at 150 rpm for approximately four days. The culture was then added to a separating funnel along with 50 mL of chloroform. The mixture was shaken well and then left to sit for 20 minutes to clear. Three phases were formed and each was collected in individual sterile flasks. Each phase was then tested against *S. aureus* and *E. coli* for antibiotic activity using the paper disk assay.

The second test again was an attempt to isolate the antibiotic from the supernatant. The experimental bacteria were incubated in 10 mL of LB at 27˚C, shaking at 150 rpm, for four days. 1 mL of the culture was centrifuged at 14,000 rpm for 5 minutes and filter sterilized. The sterilized supernatant was then tested for antibiotic activity, using the paper disk assay. Both methods were ineffective so modifications were made.

The third trial involved similar testing with slight changes. The experimental bacteria were incubated in 50 mL of LB broth in an Erlenmeyer flask at 30˚C, shaking at 150 rpm for approximately 24hrs. 30 mL of the culture were centrifuged at 20,000 x g for 15 minutes. The supernatant was added to a separating funnel along with 30 mL of chloroform. The mixture was shaken well and let stand to clear for approximately 20 minutes. The three phases were collected in separate sterile flasks and tested for antibiotic activity. The original supernatant was also tested for antibiotic activity.

From the previous experiments, it was understood that the interface was the most important phase to collect and test. A fourth trial involved the same procedure as the third. The interface was collected in a centrifuge tube and purified by removing aqueous and organic layers, which were mixed with the interface. The interface was tested against *S. aureus* and *E. coli* for antibiotic activity. After the initial activity test, the interface was centrifuged at 14,000 rpm for 5 minutes. The new interface was then removed and tested for antibiotic activity.

Extraction of Antibiotic Agent for HPLC

100 mL of LB were inoculated with a single colony of the experimental bacteria from microbe B in a 150 mL Erlenmeyer flask. The media were incubated in a shaking incubator for 24hrs at 150 rpm at 27˚C. The liquid culture was centrifuged at 20,000 x g (12,900 rpm in Sorvall SS-34 rotor) for 20 min at 4˚C. The supernatant was added to a separating funnel with 100 mL of chloroform. The mixture was shaken vigorously and let stand for approximately 20

minutes to clear. It was then shaken several more times to optimize yield and purity of supernatant. The organic and aqueous layers were collected and used as a control. The interface was collected and allowed to resolve overnight in the refrigerator. Removing excess organic and aqueous layers using a P200 pipette, which were collected along with the interface, further purified the interface. 300µL of hexanes were added to the interface to obtain a clear phase containing solely the antibiotic agent.

High Performance Liquid Chromatography (HPLC)

An HPLC procedure for antibiotic analysis was adapted from previous (Al-Ajlani 2006). The clear phase containing the antibiotic compound was applied to a C_{18} column (250mm length, 4.6 mm inner diameter) with eluent A (0.1%(vol/vol) trifluroacetic acid and 20%(vol/vol) acetonitrile) and eluted with segmented gradients of eluent B (0.1%(vol/vol) trifluoracetic acid and 80%(vol/vol) acetonitrile). Eluents were made using Milli-Q HPLC grade water. The gradient was applied using 40% of eluent B for 30 min and followed by 40% to 100% eluent B for 10min.

Results

Sampling for Antibiotic Activity, identification, and 16S rDNA Analysis

Environmental isolates were first cultured from a variety of locations [Table 1]. Each was immediately tested for the production of an antibiotic agent using the paper-disk assay. The microbes were tested against both *S. aureus* and *E. coli*.

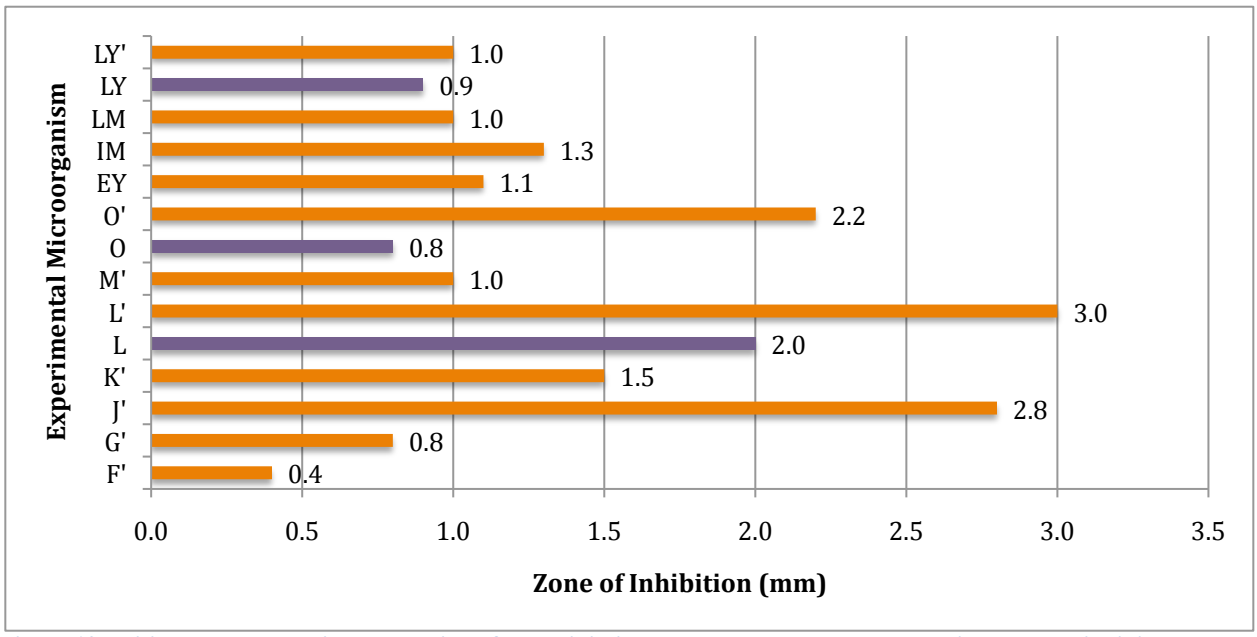

Figure 10: Initial test to determine production of an antibiotic agent. Purple bars represent microbes that inhibited the production of *E. coli* and orange bars represent microbes that inhibited the growth of *S. aureus*. Zones of inhibition of were measured in millimeters and a larger zone of inhibition indicates more potential antibiotic produced.

Zone of inhibition were determined for each microbe observed to be preventing the growth of one of the model organisms. Three isolates (LY, O, and L) inhibited the growth of *E. coli*, while the remaining isolates (LY', LM, IM, EY, O', M', L', K', J', G', and F') inhibited the growth of *S. aureus* [Figure 10]. No experimental microbe was found to produce a compound which inhibited both *E. coli* and *S. aureus*.

Table 2: Recorded morphology of colonies of each experimental microorganism.

Colony Morphology									
Microbe	Diameter (mm)	Pigmentation	Opacity	Form	Elevation	Margin	Appearance	Texture	16S rDNA
K'	1.1	Cream	Opaque	Circular	Flat	Entire	Smooth	Butyrous	n/a
J'	0.6	Cream/orange	Opaque	Circular	Convex	Erose	Smooth	Viscous	Acinetobacter
L	3.0	Cream	Opaque	Filamentous	Umbonate	Filamentous	Rough	Viscous	Bacillus
L'	3.5	Cream	Translucent	Rhizoid	Raised	Undulate	Rough	Viscous	Bacillus
M'	0.8	Cream	Opaque	Rhizoid	Flat	Erose	Rough	Viscous	n/a
O	1.2	Cream	Opaque	Circular	Flat	Entire	Smooth	Butyrous	Bacillus
O'	1.6	Cream	Opaque	circular	Convex	Entire	Smooth	Butyrous	Bacillus
O'L	4.5	Cream	Translucent	Irregular	Umbonate	Filamentous	Smooth	Butyrous	n/a
O'l2	1.6	Cream	Opaque	Circular	Convex	Entire	Smooth	Butyrous	n/a
LY	1.2	Cream	Opaque	Irregular	Convex	Undulate	Mucoid	Viscous	Bacillus
LM	1.6	Cream	Opaque	Circular	Convex	Entire	Smooth	Butyrous	n/a
IM1	5.5	Cream	Translucent	Filamentous	Flat	Filamentous	Rough	Very viscous	Bacillus
IM2	2.0	Cream	Opaque	Spindle	Convex	Slightly undulate	Smooth	Butyrous	Bacillus
LY'	8.3	Cream	Translucent	Irregular	Very umbonate	Lobate	Rough	Very viscous	Bacillus

Microbes that presented the ability to produce an antibiotic agent were picked from the test plates and streaked onto a new plate to create a pure culture of the experimental microbe. Once the pure cultures were incubated, the colony morphology of each organism was recorded [Table 2]. In addition, DNA analysis of 16S rDNA was conducted to determine the genus of the experimental isolates. The PCR was ineffective for some isolates, labeled n/a, and thus an accurate sequence was not obtained.

Initial Extraction

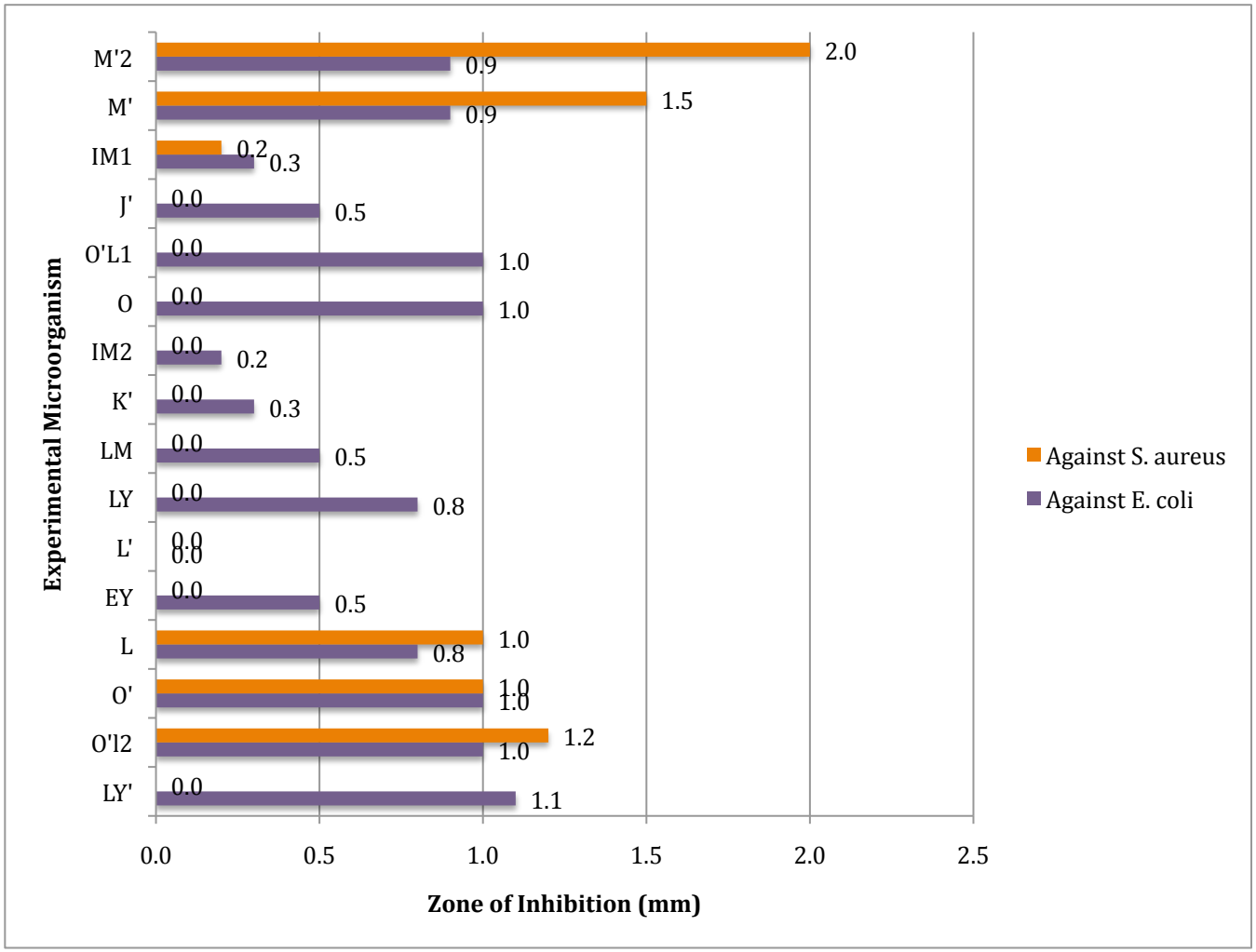

Figure 11: Initial test to extract antibiotic agent from experimental microbes. Samples were tested against both *S. aureus* and *E. coli*. Zones of inhibition against *S. aureus* are colored orange and those against *E. coli* are colored purple. Zones of inhibition were measured in millimeters.

Liquid cultures of each experimental microorganism were centrifuged to remove cells and the supernatant was tested for the presence of an antibiotic agent using the paper disk assay. The supernatant from several isolates (J', O'L1, O, IM2, K', LM, LY, EY, and LY') was found to prevent the growth of *E. coli*, while only a few (M'2, M', IM1, L, O', and O'L2) were also able to inhibit the growth of *S. aureus* as well [Figure 11]. The supernatant of L' was unable to

inhibit the growth of either model organism. Microbes exhibiting zones of inhibition greater than 1 mm were pursued in further analysis.

Extraction and Analysis of Antibiotic Compound

New cultures of the experimental isolates were grown from the freezer stocks after a pause in experimentation. The cultures were tested for antibiotic activity. It was found that the environment of the freezer caused the isolates to stop production of their antibiotic agent. In fact, only three isolates (LY', LM, and O') were observed to produce a partial zone of inhibition [Figure 12].

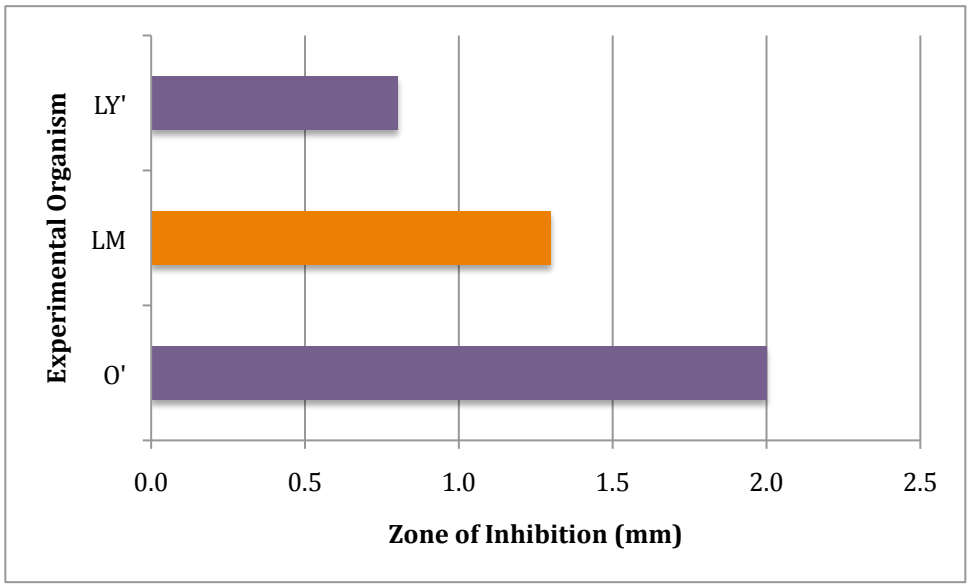

Figure 12: Test for antibiotic production from cultures incubated from freezer stock. Graph represents only microbes which produced a zone of inhibition. The purple bars represent inhibition against *E. coli* and the orange bar represents inhibition against *S. aureus*.

It was then necessary to attempt to stimulate the isolates to continue antibiotic production. Model organisms, *S. aureus* and *E. coli*, were introduced to a diluted sample of each experimental microbe in order to encourage the isolates to continue, or increase, production of their antibiotic agent.

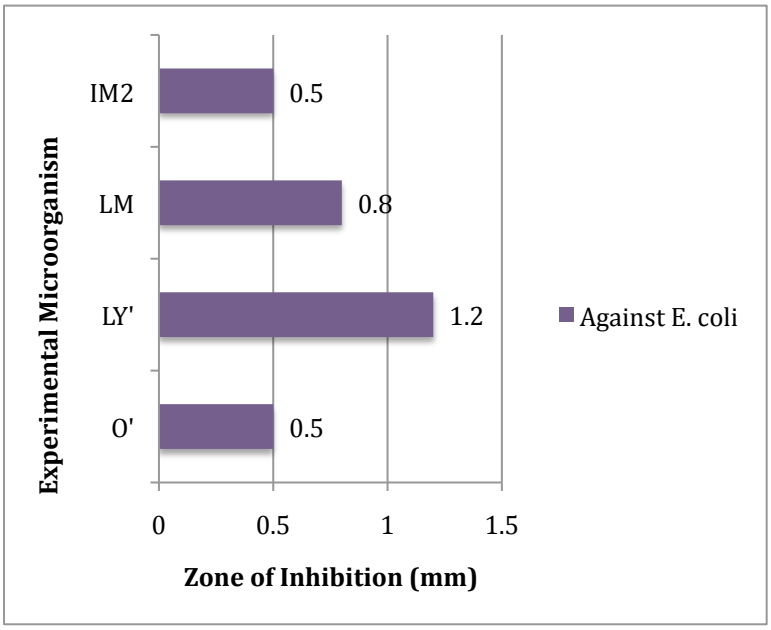

Figure 14: Attempt to encourage antibiotic production using *S. aureus* to promote production. Activity was tested against *E. coli*.

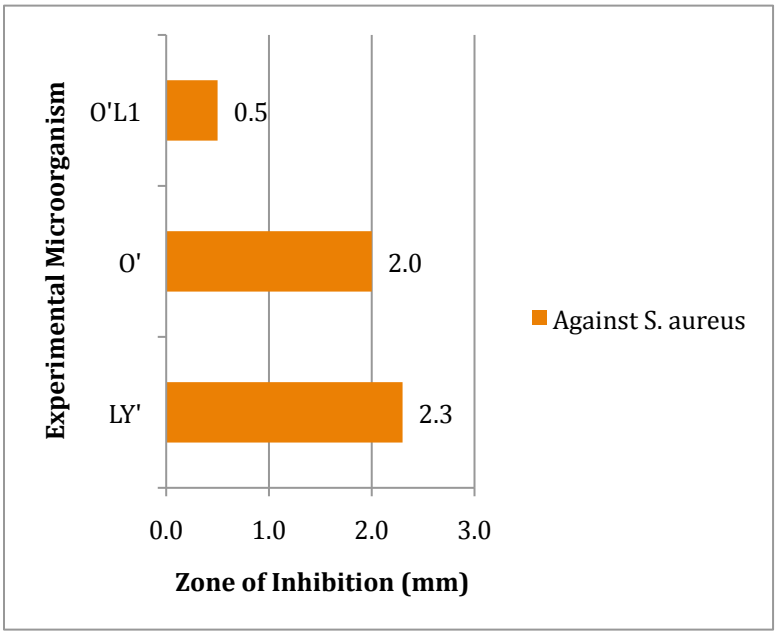

Figure 13: Attempt to encourage antibiotic production using *S. aureus* to promote production. Activity was tested against *S. aureus*.

The introduction of *S. aureus* promoted the production of an antibiotic agent for three of the experimental microbes (O'L1, O', and LY'), when tested against *S. aureus*. Only four of the experimental microbes (IM2, LM, LY', and O') were able to produce a zone of inhibition when tested against *E. coli* [Figures 12 and 13].

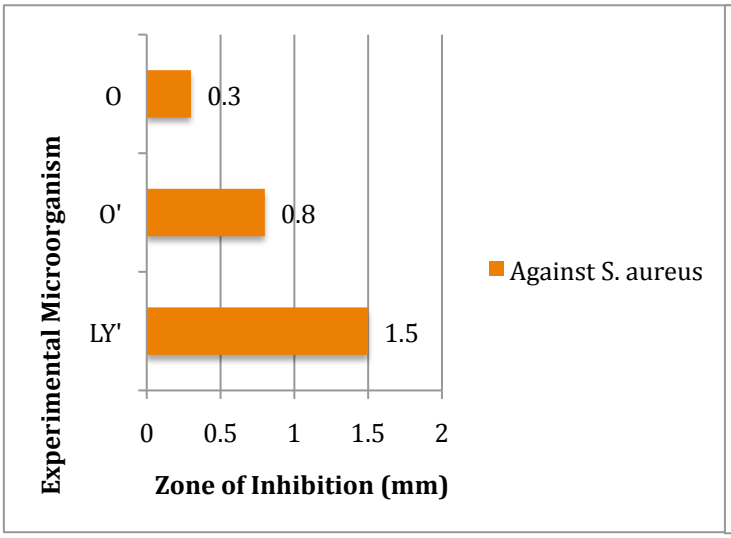

Figure 16: Attempt to encourage antibiotic production using *E. coli* to promote production. Activity was tested against *S. aureus.*

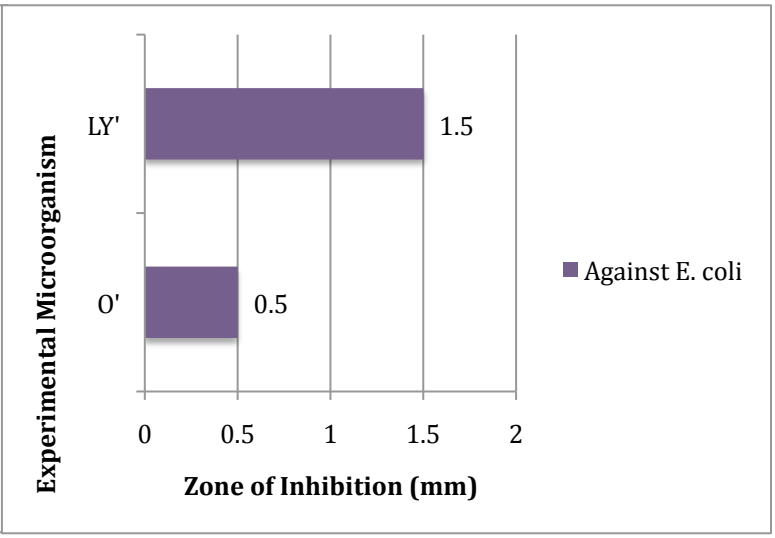

Figure 15: Attempt to encourage antibiotic production using *E. coli* to promote production. Activity was tested against *E. coli.*

In the next experiment, E. coli was used to promote the production of antimicrobial compounds. Zones of inhibition were only observed against *S. aureus* from three microbes (O, O', and LY') [Figure 15]. Only two microbes (LY' and O') were able to resume producing an antibiotic agent again that was successful at inhibiting the growth of E. coli [Figure 16].

After observing four microbes which demonstrated substantial antibiotic production, additional experiments were pursued with these isolates. Experiments were then narrowed to the three experimental microbes with the highest results of inhibition. These microbes were then renamed for simplicity purposes [Table 3].

Table 3: The renaming of the experimental microbes with the highest results of inhibition.

Renaming of Experimental Microbes			
Old Name	O’	LY’	LM
New Name	A	B	C

Extraction and Analysis

After narrowing the isolates for experimentation, pure cultures were incubated for each. Several experiments were then designed and conducted in hopes of extracting the antibiotic from the microbe and its environment. Liquid cultures were made from the pure cultures and were then extracted with an organic solvent. Each extract was tested for activity against *S. aureus* and *E. coli*.

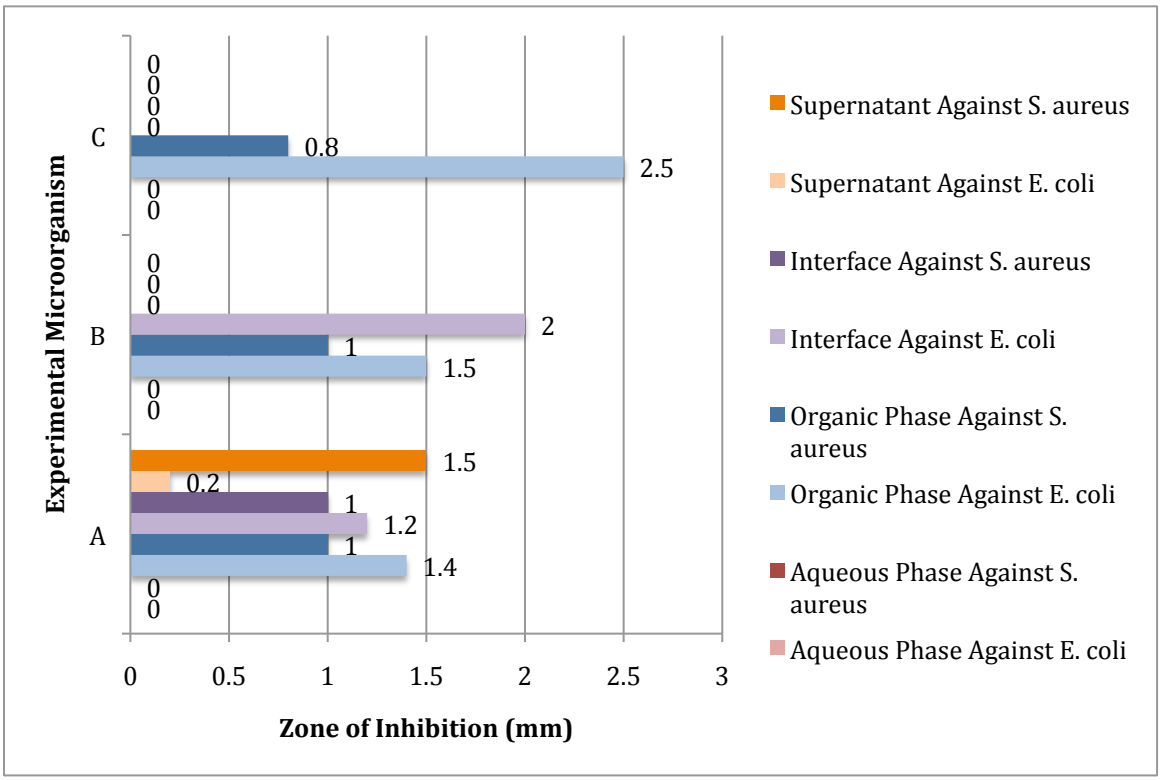

Figure 17: The first trial to design an experiment capable of extracting the antibiotic from the microbe and its environment.

The organic layers presented inhibition against both model organisms from each experimental microbe reaction [Figure 17]. The organic layer is simply leftover chloroform. Given this, a control was made using the organic solvent, chloroform, without the presence of a microbe to determine if the solvent alone would be toxic to the model organisms. Interestingly, the aqueous phase, which is the remaining supernatant, never inhibited the growth of the model organisms.

The supernatant of microbe A was the only experimental microbe to inhibit a model organism [Figure 17]. In fact, inhibition was observed against both model organisms using this supernatant. The interface of microbes A and B inhibited *E. coli*, and interface A was also able to inhibit *S. aureus* as well. The interface and supernatant of microbe C presented no signs of inhibition against either model organism.

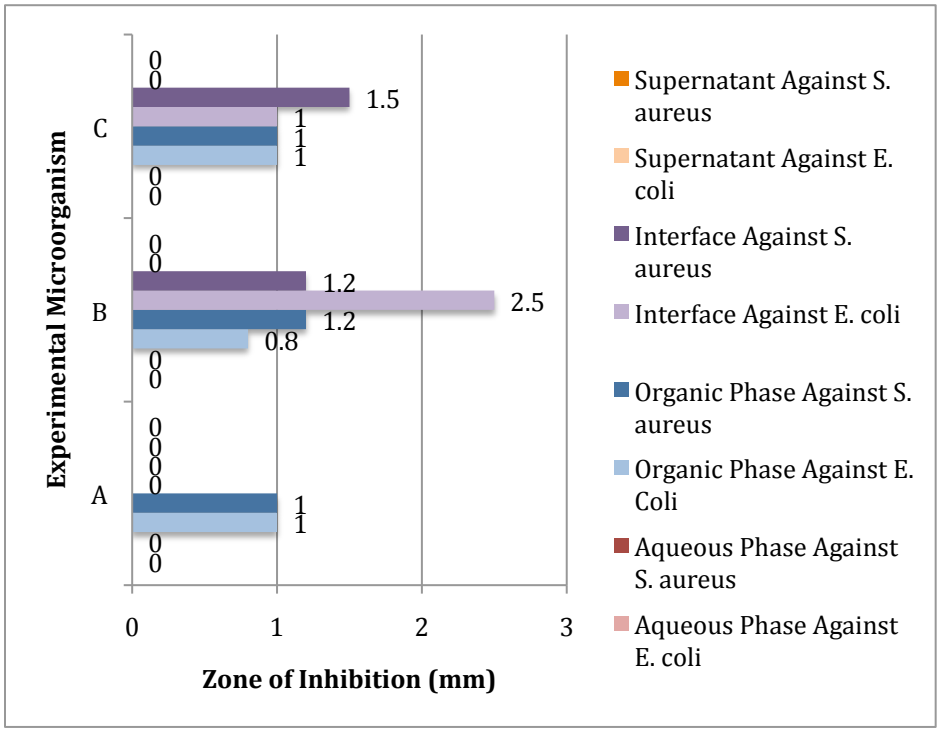

Figure18: The second trial attempting to design and implement an experiment capable of extracting the antibiotic agent from the microbe and its environment.

The second trial was derived from modifying the first trial and was found to present similar data as the first trial. As before, the organic phase caused similar inhibition, as it is simply toxic to the model organisms. The interface of microbes B and C presented strong inhibitions against each model organism. A strong inhibition from the interface of B used against *E. coli* was identified [Figure 18]. No inhibition was observed from the interface or supernatant of microbe A.

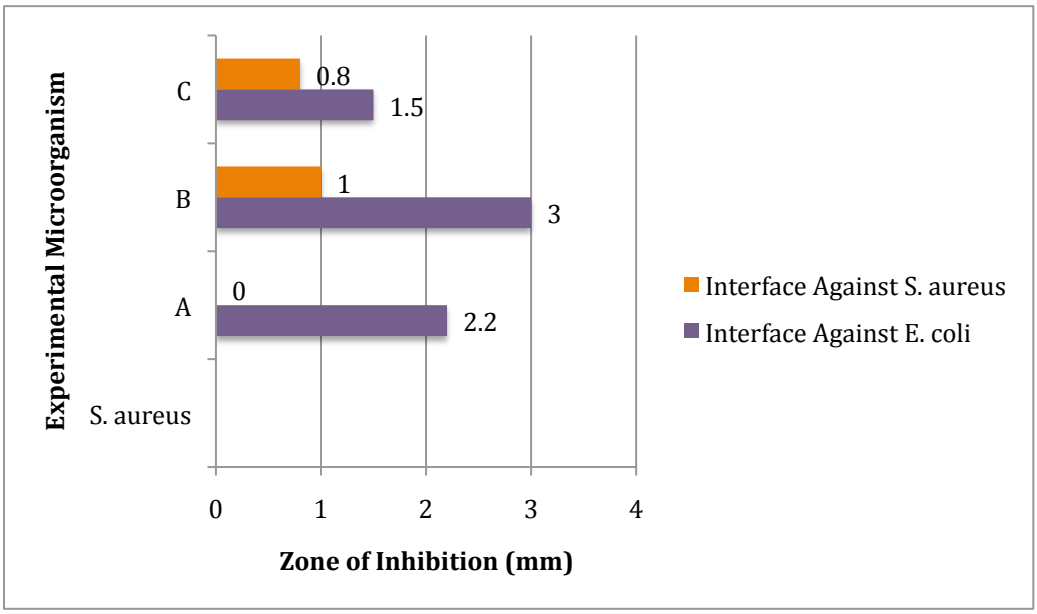

Figure 19: Trial 3 to attempt to extract the antibiotic agent from the microbe and its environment. This figure presents results of inhibition before centrifugation.

After determining that the interface demonstrated the most potent inhibition, A third trial was designed and conducted, focusing solely on the interface. Trial 3 involved testing the interface before and after centrifuging the interface, hoping to isolate the antibiotic agent in a purified, clear liquid to allow further analysis. The interface was first tested for inhibition prior to centrifugation. *S. aureus* was tested as a negative control for inhibition - assuring that there were no other molecules isolated from the supernatant and that there were no other factors causing inhibition [Figure 19]. It was observed that *S. aureus* did not make an inhibitory molecule, therefore the reaction must be isolating some molecule from the experimental isolates. The interface from each experimental microbe caused inhibition against *E. coli*. The interface from microbes B and C caused inhibition against *S. aureus* [Figure 19]. Inhibition was much stronger for each microbe against *E. coli* than *S. aureus*.

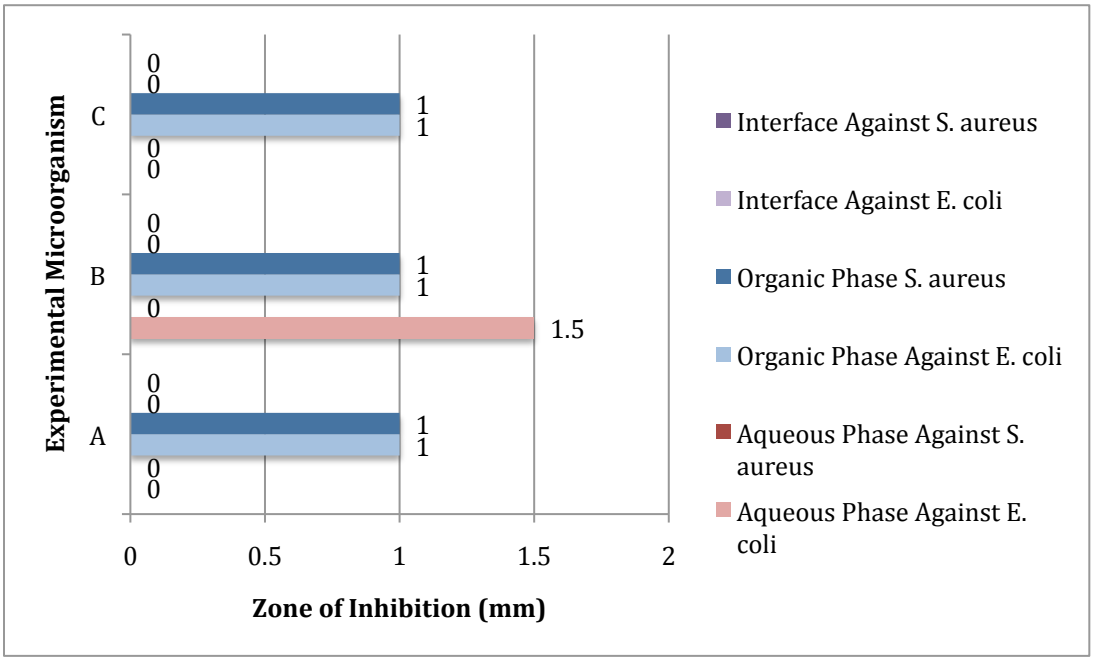

Figure 20: Trial 3 to attempt extraction of the antibiotic agent from the microbe and its environment. This figure presents results of inhibition after centrifugation.

Centrifugation caused a re-separation of the interface. Thus there were again three phases and each was tested for inhibition. As seen in previous experiments, the organic phase caused inhibition against each model organism. The new interface showed no signs of inhibition from any of the experimental microbes against either of the model organisms [Figure 20]. Interestingly, the aqueous phase of microbe B presented a strong zone of inhibition against *E. coli*.

After determining that the clear phase from the interface of microbe B inhibited the growth of *E. coli*, we analyzed that phase to attempt to identify the molecule responsible for that inhibition. HPLC was conducted to determine if a single molecule was being isolated. Therefore, if a single compound was shown, then it is probable that that is the antibiotic agent. In addition, the HPLC will not show peaks from any solvent that enters the system, so when the antibiotic compound was dissolved in hexanes to make a clear solution, then it will not affect the analysis.

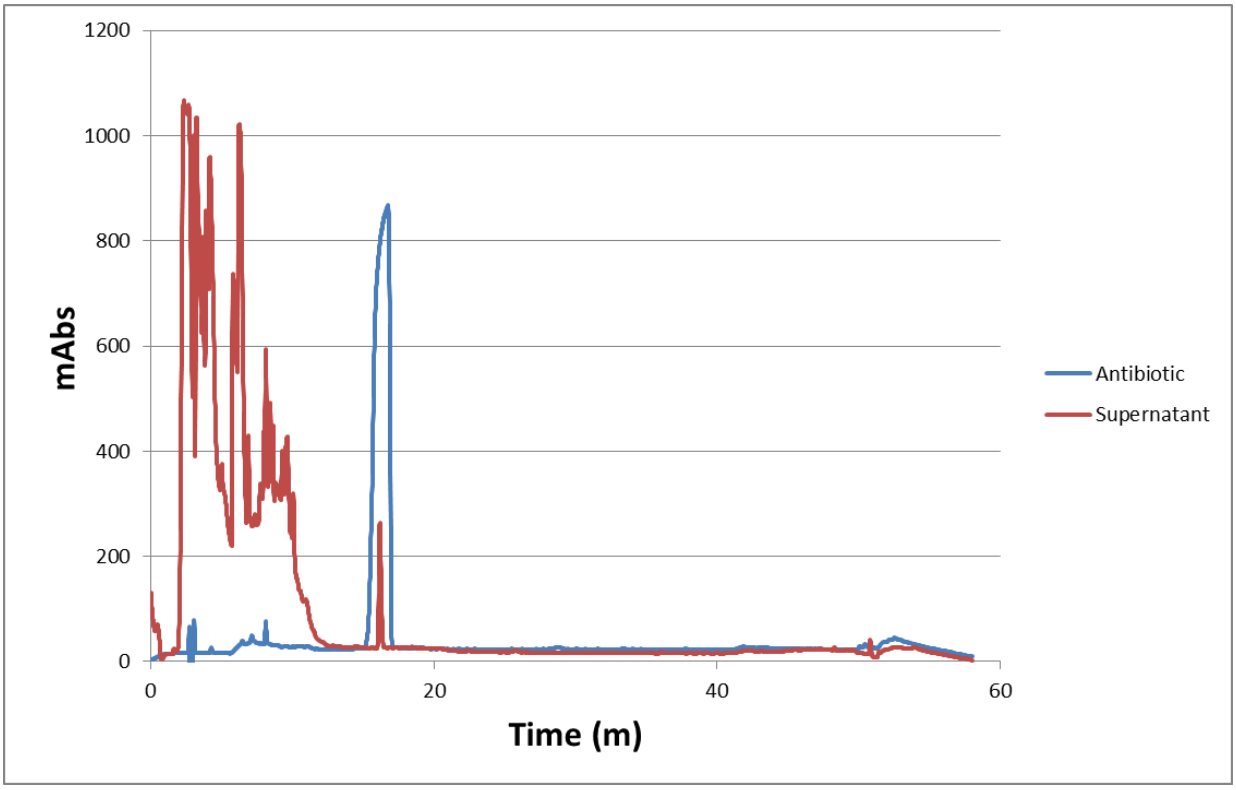

Figure 21: HPLC in absorbance by time (m) at 213 nm. Clear phase containing the antibiotic (blue) was compared to aqueous solution (red) to analyze peaks. The absorbance for the supernatant was shifted upward in order to be observed.

After extraction from the supernatant with chloroform and hexanes, the clear phase from the interface of microbe B was analyzed by HPLC to potentially identify molecules that have antimicrobial properties. The aqueous layer of microbe B was analyzed as a negative control. As it is hypothesized that the compound will be found in the interface, the aqueous layer, or excess supernatant, was tested to assure that there was no longer anything of interest in that layer. The

clear phase presented a strong peak at 16.76 min with a trace of 213 nm [Figure 21]. The supernatant presented no strong peaks. However a reduced level of the same peak from the antibiotic was observed in the supernatant. This can be expected from previous results.

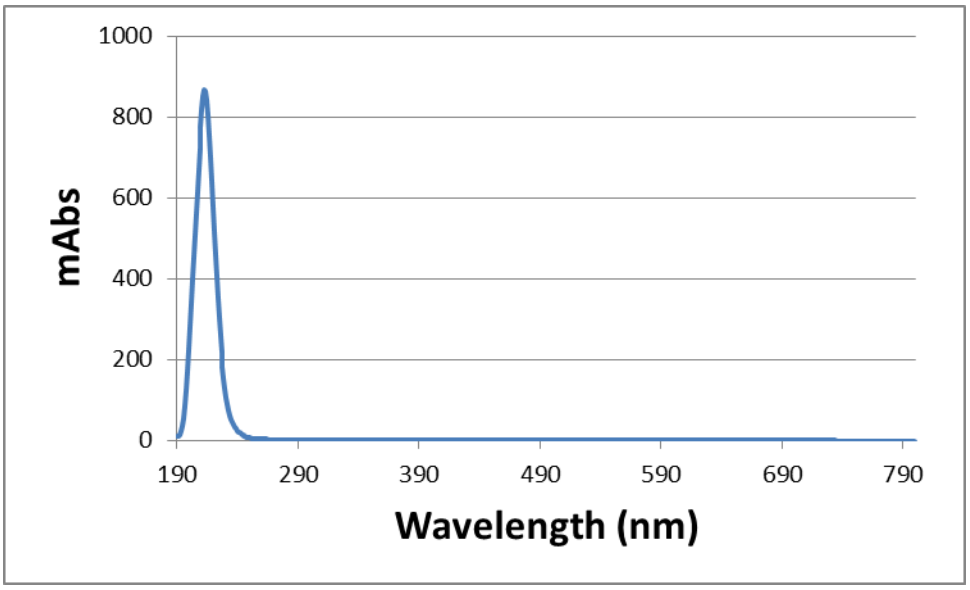

Figure 22: HPLC in absorbance by wavelength at time 16.76 min. Absorbance of clear phase containing the antibiotic (blue) was compared to all wavelengths at 16.76 min. The absorbance for the supernatant was shifted upward in order to be observed.

The HPLC run was also analyzed at the specific wavelength where a peak was observed. This will determine if absorbance occurred at that wavelength at another moment in time during the program. An absorbance peak of the antibiotic occurred only at 213 nm at time 16.76 min [Figure 22]. There are no other observed peaks at this time for the antibiotic. The supernatant presented with no peaks at this time.

Discussion

In search for a novel antibiotic compound, I sampled the environment to cultivate a microorganism with the hypothesis that I would find an isolate which produced an antibiotic agent. I planned to use model organisms with relation to pathogenic organisms as a means to determine antibiotic activity as well as a series of methods to attempt to extract the antibiotic. I also planned to use HPLC to analyze the substance I was able to analyze.

Initial Sampling

The initial sampling found 16 promising isolates that inhibited the growth of *E. coli* or *S. aureus* [Figure 10]. Any samples that showed a clear zone of inhibition was measured. It is interesting to see that from the very first antibiotic test, there were isolates which presented very strong zones of inhibition. J', L', L, and O' had large zones of inhibition, relative to the others suggesting that the isolates were producing a large quantity of an antibiotic agent which it was secreting into its environment. This means it was prepared to defend its environment from other microbes. More isolates were able to inhibit *S. aureus* than *E. coli*. This is to be expected as *S. aureus* is gram-positive because any antimicrobial agent, secreted by a microbe, which targets cell wall synthesis will likely inhibit a gram-positive organism like *S. aureus*.

Initial Extraction

The first test to isolate the antibiotic agent was a fairly simple test as a way to begin to understand the location of the antibiotic. Meaning, it was designed to determine whether or not the antibiotic agent could be found in the supernatant or the cells. We determined that the antibiotic agent was in the supernatant and not the pellet. We also deduced that the antibiotic

agent would not be forced down the centrifuge tube and into the pellet because at this speed, the centrifugation would not separate molecules - they would all be in the supernatant. Only cells are left in the pellet. From this extraction test, it can be seen that most of the isolates' supernatants inhibited a model organism. Most organisms inhibited *E. coli*, while only a few were also able to inhibit *S. aureus*. Only the supernatant of one isolate, L', was unable to inhibit either organism. The inhibition of both model organisms by six supernatants is interesting because it indicates that the antibacterial agent of these isolates was targeting a characteristic that both organisms possess. This is an important find as further analysis of these organisms can lead the discovery of a broad-spectrum antibiotic.

Promoting production of the antibiotic agent

Pure culture plates are only viable for a few months. When experimentation was paused, it caused a need to create new cultures from the freezer stock – the very reason these stocks were made. Given that the isolates were forced into a very harsh environment, it is possible that the isolates would stop producing the antibiotic agent. Losing production can be due to focusing all energy left on surviving in the extreme environment. In addition, there were no competing microbes within the media, thus there was no advantage to continuing to produce an antibiotic. Given this, when creating liquid cultures from the freezer stock, it was necessary to determine if the isolates were still producing the antibiotic agent. After incubating the new cultures, it was found that most of the isolates had indeed lost their ability to produce an antibiotic agent. However, three isolates maintained the function even after freezing [Figure 12].

Once it was observed that most of the isolates lost the function to produce the antibiotic, a procedure was designed to promote the production of the antibiotic agent. The procedure was effective for only a few of the isolates. When *S. aureus* was introduced to the isolate, it promoted the production of the agent for O'L1 to inhibit *S. aureus* on an LB agar plate. It also encouraged

production for LM and IM2 to inhibit the growth of *E. coli* [Figure 13]. Two isolates, O' and LY', were able to inhibit both model organisms [Figures 13 and 14].

This experiment was repeated using *E. coli* as the stimulating organism. When tested against *S. aureus*, O was able to inhibit the growth of *S. aureus* on the LB agar plate. Again, LY' and O' were able to inhibit both model organisms. These experiments have provided promising results. From the experiment to determine if antibiotic production was still occurring O', LM, and LY' were still producing the agent. Attempting to encourage production again in all of the original isolates, O', LY' and LM were again able to inhibit the model organisms again [Figure 15 and 16]. These microbes consistently produced antibiotic throughout experimentation, so I focused on these isolates to attempt to characterize a novel antibiotic. Moreover, the freezer stocks of all of the isolates were saved, so experimentation could also occur with these microbes, especially after some experiments were well-refined.

Trials of extraction

Each trial conducted to extract the antibiotic agent produced results which led to the modifications in the next trial. The first trial to extract the antibiotic through the agar had several difficulties. The various sizes of the zones which were cut out made it difficult to determine how much acetone to add in order to completely dissolve the agar. The procedure was performed before the pause in experimentation and thus was performed on all isolates cultivated from the beginning. The final supernatant was left to dry, but it was never determined which solvent would be optimal to re-suspend the antibiotic without it inhibiting the model organisms. In congruence with this procedure, a second one was designed to determine if the antibiotic agent was found in the supernatant. This procedure was quicker to perform and therefore the results of this experiment were obtained first. Testing the supernatant showed inhibition for most of the isolates against the model organisms. Thus it was determined to continue on the route of using

the supernatant instead of dealing with the agar.

The next experiments designed and conducted focused on isolating the antibiotic compound from the rest of the materials in the supernatant. These experiments occurred after the pause in experimentation and thus were only conducted on the three most promising isolates. The first experiment again tested the supernatant but also attempted to extract the compound from liquid cultures of the isolates using a solvent, chloroform. Extraction was unsuccessful for isolate C. This isolate showed no zones of inhibition except for the organic phase, or the chloroform, which was determined to be toxic to model organisms by itself. The interface of isolate B was found to inhibit *E. coli* pretty successfully with a zone of inhibition of 2 mm. Isolate A was able to inhibit both model organisms using the interface. A strong zone from the aqueous phase, of isolate A, against *S. aureus* was also observed [Figure 17].

The second trial attempted to use the solvent to isolate the antibiotic compound from the supernatant. Once the sample was centrifuged, the supernatant underwent the same treatment as the culture in the previous experiment. When the culture was centrifuged, isolating the supernatant, the isolates observed more inhibition of the model organisms than when the culture was not centrifuged. As the supernatants were originally able to inhibit a model organism, it was expected that separating the compound using a solvent would be successful at inhibiting the model organism from either the aqueous phase or the interface. As hoped, the interface of isolates B and C were able to inhibit both model organisms [Figure 18]. This result shows that the solvent was able to separate the antibiotic agent from the rest of the supernatant. It is one step closer to identifying the supernatant. Unfortunately, isolate A showed no inhibition for any of the phases against either model organism. Once these findings are understood, it is clear that the interface is the more important result to analyze.

It is interesting that the supernatant stopped inhibiting the model organisms after the first supernatant test, especially for isolate A which inhibited both model organisms in the first trial with the solvent [Figure 16] but lost the function with later trials. One possible reason for this was the change in centrifugation. With later trials, a larger sample of the experimental isolates were centrifuged and at a different speed and duration. The larger samples were centrifuged at a slightly reduced speed and for an extra 10-15 minutes. In addition, the larger sample was kept at a cooler temperature during centrifugation. The small samples of cultures were centrifuged at room temperature, while the larger samples were cooled down to 4˚C. Since the procedure was changed so drastically, it is possible that the antibiotic was degraded.

The experiment was repeated, collecting the interfaces to make sure that the results from the previous experiment were accurate, and not a fluke. In addition, the experiment was performed on *S. aureus* as a control to make sure there were no other factors causing inhibition. One possible outside factor could be chloroform within the supernatant which was causing inhibition. This possibility was excluded as no compounds were extracted from *S. aureus* following the same procedure that inhibited either of the model organisms. This control demonstrates that the results from the experiment of the other isolates were accurate and that the previous experiment had valid results. This indicates that the microbes produced an antibiotic and was not just a general effect of the procedure. In this trial, the interface from all three isolates was able to inhibit a model organism. Isolates B and C were able to inhibit both model organisms, as seen in the previous experiment. Surprisingly, isolate A was able to inhibit *E. coli*. Reasons for this could be that this culture of A produced more of the antibiotic agent, so more of it was isolated and thus able to inhibit the model organism.

HPLC

The final experiment to extract the antibiotic compound was repeated for isolate B to prepare a sample for HPLC analysis. Isolate B was chosen as it was always found to have substantial zones of inhibition. Thus it was determined to use this isolate to make sure that the experiments were actually finding a compound of interest. In addition, the aqueous phase from isolate B was also able to inhibit *E. coli* in the final test – furthering the deduction that isolate B will have successful results [Figure 19].

Throughout the entire HPLC program for the antibiotic agent sample, a single peak was observed. In fact, the peak was quite prominent. After 16.76 minutes, the antibiotic compound ran off the column with a strong absorbance at 213 nm. The observation of only one peak means that the solvent extracted a single compound. Thus it can be deduced that the compound we isolated and observed is very likely to be an antibiotic agent.

All wavelengths were analyzed at 16.76 minutes to determine if any other wavelengths were found at that time. It was found that there were no other peaks observed at that time. It can thus be determined that the absorbance at that wavelength was isolated to only that time. Meaning that absorbance of other compounds to did not occur at that wavelength at any other point in the experiment. This furthers the conclusion that we isolated a single compound.

Conclusion

In this project, potentially-novel antibiotic compounds were extracted from a series of environmental isolates. The specific bacterium cultured was analyzed using 16S rDNA and was determined to be of the *Bacillus* genus. The experimental organism was found on a keyboard from the second floor of the Warch Campus Center, at Lawrence University in Appleton, Wisconsin. Through HPLC, it was determined that the antibiotic compound produced by this experimental microorganism could be extracted from the supernatant of the culture, using

chloroform.

Further Directions

The next direction would be to determine the chemical structure of the isolated compound. One possible method would be to use Liquid Chromatography-Mass Spectrometry (LC-MS) to determine the molecular mass of the compound. LC-MS would enable us to apply the small sample obtained through extraction using the solvent, and running the experiment with similar conditions as the HPLC. If the compound was analyzed effectively with the HPLC, using a non-polar column to separate a polar compound, it will require the same method for the LC-MS. LC-MS uses the physical separation ability of the liquid chromatography and pairs it with the capability of the mass spectrometry to analyze the masses of the various parts of the compound.

Following this analysis, and determining the chemical structure, we could then determine which antibiotic class it is closely related to. By doing so, we would determine a strong possibility of what the compound's target is within the pathogenic cells. We would then have a fairly streamlined set of experiments to isolate and analyze compounds of interest from experimental microorganisms. We could then look at the other promising isolates determine what antibiotic compounds were isolated from these microbes.

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