2019

Isolation and Characterization of Soil Bacteria from Radium Springs, GA, and Analysis of Antibiotic Secretions Under Various Conditions

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Isolation and Characterization of Soil Bacteria from Radium Springs, GA, and Analysis of

Antibiotic Secretions Under Various Conditions

A Thesis in Biomedical Sciences by Vashishtha D Patel

Submitted in Partial Fulfillment of the Requirements for the Degree of Masters in Biomedical Sciences

October 2019

This thesis has been presented to and accepted by the Associate Dean for Curriculum and Research Office of Philadelphia College of Osteopathic Medicine in partial fulfillment of the requirements for the degree of Master of Science in Biomedical Sciences.

We, the undersigned, duly appointed committee have read and examined this manuscript and certify it is adequate in scope and quality as a thesis for this master's degree. We approve the content of the thesis to be submitted for processing and acceptance.

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Abstract

Since the discovery of the first antibiotics, they have been a cornerstone of medical treatment for bacterial infections. With the evolution of resistance to these existing agents, it is becoming increasingly important to find novel antibiotics to maintain the level of care of modern medicine.

The *Small World Initiative* created at Yale University aims to tackle this problem by crowdsourcing the study of antibiotics that may be present in soil and sediment in different environments. According to the *Small World Initiative*, over two thirds of antibiotics originate from soil bacteria or fungi.

We aim to characterize the bacteria in soil obtained from Radium Springs, GA and analyze their metabolic products for antibiotic activity against *Staphylococcus aureus* and *Acinetobacter baumannii* under various conditions of environmental stress. This site was chosen because we hypothesized that the presence of trace amounts of radium in the natural water supply may give the local biome unique characteristics. We isolated bacteria from soil samples collected from several locations with differing levels of moisture. The isolates were co-cultured with *S. aureus* and *A. baumannii* (our "tester" strains). We also exposed our tester strains to the metabolites from the soil bacteria to determine if antibiotic activity was inherent to the soil bacteria even without the presence of the target bacteria.

Our research did not find any significant antibiotic activity from the metabolites of our soil bacteria against *S. aureus* or *A. baumannii*. Further tests should be conducted on these samples with different methodologies that may induce the production of other metabolites or varying levels of the same metabolites.

1 Introduction

Less than three years after the discovery of penicillin, researchers had already identified naturally occurring penicillinase[1]. It has been known for almost a century that bacteria can develop resistance to antibiotics; however, the growth in resistant species was moderate until the late 1980's. Starting in approximately 1989, the number of β-lactamase enzymes reported began an exponential growth, which may have contributed to the increased awareness of this problem.

Penicillin was thought to be a cure for all *Staphylococcus aureus* infections until more penicillinases were discovered. This led to a push in research into antibiotics tailored for certain pathogens, such as methicillin[2]. However, in less than three years after the discovery of methicillin, strains of *S. aureus* were already found that had developed resistance against it[3]. This was the beginning of methicillin-resistant *S. aureus* (MRSA), one of the "superbugs" that has received much media coverage in recent years.

MRSA was first detected in nosocomial infections. It is thought that the increased use of methicillin in hospitals contributed to a selective pressure for resistant strains to thrive[4]. Selective pressures may allow bacteria that already harbor resistant genes (r genes) to thrive relative to their counterparts without a similar r gene, though there may be other selective pressures involved[5].

Acinetobacter baumannii is thought to have had many r genes, and it also evolves quickly in reaction to its environment[3]. This leads to intrinsic resistance, where a species develops genes of resistance over time which remain in their genome, though they many not always be expressed. However, this is not the only reported method by which *A. baumannii* develops resistance. It is also capable of horizontal gene transfer through transformation, which allow the bacterium to obtain r genes from its environment and incorporate them into their own genome[6]. This combined ability to obtain r genes from external factors and rapid evolution allows *A. baumannii* to develop resistance to new drugs quickly[3].

1.1 Mechanisms of Transfer of Antibiotic Resistance

Resistance is developed either intrinsically or obtained from external factors. However, any meaningful spread in resistance, whether by horizontal or vertical transfer, requires that a selective pressure is present to make resistant cells more viable (or non-resistant cells less viable). In the last few decades, commercial use of antibiotics in the clinic, agriculture, and meat production has created this artificial selective pressure. This is perhaps the reason for the exponential growth in reported number of β-lactamase genes during the same time period. Although antibiotics are often

overused in the clinic as well as the laboratory, perhaps most surprising is that over 80% of antibiotics (by weight) in use today are used in agriculture—specifically, on cattle[7,8].

While a large portion of antibiotics are used in agriculture, it is still important to consider the effects of clinical over-prescription of these drugs, which may potentially be the root producer of "superbugs" in hospitals. Of course, the problem does not lie completely with physicians—some of whom feel pressured by their patients to write prescriptions that may not be needed. According to one publication, a survey of 1,000 physicians found that 55% had prescribed antibiotics simply to coerce patients into leaving surgery after treatment[9].

Due to the prevalence of antibiotics in the clinic as well as in industry, we are fast approaching a time when many of our drugs will be rendered obsolete for certain strains of bacteria, some of which are ubiquitous. The way researchers have begun approaching the search for novel agents is to chemically design molecules that will function in the ways that existing antibiotics work–while being immune to degradation enzymes that give these microbes their resistance. An alternative approach is to search for novel agents present in nature that have not yet been discovered, as proposed by the *Small World Initiative*. However, it may be possible to narrow down the search by looking at the mechanisms of action of currently available antibiotics.

1.2 Mechanisms of Action of Common Antibiotics

Antibiotics can be categorized into two over-arching groups based on their effect on target strains. Bactericidal antibiotics kill the bacteria they target (and many of those that they do not target), while bacteriostatic antibiotics simply prevent further replication of the bacteria they target (and some they do not target). Because of the growth in resistant bacteria, multiple antibiotics are sometimes administered to increase the likelihood of curing a patient. For example, enterococci species are becoming very difficult to treat with a single drug, so a multi-drug therapy is common[10–12].

Antibiotics can also be categorized by their specific mechanism of action by which they have their desired effect on the bacteria. Some antibiotics, such as β-lactams (e.g. penicillin), prevent synthesis of new cell walls. Other antibiotics work upstream by preventing synthesis of proteins, some of which may be vital for the bacterium's survival. These antibiotics can be categorized as either bactericidal or bacteriostatic, depending on whether they target cell mechanisms responsible for survival, or replication. Those that target mechanisms required for survival are bactericidal, while those that target replication machinery are bacteriostatic. The last major category of antibiotic drugs works by preventing DNA or RNA repair and replication[13].

1.2.1 Cell-Wall Inhibitors

Perhaps the most commonly known cell-wall inhibiting antibiotic is penicillin, a drug in the β-lactam class of antibiotics. This class of antibiotics was initially effective only against Gram-positive species, which have a thick peptidoglycan cell

wall surrounding their cell membrane (Gram-negative bacteria, conversely, have an inner and outer cell membrane, between which there is a relatively thin layer of peptidoglycan and a periplasmic space). It is this peptidoglycan layer that β-lactamase antibiotics disrupt[14]. Many of the early β-lactams are not effective against Gram-negative bacteria because they use different transpeptidases for crosslinking of their peptidoglycan layer. However, starting with the production of ampicillin and other broad-spectrum β-lactam antibiotics, there was some activity against Gram-negative species as well[14].

The peptidoglycan layer in both types of bacteria is made up of chains of alternating N-acetyl-glucosamine (NAG) and N-acetyl-muramic acid (NAM). These chains are crosslinked to one another using a transpeptidase enzyme. The enzymes covalently link peptides composed of L-lysine, L/D-alanine, and D-glutamate located on the NAM residues of the peptidoglycan layer. Penicillin and other β-lactams covalently bind with the transpeptidases (also known as penicillin-binding proteins) and prevent their proper function, thereby disrupting the crosslinking process of new peptidoglycan polymers[15]. The various antibiotics in the β-lactam class have different affinities for the various penicillin-binding proteins (PBPs) naturally found in bacteria, so each antibiotic can be effective against a slightly different spectrum of bacterial species[16].
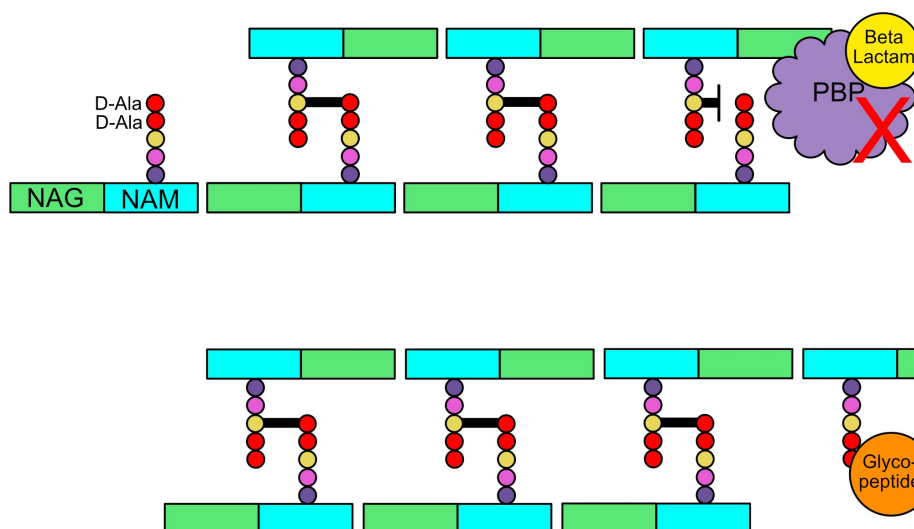
Figure 1.1: **ß-lactam mechanism of action versus glycopeptides mechanism of action.** Top: ß-lactam antibiotics bind to the transpeptidase enzyme preventing its action in crosslinking cell wall peptidoglycans. Bottom: Glycopeptides directly bind the peptidoglycans in the cell wall to prevent crosslinking.

Glycopeptide antibiotics, such as vancomycin, have a similar effect on the cell walls of Gram-positive bacteria, though their mechanism of action is different. Rather

than binding with the transpeptidase enzymes that catalyze crosslinking of peptido-glycan polymers, glycopeptide antibiotics directly bind the peptidoglycan polymers, thereby preventing the addition of new polymers or crosslinking of existing polymers (as shown in Figure 1.1)[7,17].

Therefore, the arsenal of antibiotics available for Gram-negative bacteria is significantly smaller than that for Gram-positive bacteria. However, two polypeptide antibiotics that seem to be effective are colistin and polymyxin B (the latter produced by *Paenibacillus polymyxa* and the former by *Bacillus colistinus*). Both agents have a similar structure and activity and are generally interchangeable. They have an electrostatic interaction with the phospholipid bilayer of a Gram-negative bacterium's cell wall which acts as an antagonist for cations, preventing their entry into the cell[18]. Cations such as calcium are imperative for metabolism in life, and their absence essentially "starves" bacterial cells until they die. Their mechanism of action is external to the cell therefore bacteria are slow to develop resistance. Despite the clear advantages of these drugs, they are saved as a last-resort in most clinical cases because of their relatively high nephrotoxic profile[19].

Of course, many different antibiotics exist that have activity against a spectrum of both Gram-positive and Gram-negative bacteria, with varying degrees of toxicity to humans. Those listed here are simply examples of the major classes of drugs that have at least some activity against Gram-positive bacteria (both *S. aureus* and *A. baumannii* are Gram-positive).

1.2.2 Protein Synthesis Inhibitors

Protein-synthesis inhibitors have many different mechanisms of action and some of these mechanisms are not as well understood as those for cell-wall inhibitors. This class can be further subdivided into drugs that disrupt the 50S subunit of ribosomal RNA (rRNA) and those that disrupt the 30S subunit[11]. Both of these subunits come together during translation to read messenger RNA (mRNA) and create proteins, and some drugs work by preventing this from occurring[20]. Macrolides and linezolids are examples of 50S-targeting antibiotics, while tetracyclines are the major class of antibiotics which target the 30S subunit.

It is believed that macrolides operate by inhibiting the addition of new transfer RNA (tRNA) to rRNA that is actively translating mRNA into proteins[21]. One possible target site for these drugs may be peptidyl transferase, which is responsible for catalyzing the reaction between active rRNA and incoming tRNA[22]. Chloramphenicol is another antibiotic that has a similar effect by targeting the growing peptide strand as opposed to the tRNA or rRNA[23].

The 50S and 30S inhibitors have the same result as β-lactams and glycopeptides, however they achieve it through a different route. While 50S inhibitors target peptidyl transferase, 30S inhibitors such as tetracyclines bind to incoming tRNA[11]. In effect, they "hide" the tRNA from an active rRNA so that new amino acids cannot be added to the growing peptide strand.

Protein-synthesis inhibitors are commonly used in conjunction with cell-synthesis inhibitors in clinical cases. The protein-synthesis component of a multi-drug therapy

can cause production of malfunctioning and misfolded proteins, some of which may be cell membrane proteins. These create a disruption of the cell membrane, allowing cell-synthesis inhibitors into the cell[11].

1.2.3 Nucleic Acid Repair and Replication Inhibitors

Antibiotics that inhibit the repair and replication of nucleic acid molecules can be subcategorized into DNA inhibitors and RNA inhibitors. DNA synthesis inhibitors include classes of antibiotics such as quinolones and nitroimidazole, while the major RNA synthesis inhibitor is rifampin. Rifampin does not belong to a class of antibiotics (such as quinolones or β-lactams)—rather it is its own class.

Quinolones target DNA gyrase and topoisomerase in bacterial cells, which are responsible for easing the stress of supercoiling that is caused during replication and transcription of DNA[24]. However, not all bacteria use the same DNA gyrases and topoisomerases, which limits the spectrum of activity for this class of drugs. A mutation within these enzymes that leaves them able to perform their function can also give them resistance against antibiotics[24].

Nitroimidazoles act as stress agents increasing the presence of reactive oxidative species within a cell, including host cells of the patient[25].

1.3 *Staphylococcus aureus*

1.3.1 Epidemiology of *Staphylococcus aureus* Infections

S. aureus is known to cause a multitude of infections in humans, both in the community and opportunistically in clinical settings. Skin infections, food poisoning, endocarditis, osteomyelitis, and bacteremia/sepsis have all been documented to be caused by *S. aureus*[4,26–28]. According to the CDC, 30% of the US population carries the bacteria in their noses, though it is harmless most of the time. However, certain populations are at-risk for opportunistic infections (for example, those with compromised immune systems)[29].

The incidence of multidrug-resistant *S. aureus* (MRSA) varies based on geography and demographics, however, it is believed that the origins were in Northern Australia in the 1980s or 1990s. Even today, prevalence of MRSA in Australia is significantly higher than that of other countries. In some parts of the country, 42% of the population carried some variant of MRSA[2]. Conversely, infections of MRSA remain relatively rare in Nordic countries such as Norway and Finland. However, it is thought the reason for this is attributable more to strict surveillance programs rather than demographics and hygeine[30,31].

In the US and Canada, there has been concern that MRSA was being spread through Prevnar, a pneumococcal vaccination used on children[32]. This vaccination was routinely administered beginning in 2000 in US, and 2002 in Canada, and coincided with a rise in prevalence of MRSA. The use of the vaccine was only recommended in the US at the time, and the incidence of MRSA increased disproportionally in the US relative to countries with similar demographics and geography. The timeframe

in which the increase occurred in tandem with the locale may have pointed towards some causal link between the two, as researchers have hypothesized (though no such link has been found to date). With the introduction of the vaccine, prevalence of *Streptococcus pneumoniae* has decreased substantially and it is thought that this species may have created a competitive environment for MRSA. Thus, reducing its prevalence would allow MRSA to thrive. If there is indeed a link between the two, the introduction of the vaccination to other European nations beginning in 2006 should mean they would see a similar rise in prevalence of MRSA, which is, in fact, the case[30].

Despite the harmless nature of most *S. aureus* strains, the increasing prevalence is problematic for populations across the globe because of the resistance to treatment options, as well as its ubiquity. When an infection by a resistant strain does arise, options are severely limited, which can lead to fatalities from infections that were once considered minor. These sometimes-fatal infections have a relatively robust dataset to study because of the required hospitalizations. Based on these, there are some factors on the individual level that can be correlated to the prevalence of *S. aureus*. For example, when looking at incidence of bacteremia due to *S. aureus*, the highest incidence of bacteremia in developed countries occurs in those older than 70 or younger than 1 year old[31]. Males also seem to be approximately 50% more likely to suffer from bacteremia caused by *S. aureus*, a phenomenon which has eluded explanation by researchers. Race also seems to have a strong correlation with incidence when it comes to bacteremia in the US, though it can be difficult to separate the effects of social standings and race[33]. However, when compared globally, it does seem that Caucasians have a lower incidence than some other populations (including indigenous Australians, Pacific Islanders, and Africans)[31].

1.3.2 Resistance in *Staphylococcus aureus*

In 1997, Japanese physicians reported the first strain of MRSA that had a reduced susceptibility to vancomycin, the last-line-of-defense drug for these infections at the time. The strain was found in an infant who had undergone heart surgery and developed a fever within two weeks. The physicians isolated the strain from the site of incision. Because of resistance to vancomycin, the treatment for this patient became more complex and the treatment period was lengthened[34].

Vancomycin has long been the treatment of choice for many Gram-positive bacteria that develop resistance to β-lactam drugs. With MRSA's increase in resistance to vancomycin, medicine is fast approaching a period where infections from these bacteria will be a death sentence unless other antibiotics are found.

The first strains of *S. aureus* to display resistance against methicillin preceded vancomycin-resistant strains by several decades, with the first reports appearing in 1961. These strains are thought to have obtained their resistance genes via a bacteriophage. However, these cases were isolated (in the UK) to a few hospitals[35]. By the 1970s, there were five known lineages of *S. aureus* that were known to have resistance. The reports of these strains increased throughout the following 3 decades, though they were still sparse in comparison to incidents occurring today[36]. It should be noted that the MRSA strains in Australia seem to have developed in isolation,

although they appeared at approximately the same time (1965)[3]. It is difficult to determine the exact mechanisms at play in these Australian cases because it was limited to fewer than 500 cases per million visits throughout the 1980s and 1990s. However, there was a clear disproportionality between the Eastern coast of Australia and the Western coast, where cases were few and far between[30].

1.4 *Acinetobacter baumannii*

1.4.1 Epidemiology of *Acinetobacter baumannii* Infections

Unlike *S. aureus*, which has been widely study for decades, *Acinetobacter baumannii* has only been gaining popularity with researchers in the last 20 years. It has been increasingly responsible for outbreaks of bacteremia in hospitals and hospital groups. However, like *S. aureus*, this bacterium is ubiquitous—perhaps even more so. *A. baumannii* can be found on human skin, in food, soil, and arthropods[37]. In a study done by Berlau et. al, 17% of fruits, vegetables, and fungi that were tested grew a strain of *A. baumannii*[38]. The species can be found in almost every level of the food-chain, it seems. One study found that *A. baumannii* is by-far the most common isolate from patients with bacteremia aside from *Staphylococcus epidermidis*[39].

Another study found that 41% of 77 patients who were tested were positive for *A. baumannii* in their gastrointestinal tract[40]. Research indicating its high propensity for growth on food combined with studies that show similar incidence in hospital flora could indicate that hospital food is at least partially responsible for the spread[37]. More work needs to be done to determine the true incidence of the species in the environment, as many studies have been done with small sample sizes. However, empiric research shows that when a hospital environment is created in controlled scientific conditions, *A. baumannii* shows significant improvement in survivability. Some studies have reported an increased lifespan of up to 20 days relative to controls, while others show evidence that it may be able to survive at lower humidity than found in its natural reservoirs[41,42].

Some studies published in the 1990s provide evidence that the incidence of *A. baumannii*-caused infections have been on the rise at least since then. One such publication from 1996 also found that 71% of infections in one hospital were acquired in the ICU[43]. It seems as though the phenomenon is not geographically unique, with another publication reporting a rising incidence in ICU-related infections between 1985 to 1996 in the UK[44]. A more recent study also found evidence that this was still the case as recently as 2007 in the UK, as well as in the Netherlands[45].

An interesting difference between bacteremia caused by *A. baumannii* and *S. aureus* is that the former doesn't seem to discriminate between gender groups, as does the latter. The incidence of bacteremia stemming from *A. baumannii* between males and females was roughly equal in some publications[39]. On the other hand, there does seem to be a preference for specific age groups, though it seems to almost oppose age groups preferred by *S. aureus*. In the same study, the most prevalent age group that was infected was those approximately 50 years of age[39].

1.4.2 Resistance in *Acinetobacter baumannii*

The most rudimentary way that *A. baumannii* seems to protect itself from common disinfectants is through the formation of a pilus-mediated biofilm[46]. It has been shown that these films can be produced on both plastic and glass, which further provides evidence of the nosocomial nature of most infections caused by the species[47]. Other tools that *A. baumannii* has to increase its survivability include proteins that allow for iron-acquisition while maintaining serum-resistance in the bloodstream. In this respect, it is like many *Enterobacter* species[45].

The first major class of antibiotics that *A. baumannii* was reported to have developed resistance to is the β-lactams. Similar to many other bacteria, *A. baumannii* strains have been found to have genes encoding for several β-lactamases[48,49]. The strains exhibiting many of these genes do not seem to be geographically isolated, with occurrences reported in Turkey, Kuwait, Argentina, Belgium, and France, as well as the United States[50–52]. However, there are small variations in the genetic expression that seem to be more, or less, prevalent based on geography. For example, a study of resistant strains in New York City showed reduced expression of carbapenem-resistance genes, but increased expression of class C cephalosporin-resistance genes[53]. Variation in expression aside, genes have been found that provide resistance against all classes of beta-lactams, including carbapenems[51,54].

More recently, researchers have also found genes coding for efflux pumps that seem to give some strains added resistance against carbapenems[50]. These pumps seem to also function against other classes of antibiotics, including aminoglycosides and fluoroquinolones[50,55].

S. aureus and *A. baumannii* also seem to share the Tet(M) protein, which gives them resistance against tetracyclines. These genes were found to be 100% homologous, so it is possible that there may have been some form of horizontal transfer in their lineages[56]. However, it is important to note that only one report has been published of this congruency thus far.

Though much less work has been done on the resistance genes found in A.baumannii in comparison to that done on *S. aureus* (and other heavily studied bacteria), it seems we may be running out of time as resistance increases at a faster rate than we are able to study. This outlines the challenge future physicians will face, as their arsenal of drugs to combat these infectious agents becomes smaller and smaller. As with most bacteria, they also seem to have a disproportionate impact on immuno-compromised patients, in which the array of viable drugs is already limited.

1.5 *Small World Initiative*

The rate at which resistance seems to be growing is a paramount challenge for researchers in the coming years. The best course of action, it would seem, would be to increase the number of man hours dedicated to studying novel antibiotics, as well as increasing the geographic distribution from which they may be derived. One solution, namely the **Small World Initiative** (SWI), was put forth in 2012 at Yale University.

The goal of the SWI is to "crowdsource" the search for antibiotics from soil samples from across the globe[57]. According to the initiative, two thirds of antibiotics originated from pathogens found in soil and to increase the efficiency of testing many soils simultaneously, the initiative proposed a program in which students around the world could isolate pathogens in their local soil and test for antimicrobial activity against ESKAPE pathogens (*Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), the most common sources of nosocomial infections.

The SWI released a publication in 2017 that outlined one of the many processes that could be used to isolate such antibiotic agents. However, teams working towards the goals of the initiative have chosen to go about the search using various methods based on criteria such as available materials, local soil attributes, and personal preference. The commonality for all participants is that their research focuses on the search for antibiotics in soil. Our research methodology for this publication shares some similarities with the protocols provided through the SWI, however, a significant portion of our protocol is proprietary to serve our more specific hypothesis. Though we have incorporated research methods used in other publications to suit our specific needs, we share the same over-arching goal as the **Small World Initiative** in that we hope to make progress towards discovering novel antibiotics that may already exist in the environment around us.

Radium Springs, GA was chosen as the site for soil sample collection because of the trace amounts of radium present in the water table in the area. We hypothesized that the presence of this radium may create a unique flora that is phenotypically, and perhaps genotypically, different from soil that could be found elsewhere.

2 Materials and Methods

2.1 Soil Samples

2.1.1 Collecting Soil

We obtained soil samples from Radium Springs, GA on October 12, 2015, at approximately 9:00 AM. According to the National Centers for Environmental Information, the temperatures on the day of sample collection ranged from a low of 56° F (13° C) to 68° F (20° C), and there was no precipitation for at least seven days prior to collection.

Four samples were collected from various coordinates (longitude and latitude) within 100 meters of the water (Table 2.1). We used a soil probe (*AMS 01.40 Plated Step Probe with Handle* purchased from *Amazon.com*) to obtain plugs of soil that extended from the surface to approximately 60 cm. These were then divided into two separate samples: the upper 20 cm were considered surface soil, while anything below 50 cm was considered as deep soil. Arbitrary names were given to each sample to allow for easy labelling in later experiments.

Table 2.1: Soil Sample Locations. Samples were collected from two locations at Radium Springs, GA, at two different depths to account for both aerobic environments and anaerobic environments.

Sample	Lattitude	Longitude	Depth (cm)	Distance from Water (m)
Jurassic Park 1 (J1)	31.525	-84.135	<20	<100 m
Jurassic Park 2 (J2)	31.525	-84.135	>50	<100 m
Zoolander 1 (Z1)	31.524	-84.136	<20	<10 m
Zoolander 2 (Z2)	31.524	-84.136	>50	<10 m

2.1.2 Preparing Soil Sample Stocks

The soil was then prepared for long-term storage by removing any excess moisture. This was done by placing each sample on a sterile Kimwipe inside of a flow hood for 24 hours. Another sterile Kimwipe was placed on top of the samples to soak up excess moisture. Samples were checked for moisture visually and by texture. Jurassic Park 2 and Zoolander 2 both showed signs of retained moisture after 24 hours, and they were placed into the flow hood for another 24 hours. It was not necessary to measure the moisture precisely as this step was simply to ensure that bacterial growth was limited for approximately one week in cold storage while other preparatory steps were completed.

Once dry, each sample was bagged separately, labeled and placed into cold storage at 4° C until needed. Samples were visually inspected daily for condensation to ensure no excess moisture remained. The storage bags were not opened again until we were ready to use the soil.

2.2 Tester Strains

Our tester strains were *Staphylococcus aureus* (*ATCC# 12600*) and *Acinetobacter baumannii* (*ATCC# 19606*). Both were propagated according to the documentation from *ATCC*. After incubation of the initial stock for 24 hours, as advised in *ATCC* documentation, glycerol master stocks were made for both strains to preserve genetic integrity of the original strain before exposure to any experimentation.

2.2.1 Preparing Master (Glycerol) Stock

We prepared our glycerol stock by first streaking each strain on tryptic soy agar (TSA) and incubating for a period of eight hours at 37° C. This incubation period was long enough to allow for growth of both strains while limiting it enough so that individual colonies could be obtained. After incubation, several colonies that were easily visible but did not overlap with neighboring colonies were selected using a loop. These were then used to inoculate tryptic soy broth (TSB), which was incubated at 37° C on a shaker until turbidity was visible (NOTE: each colony was used to inoculate an individual tube of TSB).

Each tube of TSB was vortexed after incubation to ensure a homogeneous mixture. From each tube, 500 µL of broth culture were placed into cryogenic vials, along with 500 µL of 50/50 mixture of glycerol and deionized water. The cryogenic vials were labelled with the date, strain, and a letter designation of A, B, or C. The letter designation was simply to ensure usage of only one master stock at any given time.

2.2.2 Preparing Working Stock

The remaining tubes of TSB which were used to make the master stock were then used to make working stock. To do this, we took 100 µL of the broth culture for each strain and added it to 20 mL of sterile TSB. These were labelled and incubated for eight hours at 37° C. After incubation, they were moved to cold storage at 4° C until needed. Each working stock was considered viable for a period of two weeks, at which point it was used to create another batch of working stock. After 3 transfers, we discarded the working stock and created a new batch from the master stock. These guidelines were put forth by *ATCC* in their *Technical Bulletin No. 6: Reference Strains.*

The eight-hour incubation period was only used in the initial batch of working stock. After we completed growth curves for each strain, we determined the optimal incubation periods for each strain to ensure we did not have over- or under-growth.

2.2.3 Confirming Resistance/Susceptibility of Tester Strains

To confirm we had received the correct strains of both *S. aureus* and *A. baumannii*, we used a resistance and susceptibility test and compared our results against published research. Our *S. aureus* strain (*ATCC# 12600*) had been found to show resistance to 1 µg cloxacillin disks and susceptibility to 10 µg ampicillin disks by another publication[58]. A prior study also found that *A. baumannii* (*ATCC# 19606*) showed resistance to gentamicin and susceptibility to minocycline, though weight or concentration were not included in the publication[59].

Testing for resistance and susceptibility was done by plating each strain on TSA with glass beads and placing a single disk of each respective test antibiotic on top. The plates were then incubated for 12 hours at 37°C, as we were not interested in limiting overgrowth for this test.

2.2.4 Creating Growth Curves

We completed growth curves for both of our tester strains using sterile TSB as a control over a period of 18 hours. This was chosen as the upper limit as all inoculates had reached stationary phase (no change in absorbance for extended period of time). We used the BioTek Synergy (Winooski, VT, USA) HT microplate reader located at the Georgia Campus of Philadelphia College of Osteopathic Medicine to automate the process. The microplate reader was operated by BioTek's Gen5 imaging software, version 2.00.18.

We began with our working stock that had been incubated for 8 hours at 37° C, vortexed, and used to inoculate wells in a 6-well plate. Three wells were used for our samples in each run of the experiment, as follows:

1. Well 2A contained a control sample with sterile TSB
2. Well 2B contained 1 mL of TSB with 0.2 µL of *S. aureus* working stock
3. Well 2C contained 1 mL of TSB with 0.2 µL of *A. baumannii*

Due to laboratory limitations, we shared the remaining three wells in the 6-well plate with another colleague who was also creating growth curves for another *ESKAPE* pathogen (*Enterococcus faecalis*). Their sample contents are as follows:

1. Well 1A contained 1 mL of sterile TSB
2. Well 1B contained 1 mL of TSB with 0.2 µL of *E. faecalis* working stock
3. Well 1C was left empty

The addition of all components was done aseptically and each plate was immediately sealed until ready to be placed in the microplate reader.
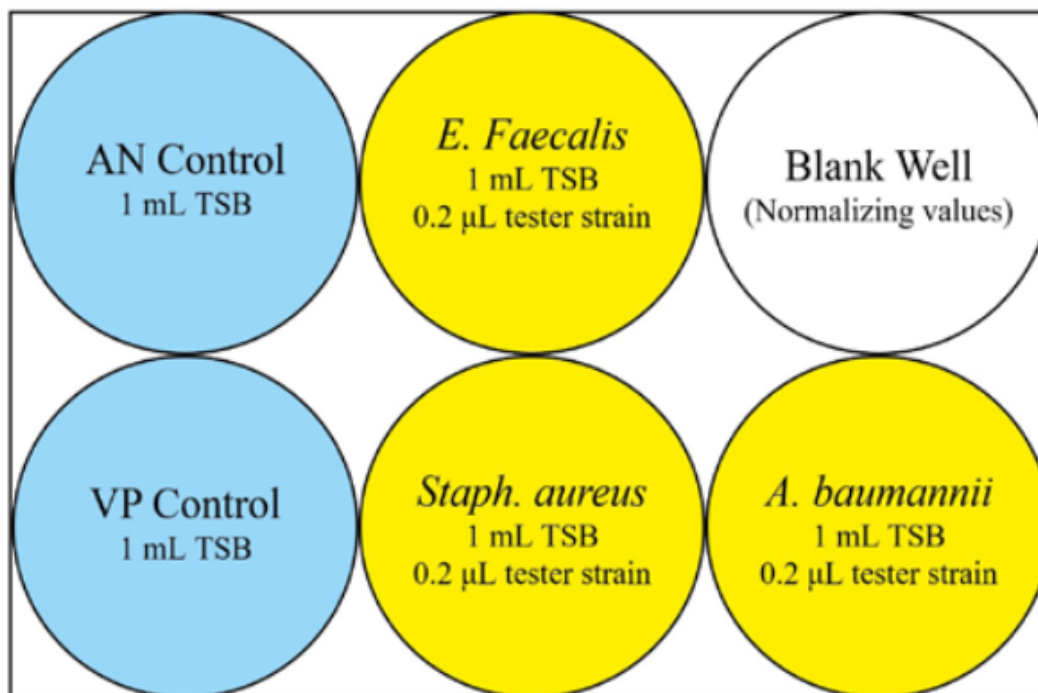
Figure 2.1: **Plate Configuration for Microplate Reader.** A 6-well plate was used for the microplate reader. Wells 1A and 1B were used by a colleague and irrelevant to our experimentation. Well 1C was filled with only deionized water to ensure proper absorbance readings (should read 0.0 at all times). Well 2A was used as our control, with only sterile TSB. Wells 2B and 2C were used for our tester strains (*S. aureus* and *A. baumannii* respectively).

Using Gen5 imaging software, we programmed the microplate reader to take a baseline reading of absorbance at 600 nm and 650 nm in each of the three wells when the process was started, followed by a reading every hour for the duration of the experiment (18 hours).

Typically, a wavelength of 600 nm is used to measure optical density of bacteria in broth[60]. However, to ensure that we were able to verify our growth curves against previously published work, it was important to use wavelengths that had been used in other studies. The only publicly available research using the specific strain of *A. baumannii* that we used in our work (*ATCC# 19606*) measured density at a wavelength of 650 nm[61,62]. Therefore, we chose to have this second measurement as a means of verifying our results against previously published work.

The first run was completed at a temperature of 37° C for 24 hours, followed by a run at 25° C for 36 hours, and a final run at 43° C for 18 hours–all with the shaker on 100 RPM. Note that the microplate was discarded after each run had completed, and a fresh one was made for the next run. This process was then repeated for all three temperatures with the shaker *disabled*.

2.3 Antibiotic Activity of Soil Filtrate Against Tester Strains

2.3.1 Creating Soil Filtrate and Propagating Soil Bacteria

While it was not feasible to determine the total biomass in each sample of soil, it was important to standardize, even approximately, the number of bacteria we began with for each experiment. For this reason, 2 grams of soil was used to inoculate every 20 mL of sterile TSB for each step in this process. Alternatively, it would have been possible to measure the optical density of soil bacteria in broth as we did for our tester strain growth curves. However, this was not done during experimentation and would no longer be possible with the original samples, as the flora may have changed over time.

The soil samples that were placed in cold storage were opened for the first time to create filtrate for this step. Each of the soil samples was subdivided into three aliquots with 2.0 grams of soil each using an analytical balance accurate to tenths of a milligram. The aliquots of each soil sample were immersed into 20 mL of sterile TSB. The inoculation of TSB was done in 50 mL conical tubes which were vortexed, allowed to settle for 30 minutes, and vortexed again. Each tube was then filtered using mesh filters with a pore size of 100 μm to remove soil particulate, leaving all microbes smaller than 100 μm in solution. Each broth culture was then incubated on a shaker at 25° C, 37° C, or 43° C (one inoculate per temperature). The 25° C samples were incubated for 16 hours, the 37° C samples for 8 hours, and the 43° C samples for 8 hours. These values are based on optimal growth periods and temperatures for our tester strains, and will be referred to as our standard incubation period. There was no reason to favor incubation for the same time periods that were optimal for tester strains for the soil bacteria—future experiments should consider other time-frames.

After incubation, we immediately moved all samples to cold storage at 4° C until they were needed. These samples were kept as working stock of soil bacteria for 2 weeks, at which point they were used to create new working stock following the procedure described above (*Preparing Working Stock*). The working stock was propagated into indefinite generations (as opposed to working stock of our tester strains, where we discarded each working stock after 3 generations of propagation).

A. There were 4 separate soil samples used. For each soil sample, 3 aliquots were made. Each aliquot contained 2 gram of soil.
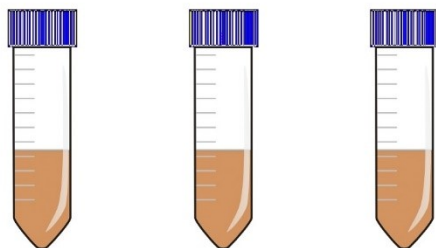
B. Aliquots are filtered through mesh filters, leaving filtrate for incubation.

25°C   37°C   43°C
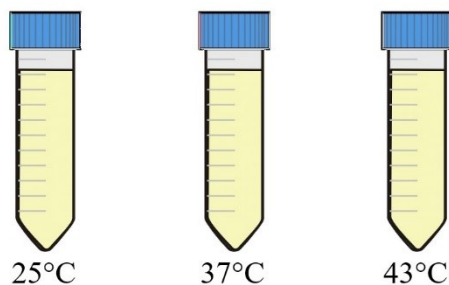
C. Each aliquot was incubated at either 25°C, 37°C, or 43°C (as shown). Growth curves of tester strains were referenced for incubation times at the desired temperatures. After incubation, aliquots were placed in cold storage at 4°C and used as working stock. Working stocks were replenished every 2 weeks.

Figure 2.2: **Creating Soil Working Stock.** Four soil samples were obtained from Radium Springs, GA. From each sample, three aliquots of two grams each were made. Each aliquot was used to inoculate a sterile conical tube with 20 mL of TSB. The mixture was properly vortexed and filtered with a pore size of 100 µm, removing all visible soil particulates. Of the three aliquots from a given soil sample, one was placed in 25° C incubation, one in 37° C, and the last in 43° C incubation. After incubation, these tubes were stored. These were considered our "soil working stock".

2.3.2 Testing Filtrate for Antibiotic Activity

Our goal for this experiment was to test the metabolites produced by soil bacteria as possible antibiotic agents against the tester strains *S. aureus* and *A. baumannii* We began with working stock created from each of the four soil samples, which had been kept in storage until this step was to be completed. From each of the soil sample stocks, we obtained 1 mL of TSB after vortexing. This was passed through a 0.22 µm syringe filter to remove all microbes, leaving only metabolites which were able to pass through the pores.

Four sterile antibiotic sensitivity disks were then soaked in each of the filtered solutions and placed on a sterile Kimwipe in a flow hood to dry. As the disks dried, we plated our tester strains, *S. aureus* and *A. baumannii* on TSA using glass beads (*Fisher Scientific #MP15000550*) to ensure an even growth, creating a "lawn". We used 60 mm plates with 10 µL of tester strain TSB. These were incubated for 15 minutes at 37° C to allow tester strains to adhere to the TSA.

Finally, both disks soaked in filtrate from a given soil working stock were placed on top of the plated tester strains. The plates were then incubated for standard incubation periods[1].

A. From the soil bacteria working stock, 1 mL of each sample (shown in dark pink) was passed through a separate 0.22µm syringe filter. This removes all microbes, leaving only metabolites (shown in light pink).

B. Sets of twelve separate antibiotic discs were soaked in each metabolite filtrate sample. Discs were dried under a fume hood after soaking.

C. Two sets of three plates per metabolite filtrate sample were prepared with a tester strain lawn, one set for *Staph. Aureus* (SA) and one for *A. Baumannii* (AB). Plates were incubated at either 25°C, 37°C, or 43°C, as shown.

Figure 2.3: **Antibiotic Activity in Soil Working Stock Filtrate.** A 0.22 µm syringe filter was used to remove all the bacteria from soil working stock, leaving only media and metabolites. Each sample of soil working stock yielded 12 antibiotic disks (half for testing against *A. baumannii* and half for *S. aureus*). We used two disks per plate of tester strain, yielding three plates of each tester strain per soil working stock. These were used for our three standard incubation configurations.

[1]Standard incubation criteria: 25° C for 16 hours, 37° C for 8 hours, or 43° C for 8 hours.

2.3.3 Co-culturing Soil Bacteria with Tester Strains using Dialysis Tubing

Co-culturing multiple bacteria can induce expression of metabolites in one species that act as antibiotic agents towards one or more of the remaining species. We adapted our co-culturing method from Marmann[63].

For this step, we obtained Biotech CE Dialysis Tubing (*Spectrum Labs #131456*) with a 300 kDa molecular weight cut-off (MWCO). It was cut into strips approximately 10 cm in length and clipped on end with a 16 mm SnakeSkin Dialysis Clip (*ThermoFisher #68011*). This allowed us to fill the tubing with 10 mL of working stock from each of our soil samples using a pipet controller. Another clip was then used to seal the opposite end of the tubing.

A 50 mL conical tube was filled with 20 mL of sterile TSB, which was inoculated using 0.2 uL of *S. aureus* working stock per 1 mL of TSB (4 uL total). Filled and sealed dialysis tubes were then transferred into the *S. aureus* TSB solution. This was carried out for each of the four soil samples in a separate conical tube, and repeated three times to achieve our standard incubation periods.

2.3.4 Testing Co-cultured Filtrate for Antibiotic Activity

After incubation, the tester strain from co-cultures was discarded and working stock from soil samples was removed from the dialysis tubing. We modified the procedure described in "Testing Filtrate for Antibiotic Activity" for the remainder of the experiment.

We removed the soil working stock from the dialysis tubing and filtered it into three separate samples: one sample was made by filtering with a 0.65 μm syringe filter, another with a 0.45 μm syringe filter, and the last with a 0.22 μm syringe filter. Note that the result of this step is the creation of three separate filtrates (each using a different pore size filter) rather than filtering the sample with each pore size in succession (Figure 2.4).

Four sterile antibiotic susceptibility disks were soaked in each filtrate and dried. We also made plates with 10 μL of *S. aureus* working stock on TSA using glass beads to create a lawn. These were incubated for 15 minutes so our tester strain could adhere to the TSA while the antibiotic disks were dried in a sterile environment to remove excess TSB. The dried disks were placed onto the *S. aureus* plates (two disks from each of the filtrates were placed on one *S. aureus* plate). The final step was to complete a standard incubation.

A. Dialysis tube is filled with soil filtrate (shown in pink) and clipped at both ends. Separate dialysis tube co-culturing was performed for each soil sample. Dialysis tube was then submerged in a 50 mL conical tube containing 20 mL of *Staph. aureus* working stock. Each dialysis tube co-culture was incubated 37°C.

B. The dialysis tube was removed from the conical tube and the *Staph. aureus* working stock was discarded. Filtrate from the dialysis tube was separated by filtering through syringe filters with different filter sizes, 0.65μm, 0.45μm, and 0.22μm. Four blank antibiotic discs were soaked in each separate filtrate sample.

C. Plates were prepared with a *Staph. aureus* lawn on the surface. Soaked antibiotic discs were dried and 2 antibiotic discs were placed on each TSA plate, two plates per filtered sample. Of each set of two plates, one was incubated at 25°C and one incubated at 37°C.

Figure 2.4: **Co-culturing Methodology.** Dialysis tubing was filled with soil working stock. The tubing allowed passage of media and metabolites smaller than 300 kDA, but created a physical barrier for bacteria. This test was done to see if the stress of our tester strains would cause our soil bacteria to create different metabolites than if they were to be incubated independently.

2.4 Antibiotic Activity of Soil Precipitate Against Tester Strains

2.4.1 Obtaining and Stamping Precipitate

For this technique, a Buchner funnel was used in association with a vacuum pump and filters with various pore sizes. The goal was to remove metabolites and media from our soil working stock, leaving behind only bacteria. These bacteria were then plated directly on top of lawns of *S. aureus*. It should be noted that *A. baumannii* was not used from this point forward due to exponential growth in samples after each consecutive step. Each of the four soil samples was filtered using filter paper with three different pore sizes. Each of these filtrates was then tested in a co-culture against the tester strains at three different temperatures. Therefore a total of 108 plates *per* tester strain *per* repitition of this test would be needed. For the same reason, we also chose not to pursue testing at 43° C from this point forward to limit the number of samples further. This was done to reduce costs of sequencing multiple samples.

The configuration of the vacuum system can be seen in Figure 2.5. The vacuum

system was used to speed up the separation of media from the bacteria, which was to be plated with our tester strain.
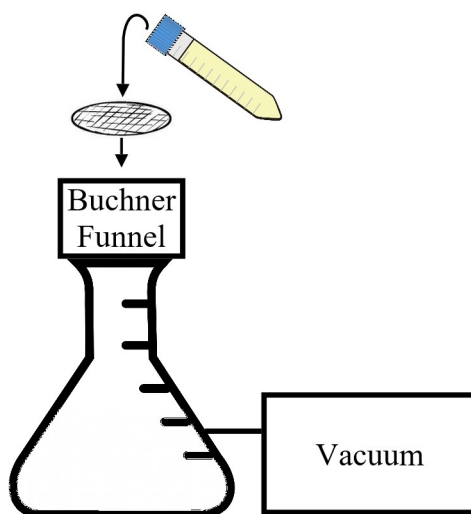
Figure 2.5: **Vacuum Pump with Buchner Funnel.** A Buchner flask was used to speed up the separating of media and metabolites from bacteria. Filters with various pore sizes were placed inside the funnel and soil working stock was poured in with the vacuum pump running. This pulled the solution through the filters, while the bacteria were caught.

Working stock from each of the four soil samples was run through a 0.65 μm filter first, followed by a 0.45 μm filter, and finally a 0.22 μm filter. Therefore, each of our soil samples rendered three filters with bacteria to be plated on *S. aureus*. These filters were to be used for incubation at 25° C, however, this process was repeated to create filters that could be used for incubation at 37° C. At completion of filtering, each soil sample yielded six filters to be plated (the total number of filters was 24, six from each of four samples)(Figure 2.6).

Note that each soil sample was filtered in filter paper of decreasing pore size (0.65μm, followed by 0.45 μm, followed by 0.22 μm). This was done to ensure that the largest bacterial cells were retained on the largest pore size, while allowing the smaller cells to pass through to the next pore size.

A. A filtration device was used to filter 10 mL of each sample of soil working stock three times. With each use, a new filter of a different pore size (0.65µm, 0.45µm, and 0.22µm) was placed inside the Buchner funnel to capture bacteria. The remaining filtrate was discarded. This process was repeated four times, once for each soil working stock, totaling 12 filters.

B. Each filter collected was stamped once and removed on two separate TSA plates, each prepared with a *Staph. aureus* lawn. One plate from each set was incubated at 25°C and the other at 37°C. From the 12 filers, 24 plates were stamped and incubated.

Figure 2.6: **Plating Precipitate from Soil Working Stock.** All four soil working stocks were filtered using a Buchner flask. The largest pore size was used first (0.65 µm), followed by 0.45 µm, and finally 0.22 µm.

2.4.2 Culturing Precipitate atop Tester Strains

The filters produced from the precipitate (as shown in Figure 2.6) were marked with a small tick mark at an arbitrary location. Each filter was then stamped, or replica plated, onto sterile TSA and a corresponding tick mark was made on the plate, corresponding with the identical tick on the filter. The same filter was then replica plated onto a plate that had been prepared with 10 µL of *S. aureus* working stock. Again, a tick mark was made on the plate corresponding to the identical tick on the filter. This was done so that we could align the plate with only precipitate with the plate of *S. aureus* AND precipitate. If a zone of inhibition was seen around a given colony on the plate with our tester strain, we could then isolate the identical colony from our precipitate plate and ensure we were getting only the unknown soil bacteria rather than a mixture of soil bacteria and *S. aureus*.

Each soil working stock yielded six filters: two 0.65 µm filters, two 0.45 µm filters, and two 0.22 µm filters. Each filter then yielded two plates: one for 25° C and one

for 37° C. Since each soil working stock yielded a total of 12 plates, we had 48 plates at the end of this step (12 plates times 4 soil samples). A hierarchy chart starting with one soil sample is shown below (Figure 2.7).
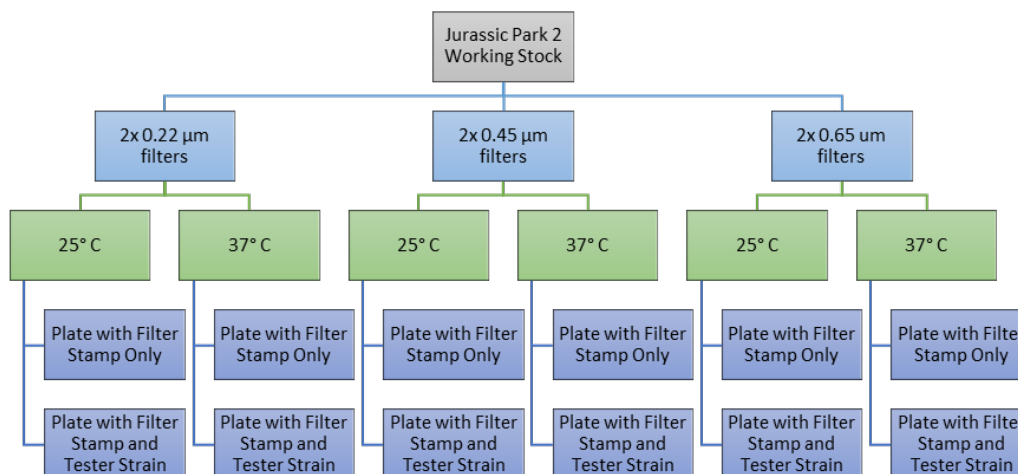
Figure 2.7: **Hierarchy of Stamping Process.** Only one soil sample is shown. This hierarchy would be identical for each soil sample.

If we had continued with *A. baumannii* and our 43° C experimentation, we would be left with 144 plates at this point, and this would multiply as the experimentation continues. For this reason, we limited our samples.

2.4.3 Selecting and Propagating Colonies with Possible Antibiotic Activity

After incubation of all the plates, we checked for zones of inhibition around the colonies of unknown bacteria that had grown on the plate with our tester strain. If a zone of inhibition was visible, we circled the location of the colony on the underside of the TSA plate. After all plates with both soil bacteria and tester strain had been inspected, we placed the plates with no visible zones of inhibition in cold storage at 4° C.

For the remaining plates (those with visible zones of inhibition), our goal was to isolate the unknown bacterial species for further testing. To do this, we first aligned the two plates that were from the same stamp using their corresponding tick marks (e.g. the co-cultured plate created with a 0.22 μm filter from Jurassic Park 2, incubated at 25° C was placed on top of the plate with only soil bacteria that was created with the same filter). The circles on the underside of the co-culture plates indicating zones of inhibition were traced over to the plates with only soil bacteria. In this way, we were able to keep track of which colonies we had isolated in the following steps.

After all prospective unknowns had been marked on the soil-bacteria-only plates, we used the aseptic technique with a loop to pick off individual colonies and used

them to inoculate sterile TSB (each unknown colony was used to inoculate its own tube of TSB). The TSB was then incubated at 37° C until turbidity was visible. It was not imperative to be more exact than "visible" at this stage because we were simply trying to grow the unknown in isolation so it could be used to streak a plate of sterile TSA. Using the loop technique, we streaked sterile TSA with each of the unknown broth cultures (again, each unknown was used to inoculate its own plate of TSA).

The last step for isolation was to select a single colony from each of the streaked plates and inoculate sterile TSB. These would be our working stock for each of the unknowns. The first batch of working stock was also used to create a master stock based on the procedure outlined in *Preparing Master (Glycerol) Stock.*

In summary, we selected colonies from the replica plates using a loop and aseptic technique and incubated them in TSB. These broth cultures were then used to create streak plates on sterile TSA to give us another opportunity to select an isolated colony with a loop. Finally, a loop was used to select a colony, which was used to inoculate another tube of TSB. This second broth culture was used to create stock (Figure 2.8).

Working stock of our unknowns was considered viable for two weeks, at which point it was used to create a new batch. After 3 generations, the working stock was discarded and new stock was created from the master stock.

A. After incubating the stamped TSA plates, plates were examined for any zones of inhibition. Bacteria collected on filters were stamped on *Staph. aureus* lawns (shown in orange). If a bacterial colony (shown in pink) inhibits the growth of the tester strain (shown in white), that colony was chosen for isolation.

B. Using a sterile loop, a sample of the chosen colony was taken for isolation. The loop was then used to inoculate a conical tube of TSB, one tube per colony. Each tube was incubated at 37°C.

C. After incubation, a sample of the culture was placed in glycerol stock and the rest was used as a working stock for identification.

Figure 2.8: **Selecting Isolates for Further Testing.** Isolates were selected if a zone of inhibition was visible. The loop technique was used on the plates with only our soil precipitate, NOT the plates with our tester strain. This was to ensure that we obtained a pure sample of unknown bacteria that was able to inhibit growth of *S. aureus*.

2.5 Identifying Bacteria from Samples Exhibiting Antibiotic Activity

2.5.1 DNA Sequencing

DNA sequencing was outsourced to EMSL Analytical Incorporated (*EMSL Test Code M192*). The samples were sent to their laboratory located at: 200 RT 130, Cinnaminson, NJ 08077. Because of the prohibitive cost of high throughput sequencing, we were not able to have all samples sequenced. We were limited to only one of the unknown samples, and we chose sample Z2 0.22 25 A (each sample is discussed further in *Results* and can be found in Table 3.2). This unknown with antibiotic activity against *S. aureus* was found less than 10 m from water's edge at Radium Springs, GA, at a depth of greater than 50 cm from surface (Table 2.1).

This sample was chosen because it was one of four isolates that produced an antibiotic activity against *S. aureus* from a single soil sample, using a single filter size (0.22 µm), at a single incubation temperature (25° C). We believed that this showed the greatest potential for further research, and our limitation of sequencing only one sample meant we were forced to choose the sample with most potential.

EMSL required that our samples be used to inoculate TSA slants and sent overnight to their lab to ensure cells were still viable on their arrival. They used 16S ribosomal RNA sequencing to determine the genus and species of our sample. The sequences they found were cross referenced with the GenBank database from the National center for Biotechnology Information (NCBI). The sequencing results from EMSL can be found in the Appendix (*DNA Sequencing Results*).

2.5.2 Gram Stain, Mannitol Fermentation, Motility, and Crystal Formation Testing

All samples with possible antibiotic activity against *S. aureus* were first Gram-stained using the protocol described in the Appendix (*Gram Stain Protocol*). Gram-stains were completed using working stock that was created in an earlier stage (*Selecting and Propagating Colonies with Possible Antibiotic Activity*). Based on the result of the Gram stain, one of two dichotomous keys (one for Gram positive species, and one for Gram negative species) would be used to carry out further identification tests; however because all of our samples were found to be Gram positive, only the Gram positive dichotomous key is included here (Figure 2.9)[64].

Gram stains were also used to determine spore formation, the second step of the dichotomous key when samples were seen to be bacillus. Spore formation was then confirmed using malachite green and safranin staining. Because spores were present in all our samples, we did not complete a catalase test. We then used a mannitol fermentation test to narrow down possibilities. This allowed us to determine that all our samples were species in "Group 1" bacilli.

Gram Positive Dichotomous Key
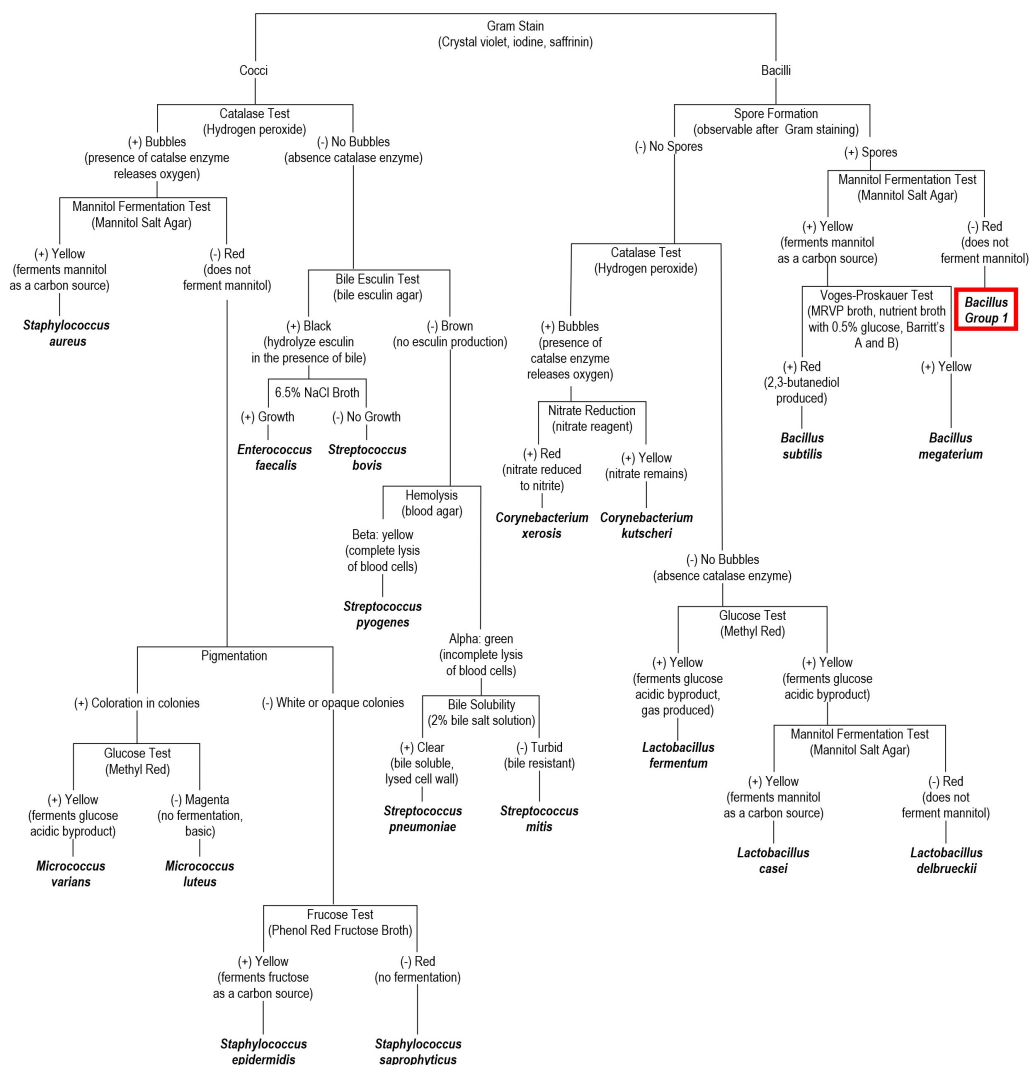
Figure 2.9: **Identification Dichotomous Key.** This key was followed for identification of our unknown samples, narrowing down the possible outcomes at each step. Key was obtained from prior publication by Amanda Nguyen.

A secondary dichotomous key was required to narrow down which species our unknowns may be, as this dichotomous key for all Gram positive bacteria did not differentiate between species within Group 1 Bacilli. This secondary key (Figure 2.10) for differentiating between Group 1 Bacilli was designed by Amanda Nguyen, a fellow student, based on guidelines put forth by Public Health England in UK Standards for Microbiology Investigations[64,65].

Bacillus Group 1 Differentiation
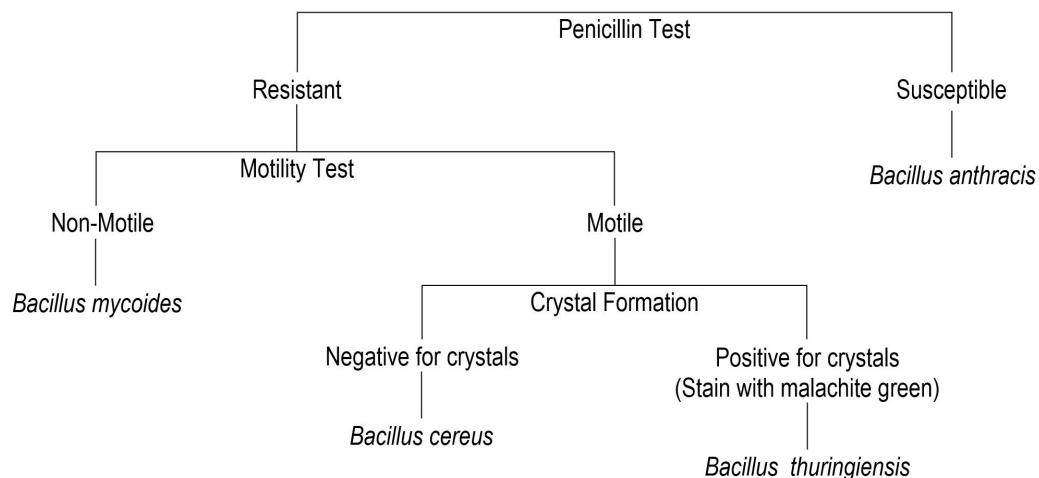
Figure 2.10: **Dichotomous Key for Group 1 Bacillus.** This key allowed further differentiation of our unknown strains. Obtained from Amanda Nguyen based on information from UK standards for Microbiology Investigations.

We then conducted penicillin susceptibility tests on all our samples and found all unknowns to be resistant. The next step in the above key was to carry out motility testing, for which we used pre-made motility, indole, and ornithine (MIO) tubes which also tested for indole production and ornithine decarboxylase, though these tests were unnecessary based on previous identification tests. Finally, some samples were tested for carboxylic acid crystal formation with malachite green stains

3 Results

3.1 Preparatory Tests

3.1.1 Confirmation of Resistance and Susceptibility of Tester Strains

We are confident that *ATCC* delivered the correct strains of bacteria to us, so this step was simply an authentication test before moving further into experimentation. A previously published study used *ATCC# 12600* (*S. aureus*) for resistance and susceptibility testing and our results were congruent with their research[55]. In both cases, the strain of *S. aureus* was found to exhibit resistance to cloxacillin and susceptibility to ampicillin. Another team conducted similar research on our strain of *A. baumannii* (*ATCC# 19606*), and our results for this strain were congruent to theirs: *A. baumannii* (*ATCC# 19606*) was found to be resistant to gentamicin and susceptible to minocycline (Table 3.1)[59]. It was not considered necessary to further authenticate that we had received the correct strains from *ATCC*.

Table 3.1: Susceptibility Testing. Results for susceptibility testing are shown here. The concentration of antibiotics was selected based on the studies referenced above.

Tester Strain	Antibiotic Disks	Resistance	Zone of Inhibition
Staphylococcus aureus (ATCC# 12600)	Cloxacillin (1 Î¼g)	Resistant	14 mm
Staphylococcus aureus (ATCC# 12600)	Ampicillin (2 Î¼g)	Susceptible	23 mm
Acinetobacter baumannii (ATCC# 19606)	Gentamicin (10 Î¼g)	Resistant	13 mm
Acinetobacter baumannii (ATCC# 19606)	Minocycline (30 Î¼g)	Susceptible	8 mm

3.1.2 Growth Curves

Growth curves were completed two times at each temperature (25° C, 37° C, 43° C) with the shaker on, and two times at each temperature with the shaker off, for a total of 12 runs. During each run, absorbance readings were taken at two wavelengths (600 nm and 650 nm). Noise introduced by TSB was removed by subtracting the absorbance of the sterile TSB at the matching wavelength and time from each reading. For example, in the first run at 37° C with the shaker on, the absorbance of *S. aureus* after eight hours at 600 nm was 0.692 (OD), while the absorbance of sterile TSB was -0.006 (OD). To normalize the value of the *S. aureus* reading, we subtracted -0.006 (OD) from 0.692 (OD), giving us an absorbance of 0.698. Note that a negative absorbance is not possible, however, the magnitude of the reading here is negligible. These curves were completed several times to ensure repeatability and yielded some anomalous negative readings in the first hour each time.

The normalized values for the two corresponding readings were averaged together. For example, the normalized absorbance of *S. aureus* during the first 37° C run with shaking after eight hours was 0.698 (OD). During the second run with the same settings, the normalized absorbance after eight hours was 0.712 (OD). These values were averaged, giving a final value of 0.705 (OD). This was done for each reading in each run.

The normalized and averaged values for each temperature, with and without the shaker, were consolidated into one table for *S. aureus*, and one for *A. baumannii*. Both of these tables can be found in the Appendix (*Staphylococcus aureus* Growth Data* and *Acinetobacter baumannii Growth Data*). For *S. aureus*, the readings taken at 600 nm were used, while the readings at 650 nm were used for *A. baumannii* The values in both tables were then charted using Microsoft Excel 2017 to obtain a final growth curve (Figure 3.1 and Figure 3.2). These values were plotted using absorbances at a wavelength of 600 nm. A consolidated table was also created for *A. baumannii* using the same process, although the readings used were taken at a wavelength of 650 nm.
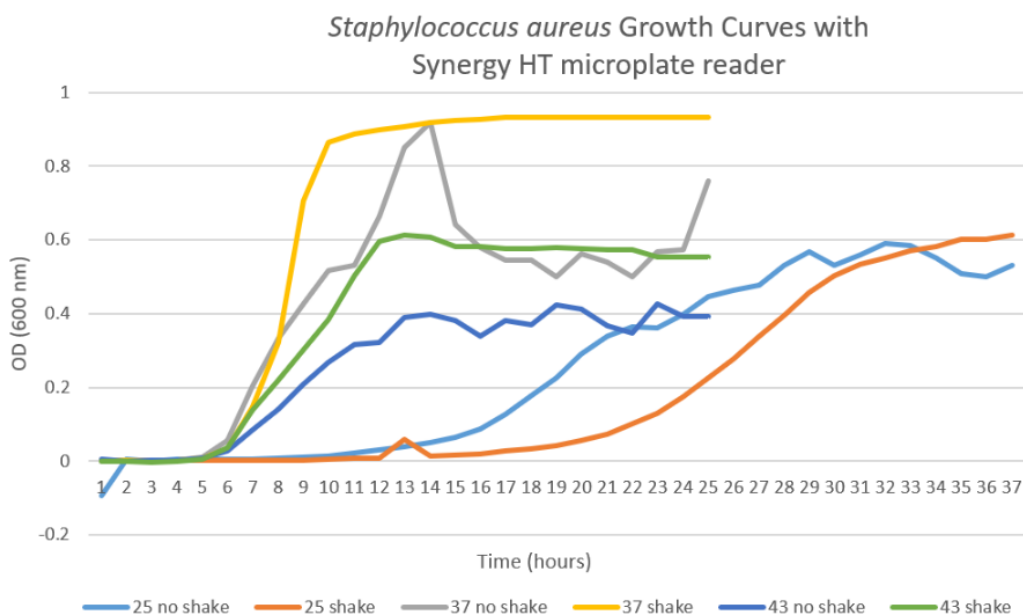
Figure 3.1: **Growth Curves for *S. aureus*.** Data can be found in Appendix A.2. Optimal incubation periods ("standard incubation") for *S. aureus* were obtained from this chart.
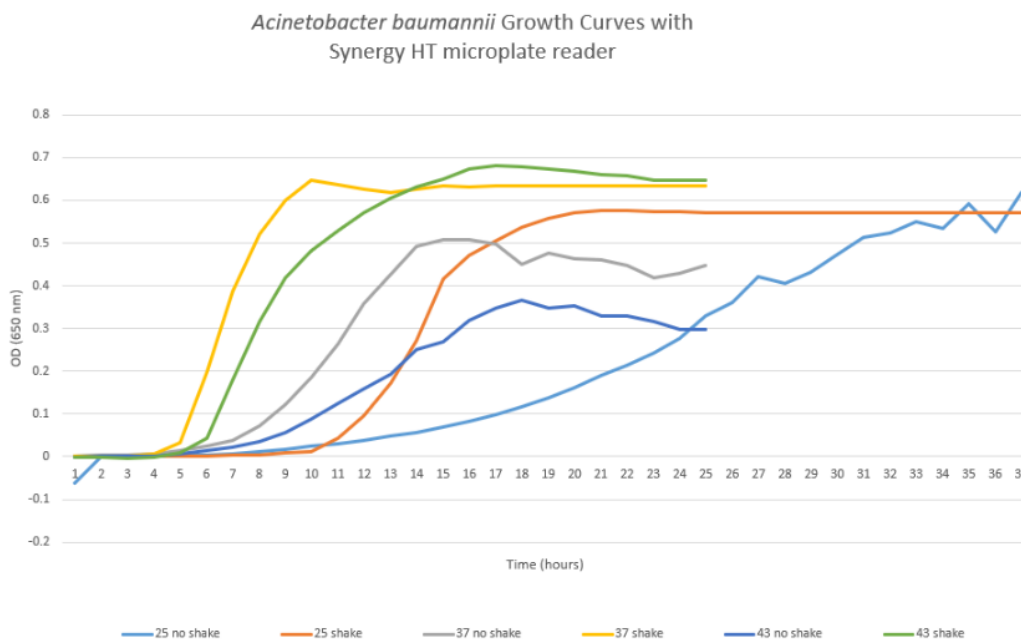
Figure 3.2: **Growth Curves for *A. baumannii.*** Data can be found in Appendix A.3. Optimal incubation periods ("standard incubation") for *A. baumannii* were obtained from this chart.

3.2 Antibiotic Viability of Soil Filtrate

3.2.1 Antibiotic Activity of Soil Filtrate Against Tester Strains

We tested filtrate for antibiotic activity a total of three times (beginning with the original soil working stock each time). Only two sets of disks showed any zones of inhibition when placed on our tester strain. In fact, some of the metabolites from various samples seemed to encourage growth for our tester strains. The two sets of disks that showed inhibition happened to be our control disks, soaked only in sterile broth (Figure 3.3). The remaining disks showed no inhibition of tester strains (Figure 3.4).
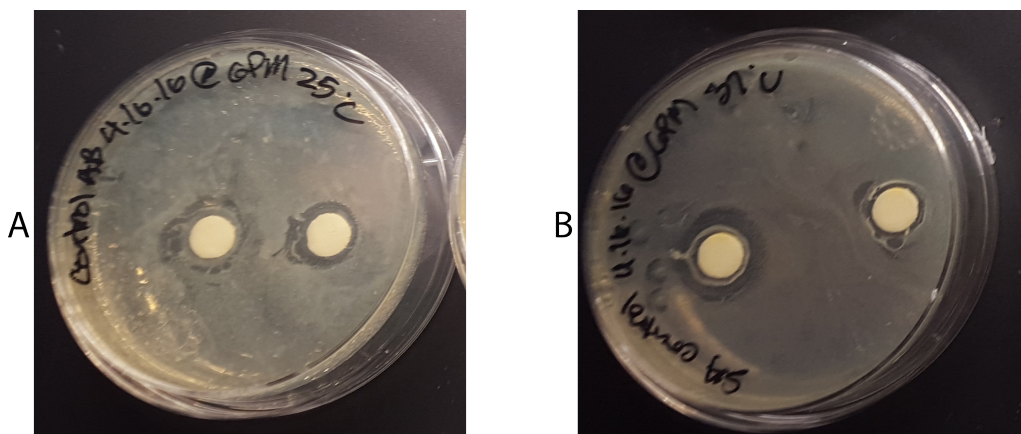
Figure 3.3: **Zones of Inhibition from Control Samples.** Left, disks soaked in sterile TSB placed on *A. baumannii* and incubated at 25° C. Right, disks soaked in sterile TSB placed on *S. aureus* and incubated at 37° C.

The disks from Zoolander 2 filtrate and Jurassic Park 2 filtrate, particularly when incubated with the tester strains at 25° C and 37° C seemed to encourage the growth of our tester strains in their vicinity. Zones of higher density growth were clearly be seen in these plates.

This step was repeated a total of three times, however only one set of results is shown. Results from the second and third repetition showed no significant differences.
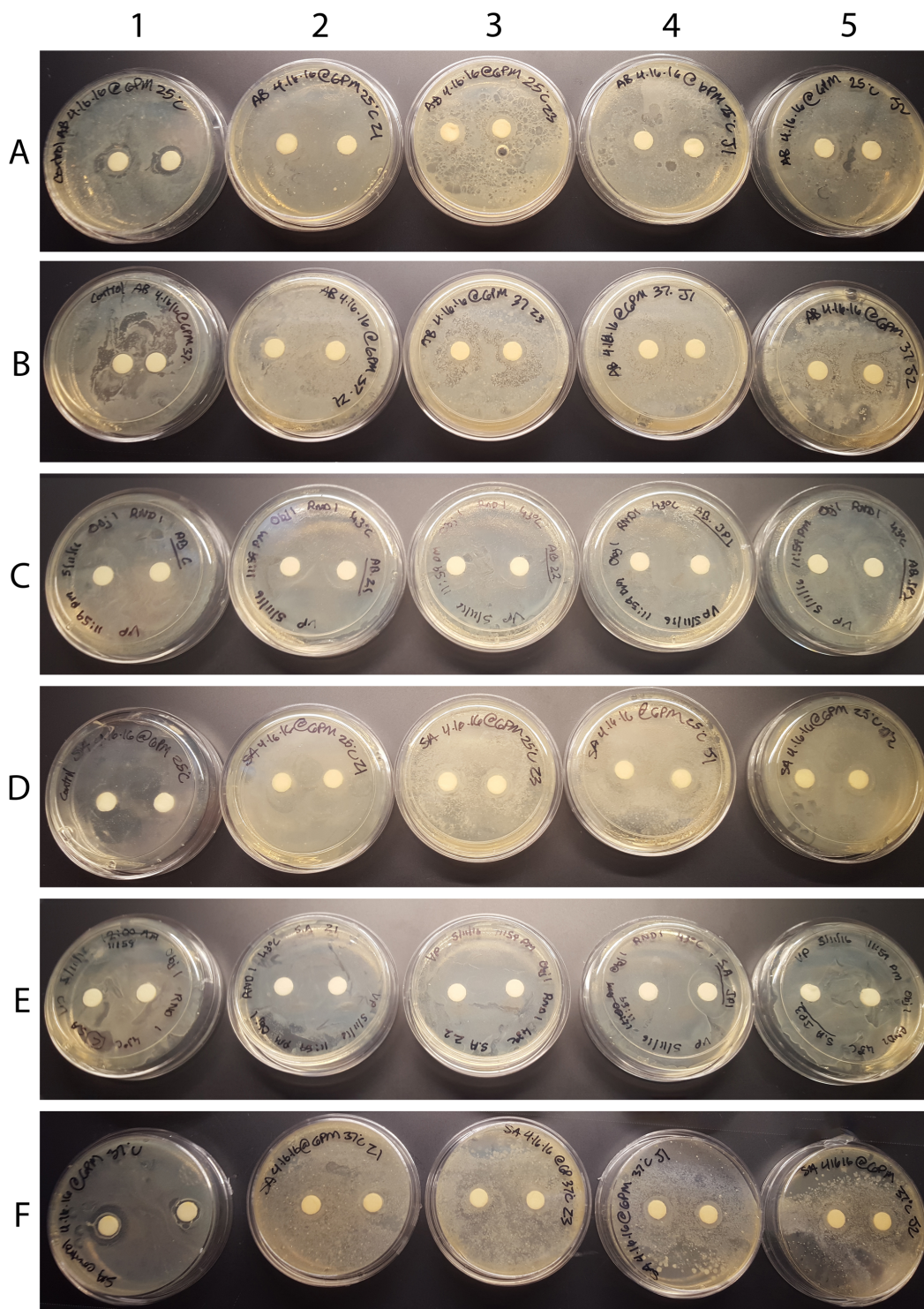
Figure 3.4: **Antibiotic Activity of Soil Working Stock Filtrates. Rows:** A) *A. baumannii* incubated at 25° C, B) *A. baumannii* incubated at 37° C, C) *A. baumannii* incubated at 43° C, D) *S. aureus* incubated at 25° C, E) *S. aureus* incubated at 37° C, F) *S. aureus* incubated at 43° C. **Columns:** Disks soaked in 1) Sterile broth, 2) Zoolander 1 filtrate, 3) Zoolander 2 filtrate, 4) Jurassic Park 1 filtrate, 5) Jurassic Park 2 filtrate.

3.2.2 Antibiotic Activity of Co-Cultured Soil Filtrate Against Tester Strains

Co-culturing the soil working stock with working stock of our tester strains did not yield significantly different results than culturing them independently. The results were largely similar to those from the previous section. Only a selected sample is shown here, as the number of samples increased dramatically in this step (Figure 3.5).

Unlike filtrate from independently cultured soil working stock, filtrate from co-cultured soil working stock did not seem to encourage growth of our tester strains.
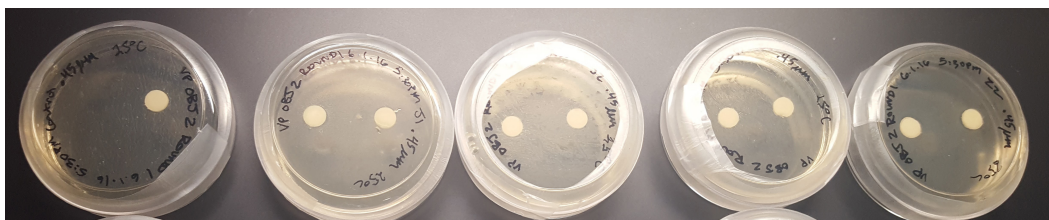
Figure 3.5: **Antibiotic Activity of Co-cultured Filtrate.** Plates from all samples in three repetitions of this step yielded similar plates to those shown here. Disks in this sample were soaked in filtrate from Jurassic Park 1 working stock that had been co-cultured with *S. aureus*. Soil working stock was filtered using .45 μm syringe filters before placing on our tester strain.

3.3 Antibiotic Viability of Soil Precipitate

3.3.1 Antibiotic Activity of Soil Precipitate Against Tester Strains

When our soil bacteria were stamped onto *S. aureus* and incubated, a total of 13 unknown colonies were found with zones of inhibition around them. The zones of inhibition are not visible in the photographs below, however they are clear and distinct when looking at the plates directly (Figure 3.7).
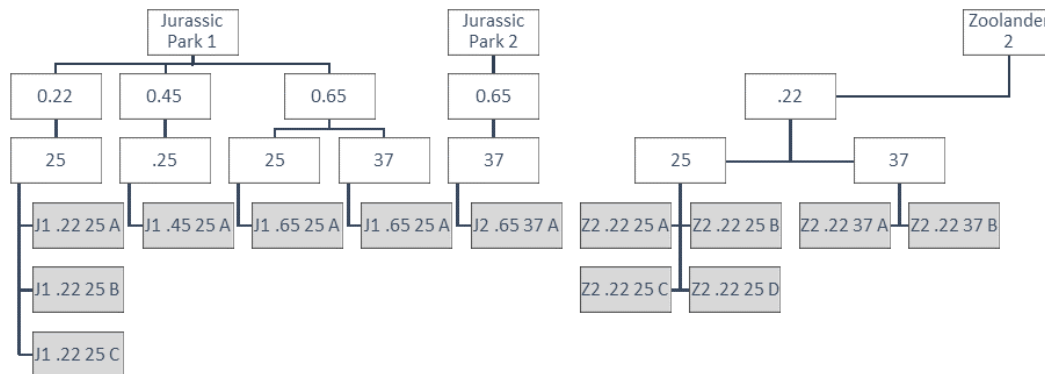
Figure 3.6: **Samples with Zones of Inhibition Visible.** Each soil sample that yielded any inhibition is shown in the top row (Jurassic Park 1, Jurassic Park 2, and Zoolander 2). Next, the filter size that yielded the inhibitive sample is shown (in μm). Next, the incubation temperature used to arrive at the inhibitive sample is shown. In the grey rectangles, each sample is given an identifying name for convenience.
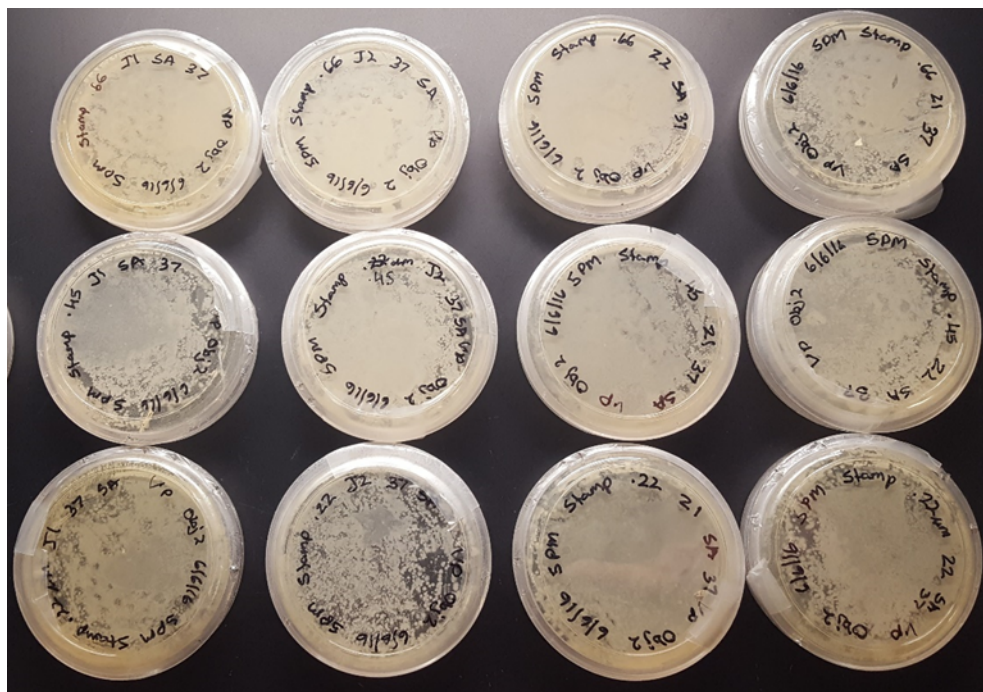
Figure 3.7: **Soil Precipitate Stamps on *S. aureus*.** The plates shown here were the ones incubated at 37° C with the soil bacteria on top of the tester strain. The soil bacteria on sterile TSA is not shown. Zones of inhibition are present but not visible on photographs.

3.3.2 Propagation of Colonies with Indications of Antibiotic Activities

The colonies selected as possible antibiotic emitters (as indicated in Figure 3.6) were propagated in sterile TSB. The plates are also shown in Figure 3.7, however zones of inhibition are not visible in the photographs.

From the broth cultures, the unknown bacteria were streaked onto individual plates and incubated again. Finally, a single colony was chosen to create working stock and master stock. This was done to create as pure a culture (monoclonal) as possible with the available tools. The labels for each of the colonies selected as progenitors for the stocks are shown below (Table 3.2).

In Figure 3.8, we included images of the colonies that were selected to create working and master stock. The selected colonies were isolated (there was no contact with neighboring colonies) and regular in shape. Only one colony was picked off with a loop to be used to inoculate sterile TSB. Our master stock, stored at -80° C, is composed of progeny from these colonies and should be identical as the cells were not passaged again after isolates were obtained from the streaks below.
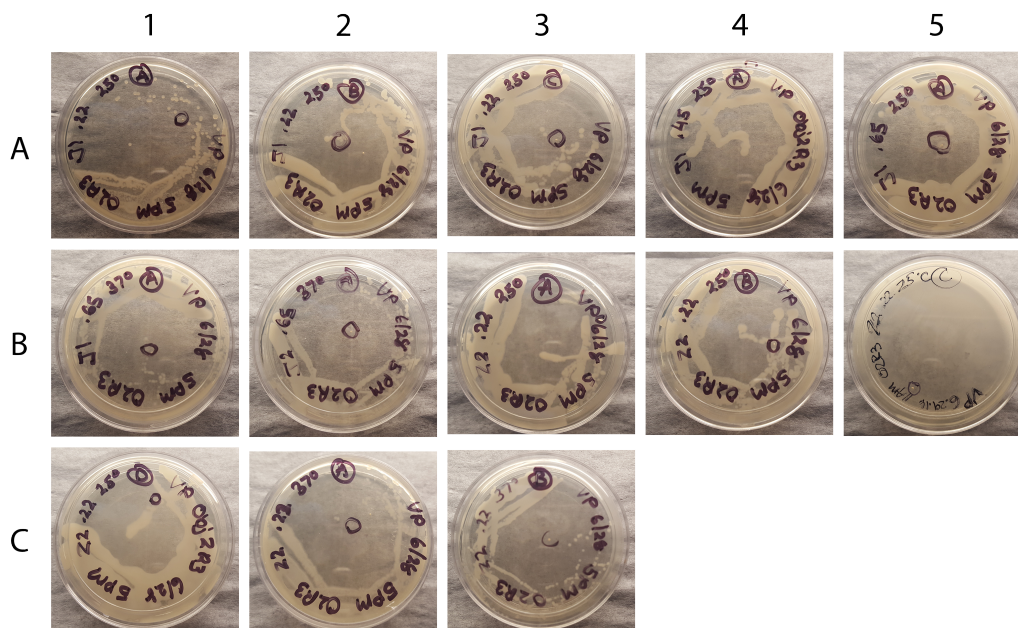
Figure 3.8: **Selected Progenitor Colonies for Potential Antibiotic-producing Bacteria.** Streak plates created using unknown bacteria from soil samples, as described in "Propagation of Colonies with Indications of Antibiotic Activities". Colonies that were selected to create stocks are circled here.

The zones of inhibition created by each of the selected isolates on *S. aureus* were all approximately 2 mm in diameter, however they were too small to effectively measure.

Table 3.2: Selected Progenitor Colonies for Potential Antibiotic-producing Bacteria (Labels).

	1	2	3	4	5
A)	J1 0.22 25 A	J1 0.22 25 B	J1 0.22 25 C	J1 0.45 25 A	J1 0.65 25 A
B)	J1 0.65 37 A	J2 0.65 37 A	Z2 0.22 25 A	Z2 0.22 25 B	Z2 0.22 25 C
C)	Z2 0.22 25 D	Z2 0.22 37 A	Z2 0.22 37 B		

3.4 Identification of Select Samples with Possible Antibiotic Activity

3.4.1 DNA Sequencing

The complete results from EMSL DNA sequencing (EMSL Test Code M192) can be found in the Appendix (*DNA Sequencing Results*). As expected based on our results from manual identification using dichotomous keys, DNA sequencing indicated that our unknown bacteria belonged to Group 1 bacilli. EMSL was able to narrow down the sample to one of three species: *Bacillus thuringiensis*, *Bacillus cereus*, and Bacillus mycoides. However, because of the homogeneity between the genomes of these bacteria they were unable to further differentiate.

EMSL's report states that identification of bacterial samples is done by sequencing the DNA coding for 16S ribosomal RNA (rRNA) and comparing with GenBank database at the National Center for Biotechnology Information (NCBI). Therefore, we can conclude based on their results that all three of the species they listed (*B. thuringiensis*, *B. cereus*, and *B. mycoides*) have a conserved 16S rRNA that is identical to one another.

The sample that was chosen to be sequenced (Z2 0.22 25 A) was also identified manually via the dichotomous keys as described in *Gram Stain, Mannitol Fermentation, Motility, and Crystal Formation Testing* in the Appendix. Manual identification results indicated that the species in question was *B. mycoides*, thus confirming EMSL's sequencing results.

3.4.2 Identification Tests and Dichotomous Key

The identification tests using the dichotomous key were conducted on all samples that produced a zone of inhibition against *S. aureus* (Figure 3.6). However, only selected results are shown for each step in the identification process. Protocols for each of the identification tests can be found in the Appendix (*Identification Tests*).

Pictures of Gram stains of our unknown bacterium from the soil samples are shown in Figure 3.9. All samples (including the ones not shown here) were Gram positive, and therefore appear purple under stain. We are also able to determine that all samples are bacilli (rod-shaped).

We were further able to confirm that samples were Gram positive by using MacConkey agar and PEA. MacConkey agar is selective for only Gram negative species and we saw no growth. PEA, conversely, inhibits growth of Gram negative species,

thus selecting for only Gram positive species. It is typically used to identify Staphylococcus species, but has also been shown to grow Bacillus species[66].

We then used malachite green staining as described in the Appendix (*Spore Formation (and Malachite Staining) Protocol*) to determine whether our samples were spore-forming bacteria. We found that all of our samples were spore-formers. Finally, we conducted motility testing in TSA filled test tubes using the procedure described in the Appendix. The results from the motility tests were confirmed by using MIO tubes which also tested for indole and ornithine testing (Figure 3.10).

We also tested each of the samples for susceptibility against penicillin to confirm that we did not have *Bacillus anthracis* in any of our samples. This was the only differentiating trait between *B. anthracis* and the other species in Group 1 Bacillus. Susceptibility tests were completed following the procedure described in Confirming Resistance/Susceptibility of Tester Strains.

The results for each of these tests are displayed in Table 3.3. Using this table along with the dichotomous keys (Figure 2.9 and Figure 2.10) above, we were able to determine the composition of each of our samples to the species level.

Table 3.3: Compiled Identification Tests.

Sample	Gram	PEA	McC	Spore	Cat.	Oxi.	Man.	Susc.	Mot.	Orn.	Likely Strain
J1 0.22 25 A	+	+	-	+	+	-	-	S	+	+	Bacillus anthracis
J1 0.22 25 B	+	+	-	+	+	-	-	S	-	-	Bacillus anthracis
J1 0.22 25 C	+	+	-	+	+	-	-	S	+	-	Baccilus cereus
J1 0.45 25 A	+	+	-	+	+	-	-	R	-	-	Bacillus mycoides
J1 0.65 25 A	+	+	-	+	+	-	-	R	-	+	Bacillus mycoides
J1 0.65 37 A	+	+	-	+	+	-	-	R	-	+	Bacillus mycoides
J2 0.65 37 A	+	+	-	+	+	-	-	R	-	+	Bacillus mycoides
Z2 0.22 25 A	+	+	-	+	+	-	-	R	-	+	Bacillus mycoides
Z2 0.22 25 B	+	+	-	+	+	-	-	R	-	+	Bacillus mycoides
Z2 0.22 25 C	+	+	-	+	+	-	-	S	+	-	Bacillus anthracis
Z2 0.22 25 D	+	+	-	+	+	-	-	R	+	+	Baccilus cereus
Z2 0.22 37 A	+	+	-	+	+	-	-	R	+	+	Baccilus cereus
Z2 0.22 37 B	+	+	-	+	+	-	-	S	-	-	Bacillus anthracis
Z2 0.22 37 C	+	+	-	+	+	-	-	S	+	+	Bacillus anthracis
Z2 0.22 37 D	+	+	-	+	+	-	-	S	+	-	Bacillus anthracis

J1 0.22 25C A | J1 0.22 25C B | J1 0.22 25C C | J1 0.45 25C A

J1 0.65 25C A | J1 0.65 37C A | J2 0.65 37C A | Z2 0.22 25C A

Z2 0.22 25C B | Z2 0.22 25C C | Z2 0.22 37C A | Z2 0.22 37C B
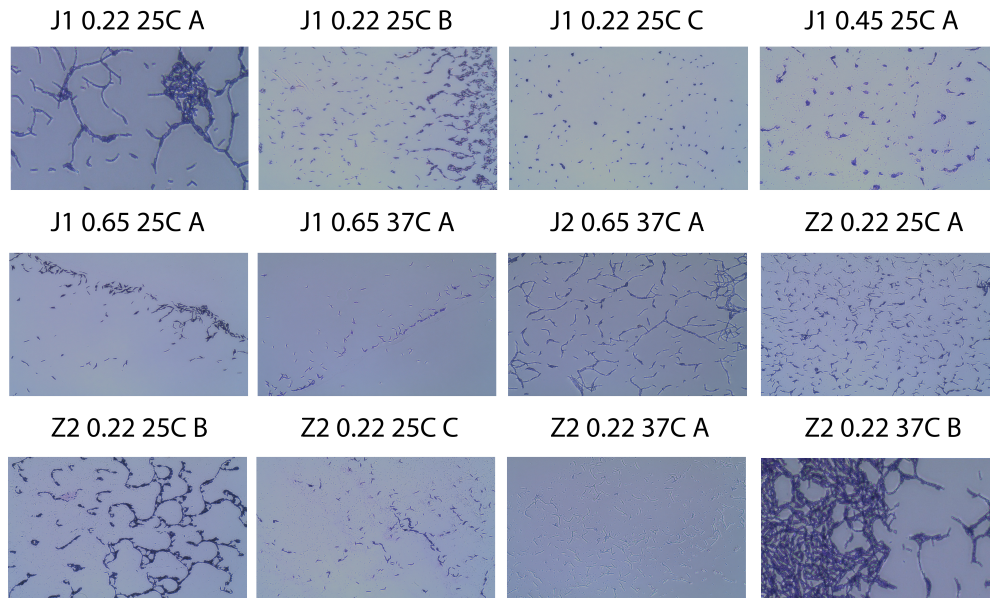
Figure 3.9: **Selected Gram Stains.** All strains were Gram stained and found to be Gram positive.

J1 0.22 25C B | J1 0.22 25C C | J1 0.45 25C A | J1 0.65 25C A | Z2 0.22 25C A
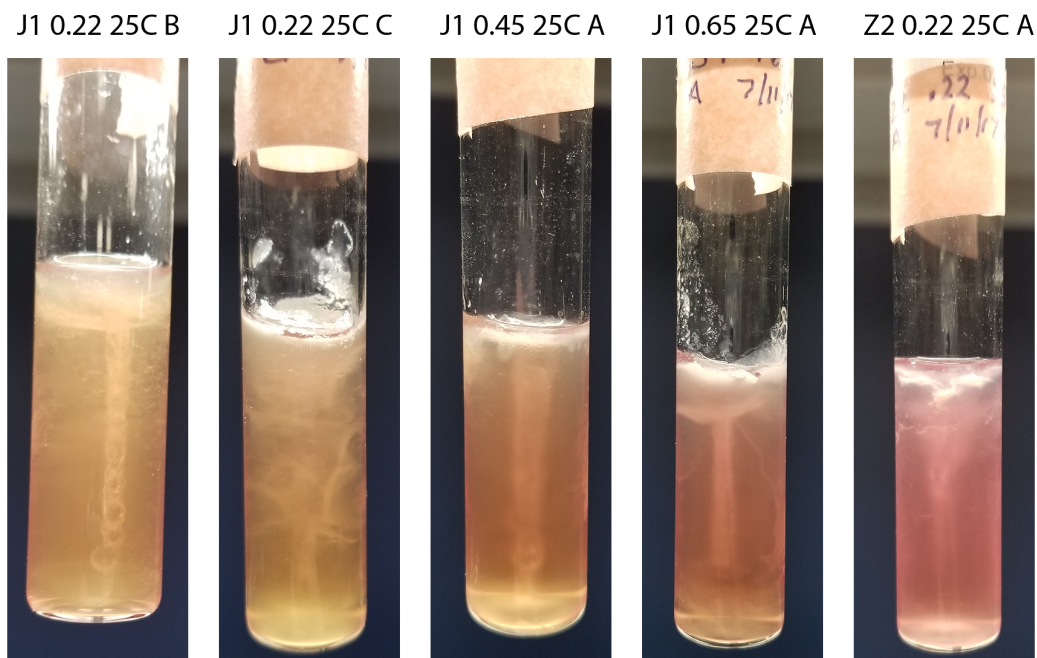
Figure 3.10: **Selected Motility Tests.** Motility tests for select samples are included above.

4 Discussion

4.1 Preparatory Tests

4.1.1 Growth Curves

Growth curves were done for both tester strains to ensure that we did not allow them to overgrow in future experiments, and as a baseline against soil bacteria. While it is unlikely that soil bacteria would exhibit a similar growth curve, there is no feasible method to determine the ideal growth period for a sample with countless distinct species in it. It was also not possible to test many different incubation periods because the number of samples grows exponentially with each consecutive step of this research. Each additional incubation period would necessitate four additional filtrates in *Creating Soil Filtrate and Propagating Soil Bacteria* and *Co-culturing Soil Bacteria with Tester Strains using Dialysis Tubing*. In the following portions of the experiment, this number would further multiply.

The optimal incubation period for our tester strains was the time it took for them to reach the center of their "exponential" or "log" phase, labelled in Figure 4.1.
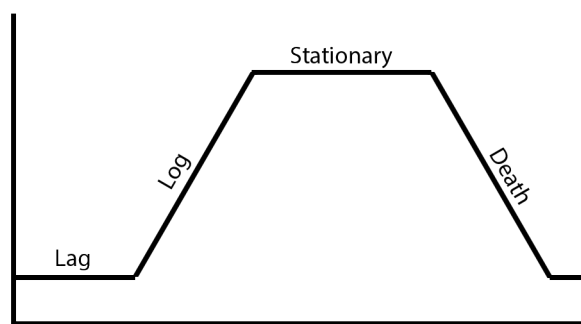
Figure 4.1: **A typical growth curve for a bacterial species.** The Y-axis shows units of growth (either CFU or absorbance) on a logarithmic scale, while the X-axis is time.

This template was used to determine an "optimal" incubation period for our tester strain, which was also used as the incubation period for our soil samples. However, it is likely that the soil samples incubated in different conditions or for different lengths of time may have rendered different results in further steps. This is a possible alteration of our research for academics who may choose to continue this research.

Figure 3.1 and Figure 3.2 show the growth curves of both tester strains. While both

strains thrived at 37° C with a shaker, some clear differences can be seen. Perhaps the most clear difference is the incubation period at 25° C with a shaker. In this configuration, *S. aureus* growth severely lags in relation to *A. baumannii.*

In the *S. aureus* growth curve, a spike in absorbance can be seen 13 hours after the beginning of the incubation period. Of the two identical runs that were done to get these data points, one showed a large deviation from the norm for this time point, while the other was normal. This indicates that it was an abnormal reading. Because the growth curve quickly resumed its normal course after hour 13, we believe there was no contamination in the samples, which would have altered the remainder of the curve.

One final thing to note for these growth curves is the small negative value for absorbance after 1 hour of shaking at 43° C. The only way a negative value would be attained is if our control had a larger absorbance than our sample. This could occur if the control had been contaminated and exhibited growth of biomass, however, this is unlikely as the absorbance of our control sample did not change throughout the incubation period. This leads us to believe that there was an error in creating the nutrient broth. A slightly higher volume of nutrient broth in relation to distilled water during preparation of the broth could have created TSB that absorbs more light. If the broth used in the control sample and the bacterial samples were of different origins, it is possible that this artifact is insignificant.

4.2 Antibiotic Activity in Soil Filtrate

As stated in the results, there was no visible zone of inhibition around any of the disks soaked in soil working stock filtrate, regardless of whether they were co-cultured with tester strain working stock or independently. The two plates that exhibited what looks like a zone of inhibition in Figure 3.3 were both control plates. The disks in these plates were soaked only in sterile TSB.

The probable cause of this "zone of inhibition" is simply incomplete drying of the disks. They may have retained TSB, and when placed on our tester strain, expelled some onto the plate. This expulsion of TSB may have carried with it, the nearby tester strain.

Perhaps more interesting than the lack of any zones of inhibition was the consistency with which filtrate from Jurassic Park 2 and Zoolander 2 samples encouraged the growth of our tester strains at both 25° C and 37° C. This experiment was done three times, and each cycle yielded a similar result with respect to these two samples. It is likely that the bacteria from these samples produce metabolites that are beneficial to the growth of both *S. aureus* and *A. baumannii*

Interestingly, both samples that encouraged growth of our tester strains were obtained from relatively anaerobic environments (>50 cm from surface soil) as shown in Table 2.1. This result also provides a stark difference from research done by Ki Chi and Jarvis Li, who found that deep-soil bacteria commonly release phenylacetic acid, a compound that acts as an antibiotic agent against both *A. baumannii* and *S. aureus*[67]. Other publications have also found that this compound increases immune

system activity against *A. baumannii* in mammals[68].

While the results for this test were underwhelming in terms of antibiotic activity, we believe the process by which we did the experimentation was novel—another publication could not be found that had a similar methodology. However, it may be advisable for researchers to modify the methodology slightly. Rather than carrying out a "shotgun" approach and testing a concoction of unknown soil bacteria for antibiotic agents, it would seem to be more efficient to look at commercially available antibiotics and work backwards to see what kinds of strains are most likely to produce similar molecules. For example, if it were determined that Gram-negative bacteria are more likely to produce a given molecule that may exhibit antibiotic activity, one could kill Gram-positive bacteria in a sample using pure singlet oxygen, an excited form of molecular oxygen[69]. With only Gram-negative bacteria in a sample, metabolites produced may be significantly different.

4.3 Antibiotic Activity in Soil Precipitate

As shown in Figure 2.4, each filter was stamped onto a plate that had been prepared with 10 μL of *S. aureus* working stock (using glass beads to spread uniformly). The goal of this exercise was to transfer the bacteria that had been retained by each filter to TSA with our tester strain. If a specific strain of bacteria is producing metabolites that are detrimental to the growth of *S. aureus*, it would be more likely to show a zone of inhibition on a plate immediately surrounding the unknown bacteria than it would in filtrate that was composed of metabolites from all the bacteria in our soil samples. It would also be possible to isolate the bacteria of interest that produced this antibiotic agent, as it would be visibly separated from other species on a plate.

A total of 13 strains of unknown soil bacteria produced inhibitory effects on *S. aureus*. Of the 13 strains, nine were found in a filter pore size of 0.22 μm, which implies that the individual cells of these bacteria were smaller in size than 0.45 μm but larger than 0.22 μm. Of the 13 strains, nine were also found in media incubated at 25° C, which may indicate that species that can inhibit *S. aureus* growth tend to thrive at room temperature more than body temperature. However, it may be that body temperature allows other species to thrive and out-compete the unknowns that have inhibitory effect. Regardless of the cause, future researchers may want to focus their efforts on a 25° C growth temperature.

One interesting finding of our research was that for samples collected near the water (<10 m), the deeper soil yielded more species with antibiotic activity against our tester bacteria. However, for soil that was collected further away from the water (<100 m), the surface soil was more likely to yield antibiotic activity against the tester strain. The soil collected from less than 10 m from the water also only exhibited antibiotic activity from species whose cells were smaller than 0.45 μm, whereas the soil that was collected from a larger distance exhibited antibiotic activity, in some cases, where the bacterial species were larger than 0.65 μm. This is another procedure in which future research may be able to target bacteria more precisely. For example, if working with soil near the water at Radium Springs, GA, one would focus on bacterial species whose cells are generally smaller than 0.45 μm. The further

the soil samples are collected, the larger the spectrum of size that may be of interest.

4.4 Prospective Species with Antibiotic Activity

After isolation of our possible candidates that may have antibiotic activity against the tester strain *S. aureus*, we needed to identify our samples. We approached this from two directions—we sent a sample to another lab to have it sequenced and compared against the NCBI database, and we used a dichotomous key to run identification tests and narrow down the possibilities with each consecutive experiment.

We found that all our possible candidates were Gram positive, exhibited spore formation, produced catalase, lacked oxidase, and could not ferment mannitol. This led us to believe that all our samples were species from Bacillus Group 1 (*Bacillus anthracis*, *Bacillus cereus*, Bacillus mycoides, and *Bacillus thuringiensis*), however we needed to conduct further tests to determine which species were present in each sample. The differentiating factors between samples were penicillin susceptibility, motility, presence of ornithine decarboxylase, and endospore formation (seen via crystal formation with a malachite stain).

Based on the dichotomous key for Group 1 Bacilli (Figure 2.10), we determined the most likely species present in each sample. According to the *UK Standards for Microbiology Investigations*, all of the Group 1 Bacilli are ubiquitous so it is no surprise that they were found in our samples. However, it was interesting that every sample with antibiotic activity was a species belonging to this group. It should also be noted that one member of this group was absent from all our samples, *Bacillus thuringiensis*. This is a possible candidate to act as a negative control in future studies that may attempt to determine the molecule(s) that may be inhibiting *S. aureus* growth.

Group 1 Bacilli are extremely similar, to the extent that our DNA sequencing was unable to differentiate between them. Other studies have had comparable results, including the work done by Ash, which showed that the 16S rRNA sequence in these species varies by less than nine nucleotides[70]. Because of the similarities, it can be hypothesized that most Group 1 Bacilli produce the same (or a similar) metabolite that may have adverse effects on the growth of *S. aureus*.

The species in Group 1 Bacilli (sometimes referred to as the *B. cereus* group), all seem to have pathologic consequences in humans in some cases, however they cause significantly different presentations. *B. anthracis* is the cause of anthrax, likely the most well-known condition caused by this group, for example[71]. Therefore, the metabolite that is producing their antibiotic activity against *S. aureus* may also produce some type of toxicity in humans.

Perhaps the most relevant "next step" in our research is to isolate and amplify the molecule or molecules of interest for further testing. The levels naturally produced by our soil bacteria were only sufficient to create zones of inhibition that were less than 3 mm in diameter. This does not inherently imply they have a weak antibiotic activity, as we cannot test for the concentration present until it is isolated. It is possible that it may be a strong antibiotic when present at higher levels.

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Appendix

1 Identification Tests

1.1 Gram Stain Protocol

1. Place a drop of TSB innoculated with unknown bacteria onto the center of a clean slide.
2. Hold 6 inches over Bunsen burner until dry.
3. Place a drop of crystal violet stain onto the now-dried bacteria and let sit for 60 seconds.
4. Flush the slide with distilled water until runoff is clear.
5. Gently flush the slide with acetone or ethanol for 5 seconds.
6. Gently flush the slide with distilled water again.
7. Place a drop of safranin counter-strain onto the center of the slide.
8. Gently flush the slide with distilled water.
9. Hold the slide 6 inches over Bunsen burner until dry.

When viewed under a microscope, Gram positive bacteria will appear purple, while Gram negative bacteria will appear red.

1.2 Phenyethyl Alcohol Agar (PEA) Protocol

Note: this protocol was completed using ThermoFisher Phenyethyl Alcohol Agar solid medium. Components included in the media are listed below, as found in ThermoFisher's documentation (http://tools.thermofisher.com/content/sfs/manuals/IFU454301.pdf).

Reagent	Weight/Volume
Casein Peptone	15.0 g
Sodium Chloride	5.0 g
Soy Peptone	5.0 g
Phenylethyl Alcohol	2.5 g
Agar	15.0 g
Water	1000.0 mL

PEA Media Creation

1. Use a lab scale to measure out 42.5 g of ThermoFisher PEA medium per 1000 mL of PEA agar required.
2. Add 1000 mL of water per 42.5 g of PEA medium to an empty beaker.

3. Place beaker on a hot plate with stirrer turned to medium settings.
4. Add PEA medium to water slowly as it is stirred into a homogenous mixture.
5. Allow mixture to heat to a boil for 15 minutes (with stirrer on).
6. Cover the beaker and autoclave the mixture to sterilize.
7. Allow mixture to cool to 45-50°C and add defibrinated sheep's blood (5% of final volume).
8. Pour mixture into sterile plates or tubes as required while it is warm.
9. Allow plates and tubes to cool into solid media before use.

Plating Unknown Bacteria on PEA

1. Prepare TSB inoculate with unknown bacteria.
2. Using aseptic technique, streak unknown bacteria onto solidified PEA agar.
3. Incubate at given temperature.

Phenylethyl alcohol agar is selective for Gram positive cocci. Therefore growth of unknown strain on PEA is evidence of Gram positive cocci. However, absence of growth can be due to many factors and should not be used as evidence to rule out that the unknown may be a Gram positive cocci.

1.3 MacConkey Agar Protocol

Note: this protocol was completed using ThermoFisher MacConkey Agar solid medium. Components included in the media are listed below, as found in ThermoFisher's documentation (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/IFU453801.pdf).

Reagent	Weight/Volume
Gelatin Pepton	17.0 g
Lactose	10.0 g
Sodium Chloride	5.0 g
Bile Salts	1.5 g
Casein Peptone	1.5 g
Meat Peptone	1.5 g
Neutral Red	30.0 mg
Crystal Violet	1.0 mg
Agar	13.5 hg
Water	1000.0 mL

MacConkey Media Creation

1. Use a lab scale to measure out 50.0 g of ThermoFisher MacConkey medium per 1000 mL of MacConkey agar required.
2. Add 1000 mL of water per 50.0 g of MacConkey medium to an empty beaker.
3. Place beaker on a hot plate with stirrer turned to medium settings.

4. Add MacConkey medium to water slowly as it is stirred into a homogenous mixture.
5. Allow mixture to heat to a boil for 15 minutes (with stirrer on).
6. Cover the beaker and autoclave the mixture to sterilize.
7. Pour mixture into sterile plates or tubes as required while it is warm.
8. Allow plates and tubes to cool into solid media before use.

Plating Unknown Bacteria on MacConkey Agar

1. Prepare TSB inoculate with unknown bacteria.
2. Using aseptic technique, streak unknown bacteria onto solidified MacConkey agar.
3. Incubate at given temperature.

MacConkey agar is selective for Gram negative bacilli. Visible growth indicates that the unknown bacterium is likely a Gram negative bacillus. However, lack of growth is not necessarily indicative that the unknown is *not* a Gram negative bacillus, as other extraneous factors may prevent a given species from growing.

1.4 Spore Formation (and Malachite Staining) Protocol

1. Place a drop of TSB inoculated with unknown bacteria onto the center of a clean slide.
2. Place a small piece of blotting paper or filter paper over the center of the slide.
3. Soak the blotting paper with malachite green stain.
4. Heat-dry by waving over a Bunsen burner (approximately 6 inches above the flame).
5. Remove the blotting paper and rinse the slide with distilled water.
6. Place a drop of safranin counter-strain onto the center of the slide and let sit for 60 seconds.
7. Gently flush the slide with distilled water until runoff is clear.
8. Using another piece of blotting paper, blot any excess moisture from the slide.

When viewed under a microscope, spores will stain green while the bacterial cells will retain the safranin-red. Therefore, the presence of green on the slide indicates that the unknown bacteria may be a spore-former.

1.5 Catalase Testing Protocol

1. Place a drop of TSB inoculated with unknown bacteria onto the center of a clean slide.
2. Allow slide to dry for 5 minutes (wave slide over Bunsen burner to speed up the process, being careful not to kill the bacteria due to excess heat).
3. Place a drop of hydrogen peroxide (3$) onto the center of the slide.
4. Watch for rapid formation of gas bubbles.

Rapid formation of gas bubbles indicates that the bacteria on the slide are breaking down hydrogen peroxide into water and oxygen quickly. In other words, these bacteria are catalase positive.

1.6 Mannitol Agar Protocol

Note: this protocol was completed using ThermoFisher Mannitol Salt Agar solid medium. Components included in the media are listed below, as found in ThermoFisher's documentation (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/IFU1580.pdf).

Reagent	Weight/Volume
Sodium Chloride	75.0 g
D-Mannitol	10.0 g
Casein Peptone	5.0 g
Meat Peptone	5.0 g
Beef Extract	1.0 g
Phenol Red	25.0 mg
Agar	15.0 h
Water	1000.0 mL

Mannitol Media Creation

1. Use a lab scale to measure out 110.0 g of ThermoFisher Mannitol medium per 1000 mL of Mannitol agar required.
2. Add 1000 mL of water per 110.0 g of Mannitol medium to an empty beaker.
3. Place beaker on a hot plate with stirrer turned to medium settings.
4. Add Mannitol medium to water slowly as it is stirred into a homogenous mixture.
5. Allow mixture to heat to a boil for 15 minutes (with stirrer on).
6. Cover the beaker and autoclave the mixture to sterilize.
7. Pour mixture into sterile plates or tubes as required while it is warm.
8. Allow plates and tubes to cool into solid media before use.

Plating Unknown Bacteria on Mannitol

1. Prepare TSB inoculate with unknown bacteria.
2. Using aseptic technique, streak unknown bacteria onto solidified Mannitol agar.
3. Incubate at given temperature.

Mannitol agar is a selective *and* differential growth medium. It selects for species of bacteria that are able to grow with high salt concentrations, such as *Staphylococcus* species.

However, it also contains an indicator that detects the acidic byproducts of mannitol fermentation. Therefore, species that are able to ferment mannitol, such as *Staphylococcus aureus* will create a yellow zone in their immediate vicinity.

2 *Staphylococcus aureus* Growth Data

Table 1: Absorbance readings at 600 nm.

Hours	Non-Shaker			Shaker		
	25 C	37 C	43 C	25 C	37 C	43 C
0	-0.093	0.002	0.001	0.002	0.006	-0.001
1	0.004	0.002	0.002	0.002	0.001	-0.001
2	-0.001	0.002	0.001	0.001	0.002	-0.002
3	0.004	0.002	0.002	0.002	0.003	0.001
4	0.004	0.002	0.010	0.007	0.009	0.006
5	0.004	0.002	0.056	0.034	0.029	0.036
6	0.005	0.002	0.202	0.147	0.084	0.139
7	0.007	0.003	0.333	0.322	0.141	0.220
8	0.011	0.003	0.426	0.705	0.208	0.302
9	0.015	0.005	0.517	0.864	0.269	0.384
10	0.022	0.007	0.530	0.887	0.317	0.504
11	0.030	0.009	0.664	0.898	0.321	0.597
12	0.039	0.058	0.849	0.908	0.390	0.612
13	0.051	0.014	0.918	0.918	0.398	0.606
14	0.066	0.016	0.640	0.924	0.382	0.582
15	0.088	0.019	0.579	0.927	0.338	0.582
16	0.126	0.027	0.544	0.931	0.382	0.577
17	0.178	0.033	0.546	0.931	0.369	0.577
18	0.227	0.043	0.500	0.931	0.423	0.578
19	0.292	0.057	0.562	0.931	0.412	0.576
20	0.340	0.074	0.540	0.931	0.368	0.573
21	0.365	0.101	0.499	0.931	0.348	0.574
22	0.362	0.130	0.569	0.931	0.426	0.555
23	0.399	0.174	0.573	0.931	0.392	0.555
24	0.445	0.225	0.759	0.931	0.392	0.555
25	0.462	0.278	NA	NA	NA	NA
26	0.476	0.338	NA	NA	NA	NA
27	0.530	0.396	NA	NA	NA	NA
28	0.568	0.457	NA	NA	NA	NA
29	0.532	0.503	NA	NA	NA	NA
30	0.558	0.534	NA	NA	NA	NA
31	0.591	0.551	NA	NA	NA	NA
32	0.584	0.570	NA	NA	NA	NA
33	0.550	0.581	NA	NA	NA	NA
34	0.509	0.602	NA	NA	NA	NA
35	0.500	0.603	NA	NA	NA	NA
36	0.531	0.613	NA	NA	NA	NA

3 *Acinetobacter baumannii* Growth Data

Table 2: Absorbance readings at 600 nm.

	Non-Shaker			Shaker		
Hours	25 C	37 C	43 C	25 C	37 C	43 C
0	-0.063	0.002	-0.001	0.002	0.000	-0.002
1	0.001	0.003	0.000	0.002	0.000	-0.002
2	0.000	0.004	0.000	0.002	0.001	-0.003
3	0.004	0.006	0.002	0.002	0.006	-0.001
4	0.005	0.014	0.007	0.002	0.033	0.008
5	0.005	0.024	0.014	0.002	0.199	0.043
6	0.006	0.039	0.021	0.003	0.387	0.180
7	0.011	0.072	0.035	0.003	0.521	0.316
8	0.018	0.122	0.057	0.008	0.600	0.419
9	0.024	0.185	0.088	0.012	0.646	0.481
10	0.031	0.265	0.124	0.044	0.637	0.529
11	0.039	0.359	0.159	0.097	0.625	0.572
12	0.048	0.426	0.193	0.173	0.619	0.606
13	0.057	0.493	0.251	0.272	0.626	0.632
14	0.069	0.507	0.270	0.416	0.635	0.650
15	0.083	0.509	0.319	0.470	0.631	0.672
16	0.099	0.498	0.347	0.505	0.633	0.681
17	0.116	0.451	0.366	0.536	0.633	0.679
18	0.138	0.477	0.349	0.557	0.633	0.673
19	0.161	0.464	0.354	0.571	0.633	0.669
20	0.189	0.460	0.329	0.577	0.633	0.661
21	0.214	0.448	0.329	0.575	0.633	0.657
22	0.242	0.419	0.317	0.573	0.633	0.648
23	0.276	0.428	0.298	0.573	0.633	0.648
24	0.329	0.448	0.298	0.572	0.633	0.648
25	0.361	NA	NA	0.571	NA	NA
26	0.422	NA	NA	0.571	NA	NA
27	0.405	NA	NA	0.572	NA	NA
28	0.433	NA	NA	0.571	NA	NA
29	0.475	NA	NA	0.570	NA	NA
30	0.514	NA	NA	0.570	NA	NA
31	0.523	NA	NA	0.570	NA	NA
32	0.551	NA	NA	0.571	NA	NA
33	0.533	NA	NA	0.572	NA	NA
34	0.592	NA	NA	0.571	NA	NA
35	0.525	NA	NA	0.572	NA	NA
36	0.617	NA	NA	0.571	NA	NA

4 DNA Sequencing Results

Client:	GA – PCOM	EMSL Order ID:	611601036
	625 Old Peach Tree Rd NW	Date Received:	7/21/2016
	Suwanee, GA 30024	Date Analyzed:	7/28/2016
Attention:	Vash Patel	Date Reported:	7/28/2016
Project:	Z2A .22 25 A/1A	Date Amended:	

M192 DNA Sequencing Analysis for Bacterial Isolates

Species identification:
Species identity is based on the unknown organism's DNA sequence data for 16S ribosomal RNA gene and the comparison to the GenBank database at the National Center for Biotechnology Information (NCBI,www. ncbi.nlm.nih.gov). The PCR and DNA sequences were performed using standard bacterial DNA barcode primers: 357F and 1100R. The DNA sequences were analyzed using BLAST search at NCBI.

Summary of Analysis

Lab Sample ID	Client Sample ID	Bacteria Species Identified	Similarity (%)
1036-1	1 / 1A	*Bacillus cereus* *Bacillus anthracis* *Bacillus thuringiensis* *Bacillus mycoides*	100%
1036-2	2 / Z2A .22 25 A	*Baciluus thuringiensis* *Bacillus cereus* *Bacillus mycoides*	100%

1036-1 357F
GTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTT
ATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGA
CTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTA
ACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCG
CCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGT
GGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAC

1036-1 1100R
AATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAGGCGGAGTGCTTAATGCGTT
AACTTCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTT
TCGCGCCTCAGTGTCAGTTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTCCATATCTCTACGCATTTCACCGCTACACATGGAATTCCACTT
TCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCACCTGCGCGCGCTTTA
CGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCC
AGCTTATTCAACTAGCACTTG

1036-2 357F
CAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGC
GTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGG
AGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCT

GTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTT
CCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC
GGTGGAGCATGTGGTTTAA

1036-2 1100R

AATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAGGCGGAGTGCTTAATGCGTT
AACTTCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTT
TCGCGCCTCAGTGTCAGTTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTCCATATCTCTACGCATTTCACCGCTACACATGGAATTCCACTT
TCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCACCTGCGCGCGCTTTA
CGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCC
AGCTTATTCAACTAGCAC

Zhencai Wu, M.S.
DNA Laboratory Manager

5 National Centers for Environmental Information - Weather Data

U.S. Department of Commerce
National Oceanic & Atmospheric Administration
National Environmental Satellite, Data, and Information Service

Elev: 309 ft. Lat: 31.718° N Lon: 84.351° W

Record of Climatological Observations
These data are quality controlled and may not be
identical to the original observations.
Generated on 07/24/2017

National Centers for Environmental Information
151 Patton Avenue
Asheville, North Carolina 28801

Observation Time Temperature: Unknown Observation Time Precipitation: Unknown

Station: **SASSER 0.2 WSW, GA US GHCND:US1GATL0001**

Preliminary	Year	Month	Day	Temperature (F) 24 hrs. ending at observation time		at Observation	Precipitation 24 Hour Amounts ending at observation time				At Obs Time	Evaporation		Soil Temperature (F) 4 in depth			8 in depth		
				Max.	Min.		Rain, melted snow, etc. (in)	Flag	Snow, ice pellets, hail (in)	Flag	Snow, ice pellets, hail, ice on ground (in)	24 Hour Wind Movement (mi)	Amount of Evap. (in)	Ground Cover (see *)	Max.	Min.	Ground Cover (see *)	Max.	Min.
	2015	10	1				0.18												
	2015	10	2				0.10												
	2015	10	3				0.04												
	2015	10	4				0.03												
	2015	10	5				0.07												
	2015	10	6				0.03												
	2015	10	7				T												
	2015	10	8				T												
	2015	10	9				0.00		0.0										
	2015	10	10				0.00		0.0										
	2015	10	11				0.02												
	2015	10	12				0.00		0.0										
	2015	10	13				0.00		0.0										
	2015	10	14				0.02												
	2015	10	15				0.00		0.0										
	2015	10	16				0.00		0.0										
	2015	10	17				0.00		0.0										
	2015	10	18				0.00		0.0										
	2015	10	19				0.00		0.0										
	2015	10	20				0.00		0.0										
	2015	10	21				0.00		0.0										
	2015	10	22				0.00		0.0										
	2015	10	23				0.00		0.0										
	2015	10	24				0.00		0.0										
	2015	10	25				0.00		0.0										
	2015	10	26				0.00		0.0										
	2015	10	27				0.14												
	2015	10	28				0.25												
	2015	10	29				0.08												
	2015	10	30				0.28												
	2015	10	31				T												
			Summary				1.24		0.0										

The "**" flags in Preliminary indicate the data have not completed processing and qualitycontrol and may not be identical to the original observation
Empty, or blank, cells indicate that a data observation was not reported.
*Ground Cover: 1=Grass; 2=Fallow; 3=Bare Ground; 4=Brome grass; 5=Sod; 6=Straw mulch; 7=Grass muck; 8=Bare muck; 0=Unknown
"s" This data value failed one of NCDC's quality control tests.
"T" values in the Precipitation category above indicate a TRACE value was recorded.
"A" values in the Precipitation Flag or the Snow Flag column indicate a multiday total, accumulated since last measurement, is being used.
Data value inconsistency may be present due to rounding calculations during the conversion process from SI metric units to standard imperial units.

U.S. Department of Commerce
National Oceanic & Atmospheric Administration
National Environmental Satellite, Data, and Information Service

Elev: 197 ft. Lat: 31.618° N Lon: 84.135° W

Record of Climatological Observations
These data are quality controlled and may not be
identical to the original observations.
Generated on 07/24/2017

National Centers for Environmental Information
151 Patton Avenue
Asheville, North Carolina 28801

Observation Time Temperature: Unknown Observation Time Precipitation: Unknown

Station: **ALBANY 3.7 NE, GA US GHCND:US1GADH0001**

Preliminary	Year	Month	Day	Temperature (F) 24 hrs. ending at observation time		at Observation	Precipitation 24 Hour Amounts ending at observation time				At Obs Time	Evaporation		Soil Temperature (F) 4 in depth			8 in depth		
				Max.	Min.		Rain, melted snow, etc. (in)	Flag	Snow, ice pellets, hail (in)	Flag	Snow, ice pellets, hail, ice on ground (in)	24 Hour Wind Movement (mi)	Amount of Evap. (in)	Ground Cover (see *)	Max.	Min.	Ground Cover (see *)	Max.	Min.
	2015	10	1				0.48												
	2015	10	2				0.18												
	2015	10	3				0.58												
	2015	10	4				0.10												
	2015	10	5				0.10												
	2015	10	6				0.00		0.0										
	2015	10	7				0.00		0.0										
	2015	10	8				0.00		0.0										
	2015	10	9				0.00		0.0										
	2015	10	10				0.00		0.0										
	2015	10	11				0.00		0.0										
	2015	10	12				0.00		0.0										
	2015	10	13				0.00		0.0										
	2015	10	14				0.21												
	2015	10	15				0.00		0.0										
	2015	10	16				0.00		0.0										
	2015	10	17				0.00		0.0										
	2015	10	18				0.00		0.0										
	2015	10	19				0.00		0.0										
	2015	10	20				0.00		0.0										
	2015	10	21				0.00		0.0										
	2015	10	22				0.00		0.0										
	2015	10	23				0.00		0.0										
	2015	10	24				0.00		0.0										
	2015	10	25				0.00		0.0										
	2015	10	26				0.21												
	2015	10	27				0.21												
	2015	10	28				0.21												
	2015	10	29				0.00		0.0										
	2015	10	30				0.12												
	2015	10	31				0.00		0.0										
			Summary				2.40		0.0										

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Empty, or blank, cells indicate that a data observation was not reported.
*Ground Cover: 1=Grass; 2=Fallow; 3=Bare Ground; 4=Brome grass; 5=Sod; 6=Straw mulch; 7=Grass muck; 8=Bare muck; 0=Unknown
"s" This data value failed one of NCDC's quality control tests.
"T" values in the Precipitation category above indicate a TRACE value was recorded.
"A" values in the Precipitation Flag or the Snow Flag column indicate a multiday total, accumulated since last measurement, is being used.
Data value inconsistency may be present due to rounding calculations during the conversion process from SI metric units to standard imperial units.

U.S. Department of Commerce
National Oceanic & Atmospheric Administration
National Environmental Satellite, Data, and Information Service
Elev: 247 ft. Lat: 31.629° N Lon: 84.225° W

Record of Climatological Observations
These data are quality controlled and may not be
identical to the original observations.
Generated on 07/24/2017

National Centers for Environmental Information
151 Patton Avenue
Asheville, North Carolina 28801

Observation Time Temperature: Unknown Observation Time Precipitation:
Unknown

Station: ALBANY 4.6 NW, GA US GHCND:US1GALE0002

Preliminary	Year	Month	Day	Temperature (F) 24 hrs. ending at observation time Max.	Min.	at Observation	Precipitation 24 Hour Amounts ending at observation time Rain, melted snow, etc. (in)	Flag	Snow, ice pellets, hail (in)	Flag	At Obs Time Snow, ice pellets, hail, ice on ground (in)	Evaporation 24 Hour Wind Movement (mi)	Amount of Evap. (in)	Soil Temperature (F) 4 in depth Ground Cover (see *)	Max.	Min.	8 in depth Ground Cover (see *)	Max.	Min.
	2015	10	1				0.44												
	2015	10	2				0.75												
	2015	10	3																
	2015	10	4																
	2015	10	5				0.10												
	2015	10	6				0.02												
	2015	10	7				0.00		0.0										
	2015	10	8																
	2015	10	9																
	2015	10	10																
	2015	10	11																
	2015	10	12																
	2015	10	13																
	2015	10	14				0.20												
	2015	10	15																
	2015	10	16																
	2015	10	17																
	2015	10	18																
	2015	10	19																
	2015	10	20																
	2015	10	21																
	2015	10	22																
	2015	10	23																
	2015	10	24																
	2015	10	25																
	2015	10	26																
	2015	10	27																
	2015	10	28				0.03												
	2015	10	29																
	2015	10	30				0.23												
	2015	10	31				0.00		0.0										
			Summary				1.77		0.0										

The "*" flags in Preliminary indicate the data have not completed processing and qualitycontrol and may not be identical to the original observation
Empty, or blank, cells indicate that a data observation was not reported.
*Ground Cover: 1=Grass; 2=Fallow; 3=Bare Ground; 4=Brome grass; 5=Sod; 6=Straw mulch; 7=Grass muck; 8=Bare muck; 0=Unknown
"s" This data value failed one of NCDC's quality control tests.
"T" values in the Precipitation category above indicate a TRACE value was recorded.
"A" values in the Precipitation Flag or the Snow Flag column indicate a multiday total, accumulated since last measurement, is being used.
Data value inconsistency may be present due to rounding calculations during the conversion process from SI metric units to standard imperial units.

U.S. Department of Commerce
National Oceanic & Atmospheric Administration
National Environmental Satellite, Data, and Information Service
Elev: 180 ft. Lat: 31.534° N Lon: 84.149° W

Record of Climatological Observations
These data are quality controlled and may not be
identical to the original observations.
Generated on 07/24/2017

National Centers for Environmental Information
151 Patton Avenue
Asheville, North Carolina 28801

Observation Time Temperature: 0700 Observation Time Precipitation: 0700

Station: ALBANY 3 SE, GA US GHCND:USC00090140

Preliminary	Year	Month	Day	Temperature (F) 24 hrs. ending at observation time Max.	Min.	at Observation	Precipitation 24 Hour Amounts ending at observation time Rain, melted snow, etc. (in)	Flag	Snow, ice pellets, hail (in)	Flag	At Obs Time Snow, ice pellets, hail, ice on ground (in)	Evaporation 24 Hour Wind Movement (mi)	Amount of Evap. (in)	Soil Temperature (F) 4 in depth Ground Cover (see *)	Max.	Min.	8 in depth Ground Cover (see *)	Max.	Min.
	2015	10	1	87	70	70	0.77												
	2015	10	2	81	67	67	0.07												
	2015	10	3	69	60	60	0.38												
	2015	10	4	67	59	60	0.03												
	2015	10	5	68	59	65	0.06												
	2015	10	6	69	62	62	0.04												
	2015	10	7	77	57	57	0.00												
	2015	10	8	82	56	56	0.00												
	2015	10	9	82	56	66	0.00												
	2015	10	10	86	65	69	0.00												
	2015	10	11	82	62	63	0.00												
	2015	10	12	68	56	56	0.00												
	2015	10	13	78	56	61	0.00												
	2015	10	14	81	57	57	0.17												
	2015	10	15	80	51	51	0.00												
	2015	10	16	84	51	53	0.00												
	2015	10	17	88	53	54	0.00												
	2015	10	18	75	48	48	0.00												
	2015	10	19	70	44	45	0.00												
	2015	10	20	71	45	48	0.00												
	2015	10	21	77	48	60	0.00												
	2015	10	22	81	57	58	0.00												
	2015	10	23	83	58	58	0.00												
	2015	10	24	84	57	57	0.00												
	2015	10	25	81	57	62	0.00												
	2015	10	26	79	62	69	0.00												
	2015	10	27	76	61	61	0.15												
	2015	10	28	70	61	70	0.04												
	2015	10	29	79	60	70	0.18												
	2015	10	30	82	55	55	0.04												
	2015	10	31	77	50	51	0.00												
			Summary	78	57		1.93		0										

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Empty, or blank, cells indicate that a data observation was not reported.
*Ground Cover: 1=Grass; 2=Fallow; 3=Bare Ground; 4=Brome grass; 5=Sod; 6=Straw mulch; 7=Grass muck; 8=Bare muck; 0=Unknown
"s" This data value failed one of NCDC's quality control tests.
"T" values in the Precipitation category above indicate a TRACE value was recorded.
"A" values in the Precipitation Flag or the Snow Flag column indicate a multiday total, accumulated since last measurement, is being used.
Data value inconsistency may be present due to rounding calculations during the conversion process from SI metric units to standard imperial units.

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U.S. Department of Commerce
National Oceanic & Atmospheric Administration
National Environmental Satellite, Data, and Information Service

Record of Climatological Observations
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identical to the original observations.
Generated on 07/24/2017

National Centers for Environmental Information
151 Patton Avenue
Asheville, North Carolina 28801

Elev: 190 ft. Lat: 31.536° N Lon: 84.194° W

Observation Time Temperature: Unknown Observation Time Precipitation:
Unknown

Station: **ALBANY SW GEORGIA REGIONAL AIRPORT, GA US GHCND:USW00013869**

Preliminary	Year	Month	Day	Temperature (F) 24 hrs. ending at observation time Max.	Min.	at Obs ervation	Rain, melted snow, etc. (in)	Flag	Snow, ice pellets, hail (in)	Flag	Snow, ice pellets, hail, ice on ground (in) At Obs Time	24 Hour Wind Movement (mi)	Amount of Evap. (in)	Ground Cover (see *)	4 in depth Max.	Min.	Ground Cover (see *)	8 in depth Max.	Min.
	2015	10	1	85	70		0.01		0.0		0.0								
	2015	10	2	72	63		0.11				0.0								
	2015	10	3	69	61		0.02		0.0		0.0								
	2015	10	4	70	60		0.01		0.0		0.0								
	2015	10	5	70	64		0.01		0.0		0.0								
	2015	10	6	81	63		0.00		0.0		0.0								
	2015	10	7	84	57		0.00		0.0		0.0								
	2015	10	8	85	56		0.00		0.0		0.0								
	2015	10	9	88	66		0.00		0.0		0.0								
	2015	10	10	85	66		0.00		0.0		0.0								
	2015	10	11	70	62		0.00		0.0		0.0								
	2015	10	12	80	54		0.00		0.0		0.0								
	2015	10	13	83	60		0.20		0.0		0.0								
	2015	10	14	82	55		0.00		0.0		0.0								
	2015	10	15	86	50		0.00		0.0		0.0								
	2015	10	16	88	54		0.00		0.0		0.0								
	2015	10	17	78	53		0.00		0.0		0.0								
	2015	10	18	73	48		0.00		0.0		0.0								
	2015	10	19	73	45		0.00		0.0		0.0								
	2015	10	20	80	49		0.00		0.0		0.0								
	2015	10	21	82	54		0.00		0.0		0.0								
	2015	10	22	86	58		0.00		0.0		0.0								
	2015	10	23	86	58		0.00		0.0		0.0								
	2015	10	24	83	57		0.00		0.0		0.0								
	2015	10	25	82	63		0.00		0.0		0.0								
	2015	10	26	78	70		0.01		0.0		0.0								
	2015	10	27	71	62		0.14		0.0		0.0								
	2015	10	28	81	62		0.10		0.0		0.0								
	2015	10	29	84	58		0.04		0.0		0.0								
	2015	10	30	79	52		0.00		0.0		0.0								
	2015	10	31	83	50		T		0.0		0.0								
		Summary		80	58		0.65		0.0										

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"s" This data value failed one of NCDC's quality control tests.
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U.S. Department of Commerce
National Oceanic & Atmospheric Administration
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Record of Climatological Observations
These data are quality controlled and may not be
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Generated on 07/24/2017

National Centers for Environmental Information
151 Patton Avenue
Asheville, North Carolina 28801

Elev: 260 ft. Lat: 31.763° N Lon: 84.187° W

Observation Time Temperature: 0800 Observation Time Precipitation: 0800

Station: **LEESBURG 2, GA US GHCND:USC00095061**

Preliminary	Year	Month	Day	Temperature (F) 24 hrs. ending at observation time Max.	Min.	at Obs ervation	Rain, melted snow, etc. (in)	Flag	Snow, ice pellets, hail (in)	Flag	Snow, ice pellets, hail, ice on ground (in) At Obs Time	24 Hour Wind Movement (mi)	Amount of Evap. (in)	Ground Cover (see *)	4 in depth Max.	Min.	Ground Cover (see *)	8 in depth Max.	Min.
	2015	10	1	85	69	70	0.00												
	2015	10	2	81	64	64	1.30												
	2015	10	3	68	58	59	0.53												
	2015	10	4	68	59	59	0.18												
	2015	10	5	69	59	66	0.07												
	2015	10	6	68	62	64	0.02												
	2015	10	7	77	57	59	0.00												
	2015	10	8	80	57	61	0.00												
	2015	10	9	82	61	74	0.00												
	2015	10	10	84	67	77	0.00												
	2015	10	11	77	62	68	0.00												
	2015	10	12	68	55	57	0.00												
	2015	10	13	77	57	62	0.00												
	2015	10	14	78	53	70	0.01												
	2015	10	15	83	50	51	0.00												
	2015	10	16	82	51	64	0.00												
	2015	10	17	88	50	65	0.00												
	2015	10	18	75	46	57	0.00												
	2015	10	19	69	42	46	0.00												
	2015	10	20	70	45	55	0.00												
	2015	10	21	77	52	62	0.00												
	2015	10	22	80	57	65	0.00												
	2015	10	23	83	58	66	0.00												
	2015	10	24	83	57	65	0.00												
	2015	10	25	80	61	66	0.00												
	2015	10	26	79	66	69	0.00												
	2015	10	27	76	58	59	0.43												
	2015	10	28	73	59	73	0.06												
	2015	10	29	77	58	65	0.08												
	2015	10	30	82	53	61	0.29												
	2015	10	31	76	49	59	0.00												
		Summary		77	57		2.97		0										

The "*" flags in Preliminary indicate the data have not completed processing and qualitycontrol and may not be identical to the original observation
Empty, or blank, cells indicate that a data observation was not reported.
*Ground Cover: 1=Grass; 2=Fallow; 3=Bare Ground; 4=Brome grass; 5=Sod; 6=Straw mulch; 7=Grass muck; 8=Bare muck; 0=Unknown
"s" This data value failed one of NCDC's quality control tests.
"T" values in the Precipitation category above indicate a TRACE value was recorded.
"A" values in the Precipitation Flag or the Snow Flag column indicate a multiday total, accumulated since last measurement, is being used.
Data value inconsistency may be present due to rounding calculations during the conversion process from SI metric units to standard imperial units.