ABSTRACT

Isolation, Characterization, and Antibiotic Susceptibility Testing of Halophilic Bacteria from the Grand Saline Salt Marsh

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Halophilic bacteria have the ability to survive in hypersaline environments and are of interest because of the unique mechanisms they utilize to survive in high salt concentrations. However, environmental halophiles have not been well characterized in the past, especially regarding antibiotic susceptibility testing. A few recent studies have been conducted that indicate the presence of antibiotic resistance genes in bacteria that have been around since before the widespread use of antibiotics (Martinez 2012; Dennis 2014). By examining the antibiotics susceptibility of halophilic bacteria isolated from the Grand Saline Salt Marsh, this could give further clues as to how the purpose of antibiotic resistance genes found in nature have changed over time. For this project, three wet soil samples were collected at different sites around the salt marsh. Isolates were assessed with 16s rRNA gene sequencing, biochemical testing, and optimum salt tolerance determination in order to identify the isolates. Antibiotics susceptibility testing of the isolates was performed using the Kirby-Bauer method.

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ISOLATION, CHARACTERIZATION, AND ANTIBIOTIC SUSCEPTIBILITY TESTING OF HALOPHILIC BACTERIA FROM THE GRAND SALINE SALT MARSH

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CHAPTER ONE

Introduction

Within the world of microorganisms, there exists a range of phylogenetic diversity that greatly exceeds that of macroorganisms. Examples of this diversity include extremophiles that have adapted to survive in various harsh environments (DeLong 2001). Some extremophiles include thermophiles that thrive in extremely high temperatures, acidophiles that can survive in acidic environments, and halophiles, or salt lovers, that thrive in hypersaline environments. In particular, halophiles are of interest to scientists due to their unique mechanisms of survival, applications in medicine, and uses in biotechnology and the food industry (DasSarma 2012). Thus, it is important that halophiles be further studied and understood in relation to these topics in order to better understand the microscopic realm
of extremophiles.

Halophiles exists in all three domains of life: archaea, prokarya, and eukarya. As the salinity of the environment increases, the diversity of viable organisms decreases. Of interest are the prokaryotic moderate halophiles that can survive in a wide range of salt concentrations and have adopted various unique methods of survival.

Halophiles can be classified as slight, moderate, or extreme. While slight halophiles grow best in an environment containing the range of 1-5% sodium chloride concentration, moderate halophiles thrive in environments containing 5-20% sodium chloride concentration. The moderate halophiles make up the most diverse group of halophiles, including various methanogenic archaea and anaerobic bacteria. Most of these

organisms are gram positive or gram negative aerobes or facultative anaerobes (Ventosa 1998). Extreme halophiles require environments containing up to 30% sodium chloride concentration (DasSarma 2012). Halotolerant organisms, in contrast to strict halophiles, can survive in the presence or absence of high salinity. Though halophilic organisms can grow and survive over a range of salinities, they typically display steeply increased growth at a specific concentration of salt, referred to as the optimum salt concentration. In contrast, one study found that the salt requirement and tolerance of different halophilic organisms varied according to other growth conditions including temperature and the presence of nutrients (Ventosa 1998). Moderate halophiles were found to have quite demanding nutritional needs at high salinities, such as the presence of compatible solutes in the environment. When isolating environmental halophiles, it is important to conduct studies on optimum environmental conditions such as salt concentration and temperature to promote the most vigorous growth of the isolates. Another study found that the organisms studied increased intracellular concentrations of various compatible solutes in response to two major stressors: increased salinity and increased temperature (Lamosa 1998). Halophilic microorganisms have developed a variety of mechanisms to survive in a harsh or changing environment.

As halophiles live in environments that would be extremely hypertonic to normal cells, they have developed two main methods of maintaining turgor pressure and preventing excessive fluid loss to the environment by creating a hypertonic cytoplasm. The first mechanism is the salt-in strategy, in which halophiles increase intracellular concentrations of ions. Since an excess of intracellular ions can affect enzyme activity, organisms using the salt-in strategy must have specially adapted salt-tolerant enzymes.

This often manifests in the form of an excess of acidic amino acids and a relative deficit of hydrophobic amino acids within the proteins produced by the cell (Oren 2002). This serves to ensure that the excess ions in the cytoplasm minimally disrupt the cell's enzyme structure and function. The second mechanism is the salt-out or compatible solute strategy, involving the synthesis or environmental uptake of organic molecules in order to maintain a hyperosmotic cytoplasm. These molecules include glycerol, sugars, and amino acids. These are often neutral or zwitterionic molecules that have little effect on intracellular processes (DasSarma 2012, Oren 2002).The advantage of the compatible solute strategy is that there is no need for highly specialized salt tolerant enzymes, as the intracellular ion concentration is not greatly increased. However, this method is often energetically costly, as ions must be continuously excreted against the concentration gradient via active transport (Oren 2002). One study found the presence of Na+/H+ antiports in halophiles to counterbalance the constant influx of sodium ions (Ventosa 1998). Though the salt-in method is more energetically favorable, the salt-out method is more common, indicating that enzyme function in a wide range of environments is vital despite energy expenditure (Oren 1999).

In addition to these methods, halophiles have developed additional adaptations to environments that have an alkaline pH, intense light, and electron acceptors other than oxygen. As a result of the slight pH difference between hypersaline environments, halophilic organisms must utilize enzymes that function within the range of their natural environment. This is especially important in preparing microbiological media for the study of halophiles, as the pH of the media used should reflect that of optimum growth conditions of the organism. The production of various pigments that serve to protect

against damage from ultraviolet light. Many halophiles are strict or facultative anaerobes, meaning they can survive in environments with little to no oxygen. In addition, some halophiles possess the ability to reduce sulfates and nitrates in order to harness extra energy using an electron acceptor other than oxygen (Litchfield 1998).

As halophilic organisms thrive in hypersaline environments ranging from 1-30% sodium chloride concentration, it is important to know the general salt composition of various salty bodies of water when isolating halophiles for study. For years, "hypersaline environments have been significant reservoirs for the long-term evolution of specifically adapted microorganisms" (McGenity 2000). Thus, the study of these organisms lend clues as to their evolutionary origins and the purposes served by various adaptations developed over time. While ocean waters generally contain about 3.5% salt concentration, hypersaline environments generally possess a significantly higher salt concentration. Hypersaline environments are often referred to as either thalassic or athalassic. Thalassic hypersaline environments are created by the evaporation of seawater and the retention of relative proportion of sea salts in higher concentration (DasSarma 2012). Athalassic environments were created by means other than the evaporation of sea salt and thus do not contain a relative proportion of salts that is similar to seawater. An example of how an athalassic environment could form is the dissolution of salts from continental or meteoric waters that become increasingly hypersaline upon evaporation of water (Vreeland 1992). A well-known example of a thalassic environment is the Great Salt Lake located in Utah, USA. An example of an athalassic environment is the Dead Sea located in eastern Israel. While thalassic environments mainly contain sodium and potassium salts, athalassic environments often contain a wider range of salts including

calcium carbonate, calcium sulfate, and magnesium chloride. Thus, in studies of halophiles, microbiological media must be prepared to imitate the salt content of the environment as closely as possible. Hypersaline environments are not limited to large bodies of water, but include ponds, solar salterns, and salt marshes. Soil can have a wide range of salinities, therefore saline soil has the potential to provide a unique environment in which an extremely diverse group of halophilic organisms thrive (Ventosa 1998).

Salt marshes serve as an ideal sample site for the study of moderately halophilic microorganisms. Salt marshes are defined as coastal wetlands that serve as a transition between the ocean and land. Salt marshes are often flooded and drained by salt water washed in by the tides (National Ocean Service). The soil is composed mainly of mud and peat, a spongy layer of decaying organic material that can be several feet thick. Salt marshes protect coasts from eroding by trapping sediment. They also serve to prevent major flooding by absorbing excess rainwater and metabolizing excess nutrients. Not all salt marshes are coastal, but can be produced due to the dissolution of underground salt deposits into marshy bodies of water. Salt marshes come into contact with many types of animals, such as migratory birds, saltwater fish, and reptiles such as turtles and alligators. Thus, salt marshes not only support the life of a wide diversity of halophilic and halotolerant microorganisms, but they are also home to many macroorganisms and allow for unique and complex interactions.

Halophiles have several applications in the fields of medicine, biotechnology, biodegradation and even the food industry. The products of some halophilic microorganisms have medical applications, such as the use of retinal-containing chromoproteins in the treatment of retinitis pigmentosa (DasSarma 2012).

Chromoproteins produced by halophiles are used in biocomputing and neurological probes (DasSarma 2012). The production of polyhydroxyalkanoates, which are products of bacterial fermentation of carbohydrates or lipids, can be used as tissue-engineering materials, drug delivery methods, and nutritional supplements (Chen 2005, Wu 2009). Halophile solutes and products have also been used in cosmetics, food, and nutritional supplements, including the fermentation of soy sauce and the production of β-carotene. Recent research has focused on the use of halophiles for bioremediation, or the removal or neutralization of pollutants by microorganisms. Halophiles have a profound impact on humans and the environment beyond their extreme habitats.

Previous studies focused on the environmental isolation and characterization of halophiles. These studies isolated halophiles from solar salterns, or evaporative pools of seawater that contain salt concentrations five to ten times higher than and provided crucial insight into the survival mechanisms of halophiles, the unique characteristic of phosphate-solubilization, and antibiotic sensitivity of halophiles seawater (Mathrani 1985, Zhu 2011).

When studying halophiles, the salt content of biochemical media may need to be adjusted to obtain accurate test results. For example, a previous study performed neglected to adjust the salt content of biochemical media and the results obtained reflected an unusually high number of negative test results (Azhar 2014). While another study has found that high salt content may affect the integrity of certain biochemical tests, adding a moderate amount of salt is unlikely to greatly affect the accuracy of results read on biochemical media (Gibbons 1957). In previous studies involving the antibiotic resistance profiles of halophilic microorganisms, the Mueller-Hinton media used to

perform antibiotics susceptibility testing was adjusted to the optimum salt concentration of the organisms.

Halophiles can also infect the human body. Although halophiles are typically thought to inhabit "extreme" environments, many can inhabit and infect various areas of the human body. The first pathogenic halophile, *Pasteurella haemolytica*, was identified in relation to a case of food poisoning in 1955 (Yamazi 1958). Another common potentially pathogenic halophile is *Staphylococcus aureus,* which is often associated with hospitals and can cause staph infections of the skin, boils and stys, and food intoxication via an enterotoxin. The growing prevalence of methicillin-resistant *Staphylococcus aureus*, or MRSA, exemplifies the growing problem of antibiotic resistance due to the overuse of antibiotics.

Some environmental microorganisms are capable of producing natural antibiotics, and this has selected for the development of resistance genes within other microbes. Recent studies have found that synthetic quinolone antibiotic resistance genes also exist in natural environments that have not been exposed to quinolones, alluding to a different original function for the gene (Martinez 2012). Interesting parallels can be made between the antibiotic resistance profiles of environmentally isolated organisms as compared to halophiles isolated from the human body or from a hospital setting. Arguably, because antibiotic resistance genes can be found in nature, it is likely that they originally evolved to serve some other purpose to promote survival of the organism. By gaining a better understanding of how these genes first evolved and functioned, scientists will become better equipped to combat the growing problem of infections and diseases caused by antibiotic-resistant microorganisms.

Although many environmental isolation and characterization studies involving halophiles have been conducted, there has been very little research regarding the antibiotic resistance profiles of these organisms. A previous study on a halophilic organism isolated from a solar saltern displayed resistance to penicillin, carbenicillin, cycloserine, erythromycin, and tetracycline when grown of an adjusted-salt medium (Mathrani 1985). It is important that the antibiotic resistance profiles of halophiles continues to be studied, as different hypersaline environments could contain a diverse population of microorganisms and varying levels of human contact.

A recent study stresses the importance of not confining antibiotic resistance studies to clinical-associated environments due to the widespread presence of therapeutic antibiotic resistance genes in nature (Martinez 2012). In this study, R-factors, or resistance plasmids, were obtained from pristine environments with no previous contact with antibiotics. Although antibiotic resistance has classically been associated with the overuse of antibiotics in clinical settings creating a selective pressure toward organisms containing antibiotic resistance genes, it is likely that environmental organisms are another link to the origin of antibiotic resistance. Resistance to both natural antibiotics produced by microorganisms and synthetic quinolones have been observed in environmental organisms. While the function of resistance to natural antibiotics could clearly serve a protective function for the organism, the function of the quinolone resistance gene, QnrB, is somewhat unclear, as these organisms had no previous exposure to synthetic quinolones (Martinez 2012). Antibiotic resistance genes have been found in environments in which there is no selective pressure due to previous exposure to antibiotics. It is hypothesized that genes such as QnrB served to detoxify a

microorganism from its own production of antibiotics, detoxify metabolic intermediates, aid in building the cell wall, and function in the trafficking of signal molecules into and out of the cell. The introduction of these types of genes into organisms with different needs and pressures conferred an additional property, antibiotic resistance. This phenomenon is referred to as exaptation, or the change of function of a gene without changing its sequence.

It has been found that environmental microbes contain a higher number of antibiotic resistance genes than bacterial pathogens found in clinical environments. In addition, soil archives dating back to 1940 have been analyzed and it has been found that the more recent samples contain a higher number of antibiotic resistance genes, indicating that antibiotic resistance is increasing in the environment. There is a diversity of different resistance genes between different environments, so it is critical that a wide variety of environments are studied in order to better understand the purpose and origins of antibiotic resistance genes (Martinez 2012).

Hypersaline bodies of water serve as a good model of a pristine environment that has not had previous contact with humans and therapeutic antibiotics. However, there have not been many studies conducted on the prevalence of antibiotic resistance genes in hypersaline environments. Thus, it is important that halophile antibiotic resistance profiles be obtained in order to understand the prevalence and function of antibiotic resistance genes in hypersaline environments. This could lead to a better understanding of mechanisms of survival for halophiles as well as the potential to discover new antibiotic resistance genes. Studying the antibiotic resistance genes within halophilic organisms and

hypersaline environments could serve to further uncover the mechanisms involved in the emergence, spread, maintenance, and evolution of antibiotic resistance genes (Martinez 2012).

CHAPTER TWO

Materials and Methods

Study Site and Collection of Samples

In this project, three samples were taken from three different sites on the Grand Saline Salt Marsh in Grand Saline, Texas. This sample site has a rich history in the salt industry and comes into contact with many species of migratory birds. The Grand Saline Salt Marsh lies on top of a salt dome that was formed when salt was forced through the earth's strata from subterranean deposits (Kleiner 2010). The deposits were most likely the remnants of a sea that dried up as many as 250 million years ago. The marshy soil overlying the salt domes contains high levels of sodium chloride and calcium sulfate and therefore provides an excellent habitat for halophilic microorganisms. Salt has been extracted from the area since 1834 when the Cherokee Indians made evaporative salt from the marsh. Today, Grand Saline is home for the Morton Salt Company and salt continues to be mined directly from the underground deposits as well as being extracted using evaporative methods. The Grand Saline Salt Marsh was chosen for its history within the food industry as well as long-standing proximity to humans that could have possible medical implications.

Three wet soil samples were taken near the periphery of the body of water and placed in small sterile containers. During transport back to the lab facilities, the samples were temperature controlled on ice in order to preserve the viability of microorganisms present in the samples.

Serial Dilutions and Viable Plate Count

Upon return to the lab facilities, serial dilutions were immediately performed. 20 grams of each soil sample were added to 180 mL of sterile distilled (DI) water to form a dilution of 1:100. Next, 1 mL of each sample dilution was added to 9 mL of sterile DI water using a micropipettor to form a dilution of 1:1000. This process was repeated until dilutions of 1:10,000 and 1:100,000 were obtained. Next, 0.1 mL of each dilution were plated on 2 plates each of tryptic soy agar (0.5% sodium chloride), mannitol salt agar (7.5% sodium chloride) and 12% mixed salt concentration modified growth medium to form dilutions of 1:1000, 1:10,000, 1:100,000, and 1:1,000,000. A 30% salt water stock solution was prepared using 30 g of magnesium chloride hexahydrate, 35 g of magnesium sulfate heptahydrate, 7 g of potassium chloride, 5 mL of 1 M calcium chloride, and 240.2 g of sodium chloride per 1 L distilled water. The modified growth medium was prepared by adding 5 g of peptone, 1 g of yeast extract, 567 mL of pure DI water, tris base to adjust the pH to 7.5, and 15 g of granulated agar to the 30% salt water stock solution. For simplicity, all media adjusted using the stock salt solution will be referred to as mixed salt.

The agar plates were incubated at 37ºC to reflect the environmental temperature at the time of sampling. Growth was checked at 48 hours and viable plate counts were performed using a light box and colony counter pen. Plates containing less than 30 colonies were considered too few to count, and plates containing more than 300 colonies were considered too numerous to count. The viable counts for each medium from the three sample sites were averaged to find an estimate of the various numbers of non-

halophiles and slight halophiles surviving at concentrations of 0.5% sodium chloride, 7.5% sodium chloride, and 12% mixed salt.

Culturing and Isolation

Five colonies were selected from either the mannitol salt agar or modified growth medium dilution plates based on size, color, and morphology in order to obtain as diverse a group of organisms as possible. Isolates 1, 2, and 5 were taken from sample 2, Isolate 3 was taken from sample 1, and Isolate 4 was taken from sample 3. Each colony was streak plated on mannitol salt agar and 12% salt concentration MGM and incubated for 72 hours at 37ºC in order to obtain isolated, purified colonies. Colony descriptions including size, color, consistency, elevation and margins of each colony were recorded in Excel. Subcultures were performed on slants of 7.5% mixed salt concentration MGM and incubated for 72 hours at 37ºC. Subculturing was performed every two weeks during the duration of this project to ensure viability of cultures.

Salt Tolerance Analysis

The next portion of the project involved determining optimum salt concentration for growth of each colony. Each colony was streak plated on TSA (0.5% sodium chloride), MSA (7.5% sodium chloride), 10% mixed salt MGM, and 12% mixed salt MGM. The plates were incubated for 72 hours at 37ºC and then colony and streak line size and appearance noted at each salt concentration. The optimum salt concentration for each colony was used to adjust the salt concentration of certain biochemical testing media.

rRNA PCR, Restriction Digest, and Sequencing

One-step reverse transcription polymerase chain reaction (PCR) was used to amplify the 16s rRNA gene and gel electrophoresis was performed to confirm the presence of the correct DNA product. The 16s rRNA gene serves as a marker for bacterial isolates and is often useful in sequencing and constructing phylogenies due to the slow evolution of this gene over time (Weisburg). It is also a relatively small sequence of about 1500 base pairs and can be quickly sequenced.

The first PCR was used only for a restriction digest. Isolates were streak plated on 7.5% mixed salt MGM and incubated for 24 hours at 37ºC. PuRe Taq RTG PCR beads were used along with 16s rRNA ReadyMade forward and reverse primers to amplify the DNA of the organisms during PCR. Organisms were added directly to the PCR tube by touching a sterile pipette tip to a colony from the streak plate and swirling in the contents of the PCR tube. The PCR program was set to have an initial denaturation only before the first cycle for five minutes at 95ºC, denaturation for 15 seconds at 95ºC, annealing at 52ºC for 15 seconds, and elongation at 72ºC for 60 seconds. This denaturation, elongation, and annealing settings were repeated for 30 cycles until a final elongation period of 5 minutes at 72ºC ended the program. 10 µL of the PCR products were run on a 1% agarose gel with electrophoresis at 140 V to ensure that the PCR had worked correctly. Next a restriction digest was performed using HindIII and EcoRI in order to make cuts in the sequences. 7 µL of product was used along with 1 µL of 10x NEB buffer, 1 µL of BSA, and 0.5 µL of each restriction enzyme. The restriction digest tubes were incubated in a 37ºC water bath for one hour. The restriction digest products were

then run on a 1% agarose gel at 140 V for 50 minutes. The bands were visualized using a UV light and then photographed. Band sizes were compared for each isolate to compare the cuts made within the sequence for each organism and to determine that no two isolates were the same organism. Another restriction digest was performed using the same protocol and the products were run on a higher-density 2% agarose gel in order to improve the resolution of the bands formed during the digest.

A second PCR was performed for each isolate using the same protocol and materials for 30 cycles and 10 µL of product were run on a 10% agarose gel to check the products. The remaining 15 µL of product were sent to Macrogen to be sequenced. The results of the sequencing were searched in BLAST, an online database, in order to determine the most likely genus and species of each isolate. Specimens aligning with greater than 98% accuracy were considered to be very good matches. Biochemical testing results from this project were compared to known biochemical profiles of the highly aligned organisms determined through BLAST in order to evaluate the most likely identity of the organisms.

Gram Stain, Endospore Stain, and Biochemical Testing

All bacterial isolates were Gram stained to determine morphology and arrangement. Gram positive organisms contained a thick layer of peptidoglycan in the cell wall and stained purple whereas gram negative organisms contained only a thin layer of peptidoglycan between the inner and outer cell membrane and stained pink. First, bacterial smears were prepared by placing one drop of DI water onto a microscope slide, touching an inoculating loop to the bacterial growth on a slant, and mixing the organism into the water. The mixture must completely air dry and then the slide is heat fixed by

placing the slide face up on top of a bacinerator for 15 seconds. The slide was not exposed to heat for more than 15 seconds, as this can damage the organism's cell wall. The first step of the Gram stain is the addition of crystal violet which stains all cells purple. After one minute, DI water was used to rinse the slide until the water runs clear. Next, Gram's iodine was used as a mordant to intensify the violet stain. After one minute DI water was used to rinse the slide until the water runs clear. The next step is the application of a few drops of ethanol, a decolorizing agent that breaks down the outer cell membrane and releases the crystal violet/iodine complex from the thin peptidoglycan layer of gram negative organisms. It is imperative that the ethanol only be applied for 3-5 seconds. Applying ethanol for less than this time will not allow for sufficient decolorization of gram negative cells and could lead to a false gram positive reading. Applying the ethanol for greater than 5 seconds will begin to break down the thick peptidoglycan layer of gram positive organisms and may lead to a false gram negative reading. The last step of the Gram stain procedure was the application of safranin, a counterstain to aid visualization of gram negative organisms as pink to red. Safranin is removed from the slide after one minute using DI water Light microscopy was used to observe bacterial morphology and arrangement and to "read" the Gram stain.

An endospore stain was used to detect the presence of endospores within a bacterium. Endospores can be produced by *Bacillus* and *Clostridium* organisms in response to a stressor such as heat. First, bacterial smears were prepared with the same method used for Gram staining. Next, malachite green was applied to the slide and a paper towel was placed on top of the stain. The slide was placed on top of a beaker of boiling water to force the stain into the spore casing and malachite green was

continuously applied to the paper towel to keep it damp. Endospores took up the malachite green and appeared as a small round green dot upon light microscopy. DI water was used as the decolorizing agent and removes the malachite green from the bacterial cells. Safranin was applied as a counterstain to cause the vegetative bacterial cells to appear pink to red upon light microscopy. Lastly, distilled water was used to remove excess safranin from the slides. If the bacteria stained only red or pink, no endospores were present. If there were small green dots within the bacterial cells, the organism was an endospore former.

Biochemical characterization was carried out in the next portion of the experiment. Catalase production was tested by adding 3% hydrogen peroxide to colonies grown on 7.5% salt MGM slants. Catalase is an enzyme produced by many microorganisms that catalyzes the breakdown of hydrogen peroxide to water and molecular oxygen. The production of bubbles within 5-10 seconds indicated the organisms' production of catalase due to the release of oxygen and the absence of bubbles indicated that the organism did not produce catalase (Azhar 754).

The oxidase test was performed using sterile swabs lightly touched to each colony from a slant to which a few drops of the oxidase reagent, N-dimethyl-*p*-phenylenediamine (DMPD) is added. DMPD is an electron donor that is colorless in its reduced form and turns dark blue to violet in its oxidized form due to the formation of indophenol blue. Organisms that produced cytochrome oxidase c, part of the electron transport chain, had a color change to dark blue or violet within 10-30 seconds and organisms that did not contain this electron carrier molecule remained colorless (Azhar 755). It is important to observe the results of this test within the first 30 seconds as

DMPD can be oxidized from the oxygen in the room and undergo a splotchy color change that is not as distinct as a true positive result. The oxidase test is significant in that it indicates organisms that are aerobic and utilize oxygen as a final hydrogen acceptor in the electron transport chain.

Bacto phenol red mannitol salt agar was used to determine the isolates' ability to ferment mannitol. Mannitol salt agar is also selective for gram positive halophiles; most gram negative organisms will not grow on this medium. However, some gram negative halophiles can grow on MSA. Mannitol salt agar contains 7.5% salt and phenol red to serve as a pH indicator. Phenol red is bright red in color and turns yellow in the presence of acid, usually at a pH below 6.8. Mannitol fermenters turn the agar yellow upon acid production due to the fermentation of mannitol and non-mannitol fermenters that are halophiles display growth without changing the color of the agar. Gram negative organisms usually display no growth on this medium. A heavy solid streak line was used to inoculate this medium. Plates were incubated for 72 hours at 37ºC and then observed for color changes around the streak line.

The remaining biochemical and sugar fermentation tests were first used without added salt and then the tests were repeated with all media adjusted to contain 7.5% salt.

Phenol red broth containing lactose, sucrose, and glucose was used to determine the isolates' ability to ferment these sugars. After the first set of media without added salt was used, only one isolate could grow so the recipe was adjusted to contain 7.5% salt by mass by diluting the 30% salt stock solution. Phenol red indicated the presence of acid, a by-product of sugar fermentation, by turning yellow. A negative test for the fermentation of a certain sugar was red broth after an incubation period of 72 hours at 37ºC. Some

microorganisms also possess the ability to release a gas by-product, usually carbon dioxide, upon utilization of the carbohydrate. An inverted Durham tube was present in the broth tubes to indicate carbon dioxide production as a byproduct of sugar fermentation by the microbe. The presence of an air bubble within the Durham tube was a positive test for gas production and no air bubble indicated no gas production.

SIM medium, or sulfide, indole, motility deeps, were used to indicate the organisms' ability to produce hydrogen sulfide, motility, and production of indole from tryptophan degradation. The media was inoculated with a steady stab by an inoculating needle and incubated for 72 hours at 37ºC. It was important that the inoculation is performed in as steady a manner as possible, as wobbling of the needle can cause a false positive test result for motility. If the organism had the ability to produce hydrogen sulfide, it reacted with sodium thiosulfate present in the medium, and the indicator, ferric ammonium sulfate, turned black. The presence of hydrogen sulfide indicated that the organism has the ability to break down cysteine present in the medium to several by-products, including hydrogen sulfide. This indicated that the organism produced an enzyme called cysteine desulfanase. Therefore, the presence of a black precipitate in the SIM deeps indicated a positive test for hydrogen sulfide production, while no change in the color of the medium indicated no hydrogen sulfide production. If an organism produced indole, this indicated that it had the ability to utilize tryptophan as an energy source and broke it down to pyruvate and indole using the enzyme tryptophanase. The production of indole was determined by adding Kovac's reagent, which reacted with indole to produce a reddish-pink pigment. Therefore, if indole was produced, the layer of Kovac's reagent turned reddish pink in color, whereas the Kovac's layer remained yellow

in color if indole is not present. If the organism is motile, it will be able to grow throughout the medium rather than just at the stab line. A positive test for motility will be indicated by turbidity or cloudiness throughout the SIM deep, and a negative motility test will be indicated by growth only on the stab line used to inoculate the medium. The addition of salt did not affect the turbidity, or cloudiness, of the medium, preventing a false positive result for motility solely based on the addition of salt.

Some organisms possess the ability to utilize citrate as a carbon source to produce energy in the absence of other carbohydrates such as glucose, sucrose, or lactose. Simmon's citrate agar, a chemically defined medium, was used to determine if the organism can use citrate. If organisms contain citrate permease, which allow citrate to enter the cell, carbon dioxide is produced and combines with sodium and water in the medium to produce an alkaline product, sodium carbonate. This medium contains bromothymol blue as an indicator. If citrate utilization does not occur, the medium will remain green in color and the organism will not grow. If citrate utilization does occur, carbonate products will cause the medium to turn a deep blue color.

Triple sugar iron agar slants (TSI) were used to confirm the sugar fermentation results obtained with the phenol red broths. TSI contains 1% lactose, 1% sucrose, and 0.1% glucose. It also contains sodium thiosulfate and ferrous sulfate to determine the ability of the bacteria to produce hydrogen sulfide. The media was adjusted to contain 7.5% salt using the 30% stock salt solution. Phenol red is used as the indicator and turns yellow in the presence of acid. The media is inoculated using a stab and streak technique in order to allow the organism to grow in both the butt of the tube in the absence of oxygen and the slant of the tube in the presence of oxygen. After incubation at 37ºC for

72 hours, the tubes were observed for color changes. An alkaline/acid or K/A tube appears red along the slant and yellow in the butt of the tube. This indicates fermentation of glucose but not of sucrose or lactose. An acid/acid or A/A tube indicates fermentation of glucose plus one additional sugar. An alkaline/alkaline or K/K tube indicates no fermentation of any of the three sugars. Organisms that utilize peptones produce a darker red slant due to alkaline byproducts.

Nitrate broth was used to determine the organisms' ability to reduce nitrates to nitrites or molecular nitrogen using nitrate reductase and nitrite reductase. After inoculating the broth and incubating at 37ºC for 72 hours, 5 drops of nitrate reagent A and 5 drops of nitrate reagent B were added. If the broth turns burgundy in color then this is a positive result for the presence of nitrites due to the action of nitrate reductase. If there is no color change at this point, zinc granules are added. If the broth turns red after the addition of zinc, this indicates that unreduced nitrate is present and the organism does not contain nitrate reductase. If the organism was negative for the first test, but the broth remains tan upon the addition of zinc, then the organism is positive for nitrate reductase.

Oxidation/fermentation glucose deeps were used to determine the ability of the organisms to utilize glucose by either oxidation or fermentation mechanisms. Two tubes were inoculated using a stab technique for each organism. One tube was designated as a "closed" tube and after inoculation a 3-5 mm layer of mineral oil was added to the top of the media to block oxygen from entering the media. The other tube was designated as an "open" tube and did not contain a layer of mineral oil, therefore the growing organism still had access to oxygen. O/F glucose deeps test the ability of the organism to utilize glucose in both aerobic and anaerobic conditions. This medium contains bromothymol

blue as an indicator. If acid is produced upon utilization of glucose, the indicator changes the color of the medium from green to yellow. If the organism utilized fermentative methods, both the open and closed tubes will appear yellow after incubation at 37ºC for 72 hours. If the organism oxidized glucose, only the open tube changed to yellow; the closed tube remained green. If both tubes remained green, the organism was non-saccharolytic, or unable to use the carbohydrate source present in the media. A teal color on the surface of the open tube indicated an increase in pH due to utilization of peptones rather than sugars. The low percentage of peptone to sugar "forces" utilization of glucose.

Kirby Bauer Antibiotics Susceptibility Analysis

Next, the antibiotic susceptibility of the isolates was tested using the Kirby-Bauer method. This method entails the growth of the organisms on Mueller-Hinton agar in the presence of six antibiotic discs containing concentrations of antibiotics similar to concentrations that would be observed in human blood and tissues during injection. The first set of Mueller-Hinton plates used did not contain added salt, but only Isolate 3 was able to grow on this set. The plates were adjusted to contain 7.5% mixed salt to enable the other isolates to grow, and three sets of media were inoculated for each isolate. After an incubation period of 72 hours at 37ºC, plates were observed and the zones of inhibition were measured. Zones of inhibition are clear areas of no bacterial growth surrounding the antibiotic discs. A zone of inhibition is compared with an established chart to determine susceptibility. Zones of inhibition are interpreted as resistant (R) below a specific measurement, susceptible (S) when above a specific measurement, and intermediate (I) in between the R and S values.

Antibiotics were chosen based on a range of modes of action, potency, and effectiveness against gram positive or gram negative organisms. Antibiotics can be classified as bacteriostatic, preventing the growth and division of bacteria, or bacteriocidal, effective in killing the organism.

Erythromycin disc concentration of 15 µg was used. For erythromycin at this concentration, a zone of inhibition with a diameter 18 mm or larger indicates susceptibility while a zone of inhibition less than 13 mm in diameter indicates resistance. A zone of inhibition between 13 and 18 mm would indicate intermediate sensitivity of the organism to erythromycin. Erythromycin acts as either a bacteriostatic or a bacteriocidal agent by preventing bacterial protein synthesis. This is accomplished by the binding of the drug to the 50s ribosomal subunits, which interferes with peptidyl transferase activity and the translocation of amino acids during protein translation.

Ciprofloxacin disc concentration of 5 µg was used. A zone of inhibition of a diameter larger than 21 mm indicates sensitivity, smaller than 15 mm indicates resistance, and between 15 and 21 mm displays intermediate sensitivity of the organism to ciprofloxacin. Ciprofloxacin interferes with bacterial DNA replication, repair, and transcription by binding to and inhibiting topoisomerase II and IV. Ciprofloxacin is a broad-spectrum antibiotic that is effective against a wide range of both gram positive and gram negative organisms.

Ampicillin disc concentration of 10 µg was used. A zone of inhibition of diameter less than 20 mm indicates resistance, between 20 and 29 mm indicates intermediate sensitivity, and greater than 29 mm indicates sensitivity to the antibiotic. Ampicillin acts as a bacteriocidal agent by inhibiting cell wall synthesis, leading to cell lysis and death.

Ampicillin is effective against a wide range of Gram positive organisms and a few Gram negative organisms.

Doxycycline discs containing 30 μg were used. A zone of inhibition less than 14 mm indicates resistance, between 14 and 19 mm indicates intermediate sensitivity, and greater than 19 mm indicates sensitivity. Doxycycline is a broad spectrum antibiotic that acts as a bacteriostatic agent by interfering with protein synthesis. This is caused by binding to the 30S ribosomal subunit, preventing the binding of the aminoacyl-tRNA to the ribosome.

Gentamicin discs containing a concentration of 10 μg was used. A zone of inhibition less than 12 mm in diameter indicates resistance of the organism to the antibiotic and a zone of inhibition greater than 13 mm indicates sensitivity to the antibiotic. There is no intermediate sensitivity zone of inhibition size recognized for gentamicin. Gentamicin interferes with 16s rRNA and the 30s ribosomal subunit, causing the misreading of mRNA and the production of non-functional proteins. Gentamicin is a broad spectrum antibiotic that is effective against many gram positive and gram negative organisms.

Sulfisoxazole discs of concentration 250 μg were used. A zone of inhibition less than 12 mm in diameter indicates resistance of the organism to the antibiotic, a zone of inhibition between 13 and 16 mm indicates intermediate sensitivity, and a zone of inhibition larger than 17 mm indicates sensitivity of the organism to sulfisoxazole. Sulfisoxazole inhibits the enzyme dihydropteroate synthetase and therefore interferes with the synthesis of folic acid. Sulfisoxazole is a broad-spectrum bacteriostatic agent.

CHAPTER THREE

Results

Viable Plate Count

The average viable plate counts were recorded for all sample sites on TSA, MSA, and 12% salt concentration MGM. While it was hypothesized that there would be greater numbers of colonies on the MGM, followed by a decreased number of colonies on MSA and the smallest number of colonies on TSA, in general, the data followed a different trend. The viable plate counts for sample 1 displayed the greatest number of colony forming units surviving on MSA, followed by TSA and then 12% MGM. For sample 2, the largest number of surviving CFU was on MSA followed by TSA and then 12% MGM. Sample 3 displayed the highest number of surviving CFU on TSA followed by 12% MGM and then MSA (Figure 1).

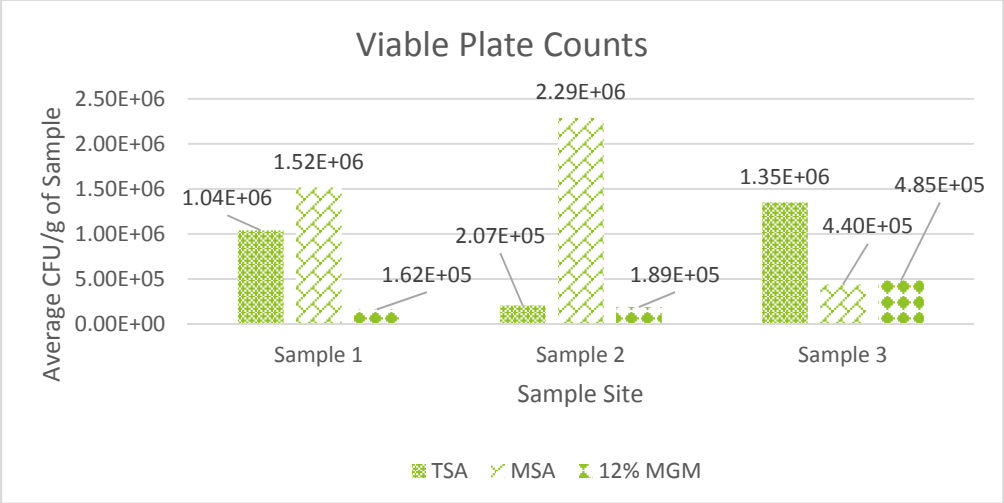


Figure 1. Viable plate counts for each sample site taken on TSA, MSA, and 12% MGM.

Isolates were chosen based on size and appearance from either the MSA plates or the 12% MGM plates.

After colonies were chosen, they were streak plated on both MSA and 12% MGM and incubated for 48 hours at 37ºC for purification purposes. Plates were checked then replaced in the incubator for another 24 hours to enhance the detectable growth of the isolates. The isolates were incubated for 72 hours at the same temperature for the remainder of the project.

Salt Tolerance Analysis

The optimum salt concentration was determined using a streak plating technique on tryptic soy agar (0.5% salt), mannitol salt agar (7.5% salt), 10% salt concentration modified growth medium and 12% salt concentration modified growth medium. The degree of growth on each media was determined based on the thickness of growth along the streak lines and the relative size of isolated colonies. All isolates displayed heavy growth on mannitol salt agar, but the degree of growth on media containing higher and lower salt concentrations varied widely.

Isolates 1 and 2 displayed no growth on TSA, heavy growth on MSA, moderate growth on 10% MGM and almost no growth on 12% MGM. Isolate 3 displayed heavy growth on both TSA and MSA with moderate growth on 10% MGM and almost no growth on 12% MGM. Isolate 4 displayed no growth on TSA, heavy growth on MSA, moderate growth on 10% MGM and light growth on 12% MGM. Isolate 5 displayed no growth on TSA, heavy growth on MSA, and light growth on 10% MGM and 12% MGM. Of note is that while isolate 5 did not display as much growth along the streak lines on the MGM plates as on the MSA plate, the colonies isolated on the MGM plates were

similar in size and appearance to those on MSA. Photographs of streak plates from the optimum salt concentration analysis portion of the experiment are contained within the appendix, A1-A5.

The appearance of the colonies of certain isolates changed between media containing different salt concentrations. While isolates 1 and 2 respectively produced an orange and red pigment on MSA, both isolates appeared white on MGM. Isolate 3 produced large tan colonies with a dry consistency on TSA, large mucoid colonies on MSA, and small white colonies on MGM. The size of the colonies also drastically decreased in size between the 7.5% and 10% salt concentration media except for isolate 5. Colony descriptions of each isolate were observed on the various media used and recorded in a table format (Table 1).

Table 1: Colony description of isolates on different media. NG designates no growth.

Isolate	Media	Color	Size (mm)	Shape	Margins	Elevation	Consistency
1	MSA	orange	3	round	entire	flat	mucoid
2	MSA	red-tan	4	round	entire	umbonate	glassy
3	MSA	yellow	8	round	entire	flat	mucoid
4	MSA	white	4	round	entire	convex	mucoid
5	MSA	white	5	round	entire	convex	mucoid
1	10% MGM	white	0.5	round	entire	flat	glassy
2	10% MGM	white	1	round	entire	convex	glassy
3	10% MGM	white	1.5	round	entire	convex	mucoid
4	10% MGM	white	0.25	round	entire	convex	mucoid
5	10% MGM	white	4.5	round	entire	convex	Mucoid
1	TSA	NG	n/a	n/a	n/a	n/a	n/a
2	TSA	NG	n/a	n/a	n/a	n/a	n/a
3	TSA	tan	8.5	round	entire	flat	dry
4	TSA	NG	n/a	n/a	n/a	n/a	n/a

Due to the heavy growth of all isolates on media containing 7.5% salt concentration, the modified growth medium was adjusted to contain 7.5% salt. The isolates were then streak plated on this media and incubated at 37ºC for 72 hours. Interestingly, when isolates were grown on MGM containing 7.5% salt, all isolates displayed a similar phenotype to those displayed when the organisms were grown on 10% MGM. Thus, the environment unique to MSA promotes the phenotypic switching observed in the isolates. This could be due to the presence of more nutrients and organic compounds such as mannitol sugar and phenol red.

Gram Stain, Endospore Stain, and Biochemical Testing

Next, the organisms were Gram stained to determine morphology and arrangement. Isolates 1 and 4 were observed to be gram negative while Isolates 2, 3, and 5 were found to be gram positive. All isolates were bacilli, or rod-shaped. Isolates 1 and 5 had a palisade arrangement while isolates 2, 3, and 4 had a strepto arrangement (Table 2).

Table 2. Gram stain results including cell morphology and arrangement.

Isolate	**1**	**2**	**3**	**4**	**5**
Gram Stain	-	+	+	-	+
Shape	bacillus	bacillus	bacillus	bacillus	bacillus
Arrangement	palisade	strepto	strepto	Strepto	palisade
Endospore	-	+(terminal)	+ (terminal)	-	-

Isolates 2 and 3 were positive for terminal endospores while Isolates 1, 4, and 5 were negative for endospores.

All isolates were found to be catalase positive and oxidase negative. Since Isolates 4 and 5 were unable to ferment glucose, it is expected that they should have been oxidase positive, indicating the use of the electron transport chain. It is possible that the excess ions present in the salt media interfered with the electron donating properties of the indicator.

Only isolate 3 was positive for the fermentation of mannitol, while all isolates were able to grow on mannitol salt agar (Table 3).

Upon the adjustment of all biochemical media to contain 7.5% salt, all isolates were inoculated in phenol red sucrose, glucose, and lactose broths, Simmon's citrate slants, TSI slants, SIM deeps, O/F glucose deeps, and nitrate broth. Isolates 1, 2, and 3 were positive for the fermentation of glucose while isolates 4 and 5 were negative for the fermentation of glucose. All isolates were negative for the fermentation of sucrose and lactose. No gas production was observed in any of the tubes (Table 3).

Table 3. Biochemical test results on media adjusted to contain 7.5% salt.

Isolate	**1**	**2**	**3**	**4**	**5**
PR G	+	+	+	-	-
PR S	-	-	-	-	-
PR L	-	-	-	-	-
TSI	K/A	K/A	K/A	K/K	K/K
MSA growth	+	+	+	+	+
Mannitol	-	-	+	-	-
Citrate	-	-	+	-	+
Nitrate	+	-	-	-	-
Motility	Motile	Non-Motile	Non-Motile	Motile	Non-Motile
Indole	-	-	-	-	-
Hydrogen sulfide	-	-	-	-	-

Isolates 3 and 5 were positive for the utilization of citrate while isolates 1, 2, and 4 were negative for citrate utilization. Isolates 1, 2, and 3 were found to be K/A on TSI, indicating the fermentation of glucose but not of lactose or sucrose, while isolates 4 and 5 were K/K, indicating no fermentation of the sugars. No gas production or hydrogen sulfide production was observed in any of the tubes (Table 3).

Only Isolate 1 was positive for the reduction of nitrates. Isolates 1 and 4 were motile while Isolates 2, 3 and 5 were non-motile (Table 3).

Upon the addition of salt to the O/F glucose deeps, the indicator, bromothymol blue, appeared to crystallize within the media. Therefore, all test results appeared negative on this medium, and the test was not accurate. Results for this test were disregarded during biochemical profiling.

16s rRNA PCR and Restriction Digest

After performing polymerase chain reaction using PureTaq PCR beads and 16s rRNA ReadyMade forward and reverse primers, 10 µL of product was run on a 1% agarose gel at 140 V for 50 minutes in order to check that the PCR worked correctly (Figure 2). After confirming the presence of a 1.5 kb band for every isolate, a restriction digest was performed using EcoRI and HindIII. 7 µL of product was run on a 1% agarose gel and the cuts made within the sequence for each isolate were compared. Isolates 1 and 2 appeared to have a cut producing two fragments of similar size, indicated by a thick band in the 750 base pair region. Isolate 3 did not appear to have any cuts within the sequence, indicated by the presence of one band in the 1500 base pair region. Isolates 4 and 5 had cuts producing two slightly different sized fragments, indicated by two distinct

band on the gel between the 1500 and 1000 base pair region (Figure 3). These results were not helpful in identifying the microbes nor determining their genus.

Sequencing and Identification

A second PCR was performed and 10 µL of product were run on a 1% agarose gel to confirm that the PCR worked. The remaining product was frozen and sent to Macrogen to be sequenced. Upon receiving the sequences, the online BLAST database was utilized to identify the most likely genus and species of the organism, when possible.

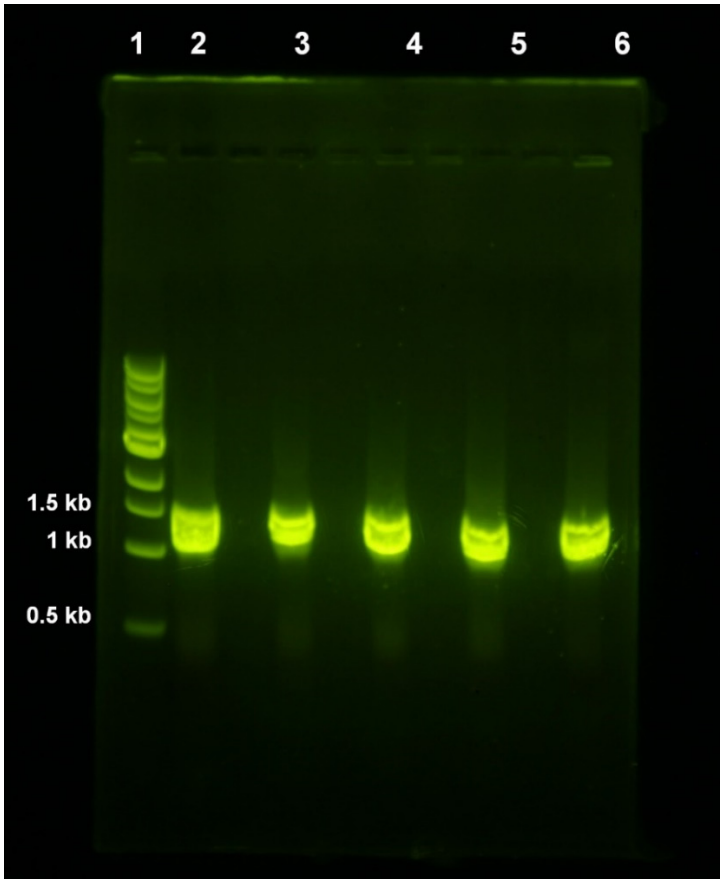


Figure 2. Confirmation of correct product after 16s rRNA PCR. All isolates had products that fell around 1.5 kb. The normal size of the 16s rRNA gene is about 1500 base pairs, with slight variations between bacterial species. Lane 1 contains a 1 kb ladder while lanes 2 through 5 contain PCR products for isolates 1 through 5.

Isolate 1 aligned with *Idiomarina seosinensis*, a halophilic bacterium found in hypersaline bodies of water. However, the sequence only aligned with an 84% match, much less than an expected value of greater than 95%. Given the nature of the sampling site, it is likely that isolate 1 at least belongs to the genus *Idiomarina*.

Isolate 2 aligned with *Thalassobacillus hwangdonensis* strain AD-1 with a 99% certainty, aligning between bases 1 and 194 of the isolate sequence and bases 562 and 756 of the database sequence. Isolate 2 also aligned with several species of *Bacillus* with

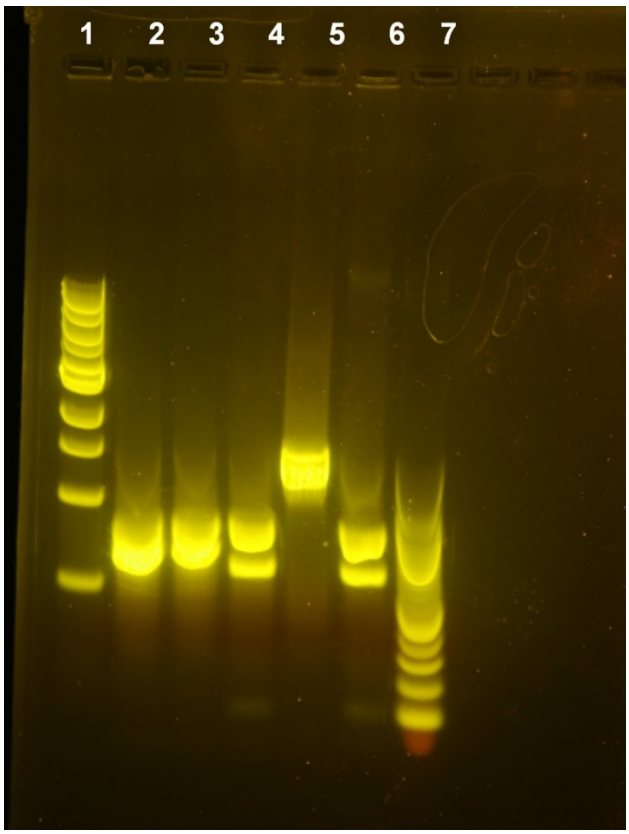


Figure 3. Product of restriction digest using the enzymes HindIII and EcoRI. Isolates 1 and 2 (lanes 2 and 3) show cuts in the sequences producing fragments of about the same size. Isolate 4 (lane 5) appears to not have any cuts within the sequence and instead shows only one band in the 1.5 kb region. Isolates 3 and 5 (lanes 4 and 6) each have cuts producing fragments of slightly different sizes between 1.5 and 1 kb. Lane 1 contains a 1 kb ladder and lane 7 contains a 100 base pair ladder.

a 98% certainty, so an online database for biochemical profiling of microorganisms was used to determine the most likely genus of the isolate.

Isolate 3 aligned with *Bacillus aryabhattai* strain BCAS11 with a 99% certainty between bases 1 and 201 of the isolate sequence and bases 513 and 713 of the database sequence. No other matches aligned with a similarly high certainty.

Isolate 4 aligned with *Idiomarina seosinensis* with a 98% certainty and with several other *Idiomarina* species with similarly high certainty, including *Idiomarina fontislapidosi, Idiomarina loihiensis,* and *Idiomarina zobellii*. Thus, it is most likely that this isolate belongs to the genus *Idiomarina*, although further comparison of existing biochemical characterizations of *Idiomarina* were used to determine the most likely species of the isolate.

The sequencing for isolate 5 was inconclusive and did not match anything in the BLAST database with certainty.

Kirby-Bauer Antibiotics Susceptibility Analysis

Upon utilization of Mueller-Hinton agar containing no added salt for antibiotics susceptibility testing, only isolate 3 displayed growth. This reflects the isolate's ability to survive in lower salt concentrations as compared to the other isolates. The zones of inhibition formed around each antibiotic disc in all 3 sets of media were measured and averaged to determine the degree of susceptibility. Isolate 3 displayed no zone of inhibition for ampicillin, and zones of sizes 28.3 mm for sulfisoxazole, 30 mm for ciprofloxacin, 27.3 mm for gentamicin, 24.7 mm for erythromycin, and 28.7 mm for doxycycline. These results are interpreted as resistance to ampicillin but sensitivity to sulfisoxazole, ciprofloxacin, gentamicin, erythromycin, and doxycycline.

Table 4: Zones of inhibition on 7.5% saline Mueller-Hinton.

Isolate	Gentamicin	Erythromycin	Doxycycline	Ampicillin	Sulfisoxazole	Ciprofloxacin
1	10.0 mm; R	32.0 mm; S	18.3 mm; S	32.8 mm; S	9.0 mm; R	13.0 mm; R
2	10.3 mm; R	33.5 mm; S	25.0 mm; S	34.8 mm; S	32.0 mm; S	15.3 mm; I
3	12.0 mm; R	29.0 mm; S	22.5 mm; S	10.8 mm; R	29.5 mm; S	17.8 mm; I
4	no zone; R	29.8 mm; S	no zone; R	12.8 mm; I	33.3 mm; S	23.8 mm; S
5	14.5 mm; I	23.8 mm; S	no zone; R	no zone: R	25.8 mm; S	25.0 mm; S

Three sets of Mueller-Hinton plates adjusted to contain 7.5% salt were used for antibiotic sensitivity testing of all of the isolates. Isolate 1 was resistant to gentamicin, sulfisoxazole, and ciprofloxacin and was susceptible to erythromycin, doxycycline, and ampicillin. Isolate 2 was resistant to gentamicin, was susceptible to erythromycin, doxycycline, ampicillin, and sulfisoxazole, and intermediately susceptible to ciprofloxacin. Isolate 3 was resistant to gentamicin and ampicillin, susceptible to erythromycin, doxycycline, and sulfisoxazole, and intermediately susceptible to ciprofloxacin. Isolate 4 was resistant to gentamicin and doxycycline, susceptible to erythromycin, sulfisoxazole, and ciprofloxacin, and intermediately susceptible to ampicillin. Isolate 5 was resistant to doxycycline and ampicillin, susceptible to erythromycin, sulfisoxazole, and ciprofloxacin, and intermediately susceptible to gentamicin.

CHAPTER FOUR

Conclusions

Moderately Halophilic and Halotolerant Bacteria Recovered

The results obtained in the initial viable plate counts display the variations in the number and type of halophiles present in the different sample sites. The first sample was taken from soil directly on the periphery of the salt marsh and displayed the highest number of organisms surviving on MSA; many halophilic organisms are able to grow in this site. This sample provided the second highest number of organisms surviving on TSA as well as the greatest phenotypic diversity of colonies, indicating a wide range of growth of non-halophiles or slight halophiles. Interestingly, the least number of surviving organisms was on the 12% MGM. The second sample contained the largest number of surviving organisms on MSA, followed by TSA and then 12% MGM. The third sample was taken across the road from the first sample site and had the most surviving organisms on TSA, followed by 12% MGM and MSA.

The ability of the isolates chosen from the dilution plates to grow faster at 37ºC rather than 25ºC reflects the temperature at the time of sampling in mid-June. Thus, the organisms surviving in the soil at this time had an optimum growth at a higher incubator temperature.

While isolates one, two, four, and five had growth restricted to a relatively low range of salinities (7.5%-10%), isolate three grew well at a wider range of salinities. Because strictly halophilic microorganisms either contain specialized enzymes that function at a given salt concentration or uptake and synthesize compatible solutes within

the cytoplasm, the organism will not grow when the salinity of the environment is either too low or too high. Organisms using the salt-in strategy will not be able to uptake adequate concentrations of ions into the cytoplasm, rendering the specialized enzymes ineffective and preventing growth of the organism. Organisms using the salt-out strategy placed in an environment with hypotonic salt concentrations would contain a cytoplasm that is extremely hyperosmotic and this would cause the bacterial cell to take in water, greatly increasing osmotic pressure. Conversely, if an organism utilizing this method of survival was grown in an environment that contained an extremely hypertonic concentrations of salt, the cytoplasm would become extremely hypotonic, causing water to leave the cell. Because isolate 3 survived in a wide range of salinities, this organism appears to utilize some strategy to quickly adjust the osmolarity of the cytoplasm in order to survive in a changing environment. This is indicative of halotolerance, rather than the other strictly halophilic isolates.

Growth of Gram Negative Organisms on Mannitol Salt Agar

Of interest is the isolation of gram negative halophiles that were able to grow on mannitol salt agar. This media contains 7.5% NaCl that inhibits the growth of most gram negative organisms due to the thin cell wall that does not effectively maintain bacterial cell shape in a hypertonic environment. Thus, this allows for shrinkage and death of gram negative organisms in a hypersaline environment. Most gram positive organisms can survive at this salt content due to the presence of a thicker layer of peptidoglycan in the cell wall, which better protects the organism against cell death in a hypertonic environment. However, two organisms of genus *Idiomarina* were isolated and able to grow well on MSA. Because these organisms were moderately halophilic and already

utilized mechanisms to maintain a hyperosmotic cytoplasm, these gram negative organisms were able to survive and even thrive in the environment provided by MSA.

Phenotypic Switching, Salt Tolerance, and Minimal Media

The wide range of phenotypes observed from colonies of each isolate grown on TSA, MSA, 10% MGM, and 12% MGM is a phenomenon called phenotypic switching. Phenotypic switching is a defense mechanism used by many microorganisms to survive under environmental stressors and it is often displayed by colony morphology variants caused by altered gene expression. An example of this phenomenon is the ability of *Serratia marcescens* to produce a red pigment when grown at room temperature but the inability of the bacteria to produce a pigment at 37ºC. Similar occurrences have also been observed in organisms such as *Candida albicans*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis* (Sousa 2012). This could be a likely explanation for the drastic colony morphology variants for isolates 1, 2, and 3. The inability of isolates 1 and 2 to produce a pigment when growing on a medium containing 10% salt or higher, as well as the extremely small colonies and thin growth as compared to the growth on MSA, could indicate that the isolates are unable to produce a pigment under environmental stressors such as increased salt concentration. Isolate 3 survived under the widest range of salinity conditions and also displayed the most drastic colony morphology variants. The difference in size, texture, consistency and color of the colonies under varying salt conditions indicates that the organism is able to adapt its phenotype in order to survive under stress.

However, phenotypic switching was not solely due to the variation of salt content, as none of the isolates produced a pigment on the adjusted 7.5% salt MGM. A proposed

explanation involves the salt-out, or compatible solute, strategy used by halophiles to maintain cytoplasmic balance. It is possible that the isolates were able to uptake mannitol sugar from the environment in order to maintain an isotonic cytoplasm in the midst of a hypersaline growth medium. While growth on 7.5% salt MGM, which contained minimal nutrients such as yeast extract and peptone for growth of the organisms, was much slower and less vigorous, growth on MSA was much faster and thicker. Thus, the presence of mannitol sugar likely allowed for a larger availability of organic molecules to uptake from the environment of the media and allowed the organisms to thrive and multiply faster and more vigorously than on the 7.5% MGM. It is also possible that the organisms were able to synthesize more intracellular organic compounds using raw materials found in the mannitol salt agar in order to maintain a cytoplasm hyperosmotic to the growth media. Isolate three was able to ferment the mannitol sugar as an energy source, and displayed very thick, mucoid growth on MSA. These results display that in addition to the salt concentration of the environment, the presence of nutrients and other organic compounds greatly affects the survival and the growth rate of halophilic microorganisms.

Biochemical Profiling of Isolates

Sets of media with no added salt were first used in order to determine if halophilic organisms could grow and therefore give accurate biochemical test results. Only the third isolate, which survived on media containing a salt concentration as low as 0.5%, survived on this set of media. While most of the biochemical media adjusted to contain 7.5% salt allowed growth of all five isolates and appeared to accurately display indicator color change to determine test results, no growth was obtained on any oxidation/fermentation glucose tubes. Upon the addition of salt, the media took on a light green color and a dark

spotted appearance that is unusual for this media. It is proposed that the addition of salt caused the indicator, bromothymol blue, to crystallize within the media, rendering it ineffective in determining an accurate test result. The same appearance was observed in the Simmon's Citrate agar to a lesser degree, but the organisms were able to grow on this media and the indicator changed color as expected.

All isolates were determined to contain the enzyme catalase and were therefore able to break down hydrogen peroxide, to oxygen and water. None of the isolates were positive for the oxidase test, indicating the absence of cytochrome oxidase c. However, Isolates 4 and 5 were negative for the fermentation of glucose so it was expected that they should be positive for cytochrome oxidase c. The ion levels present in the subculturing medium may have affected the indicator for this test.

Glucose fermentation was observed for Isolates 1, 2, and 3 while mannitol fermentation was observed for Isolate 3. The organisms are very specific in which carbohydrates they are able to utilize as an energy source, and it is likely that isolates four and five either have the ability to oxidize rather than ferment glucose, or are non-saccharolytic organisms.

Of interest is that isolates 3 and 5 were capable of using alternate energy sources such as citrate and mannitol sugar, in the case of isolate 3, and they also displayed the thickest and most hardy growth on a wide range of salt concentrations. It is possible that compared to the other isolates, these organisms are better adapted to survive pressure caused by changes in chemical composition of the environment. This is especially important for halophilic microorganisms, as hypersaline environments are often deficient in abundancy of nutrients.

Restriction Digest as a Comparative Tool

Upon performing gel electrophoresis accompanied with a 1 kb ladder following PCR, It was observed that a band of about the same size and weight, about 1.5 kb, appeared for all isolates. This is indicative that the 16s rRNA gene was correctly amplified for each isolate, as the 16s rRNA gene is about 1,542 base pairs long.

After performing a restriction digest, gel electrophoresis was used to observe the differences between cuts made in the PCR product sequence of each isolate. As the restriction digest was used purely as a differential tool to distinguish the isolates from each other, due to the sizes of bands produced there appeared to be at least three different organisms. Isolate 3 appeared to not have any cuts in the sequence due to the presence of one strong band of around 1.5 kb. Isolates 1 and 2 produced bands that were similar in position and size, indicating cuts of about the same length. However, these isolates were differentiated due to the nitrate test, as isolate 1 was positive for the production of ammonia or molecular nitrogen while isolate 2 was negative for nitrate reduction. Similar cuts were also observed between isolates 4 and 5, but these isolates were differentiated with the citrate utilization test, as isolate 4 was negative and isolate 5 was positive for citrate utilization. Thus it is likely that isolates 1 and 2 along with isolates 4 and 5 could be closely related, however, it is likely that they are different species. The restriction digest performed was not significantly definitive in determining the identity of the isolates.

Identification Process

Two different organisms belonging to genus *Idiomarina* were isolated. *Idiomarina* are gram negative rods that have been previously isolated and characterized from hypersaline bodies of water such as solar salterns or ocean water (Choi 2005, Ivanova 2000). Existing biochemical profiles for several species of *Idiomarina* were examined and compared to the biochemical results obtained in this project. Choi's characterization of *Idiomarina seosinensis* displayed negative results for the fermentation of glucose. However, isolate 1 was able to utilize glucose. The previous characterization also shows that *Idiomarina seosinensis* is unable to grow in media containing NaCl as the sole salt, but Isolate 1 was able to grow on mannitol salt agar. The optimum salt range of this organism is 7-10% and optimum temperature range from 30-35ºC is similar to the results obtained from this project. However, the biochemical profile for Isolate 1 seems more consistent with that of *Idiomarina zobellii*, which has the ability to grow on NaCl media, is negative for the reduction of nitrates and is positive for glucose utilization. Isolate 4 also aligned with *Idiomarina seosinensis* with the greatest certainty. However, the biochemical profile of Isolate 4 is more consistent with that of *Idiomarina baltica*, which has been previously characterized to be citrate negative, negative for acid production in the presence of glucose, optimum growth between 30-40ºC and growth between 0.8-10% salt (Martinez-Canovas 2004).

Isolate 2 was similar to a previous characterization of *Thalassobacillus hwangdionensis* regarding sucrose and mannitol fermentation, as well as citrate utilization, optimum temperature, and optimum salt concentration. However, Isolate 2 was found to be oxidase negative while *Thalassobacillus hwangdionensis* was previously

found to be oxidase positive (Lee 2010). This could be due to the excess ions present in the subculturing medium.

Isolate 3 was compared to existing biochemical profiles of *Bacillus aryabhattai* and differences were seen in sugar fermentation of sucrose as well as the oxidase test. However, Isolate 3 had a similar temperature and salt concentration optimum as this organism has shown growth at temperatures as high as 40ºC and grows at a wide variety of salt concentrations (Shivaji 2009).

A comparison of all isolates, including identification information, biochemical and fermentation properties, morphology, staining characteristics, and antibiotic resistance is found in table 5.

Significance of Antibiotics Resistance

According to a lab protocol specific to the concentration and potency of antibiotic discs used, isolates 3 and 5 were resistant to ampicillin, isolate 4 had an intermediate susceptibility, and isolates 1 and 2 were susceptible. Penicillin originated as a by-product of the mold *Penicillium notatum,* so it is considered a natural antibiotic. *Since* ampicillin belongs to the penicillin family of antibiotics, a common mechanism of bacterial resistance to this drug is the production of beta-lactamase, which breaks down penicillin antibiotics. Another previously characterized mechanism of resistance to penicillin drugs includes a *penA* polymorphism. This gene encodes penicillin binding protein, which disrupts and covalently bonds the beta-lactam amide bond, rendering the antibiotic ineffective (Jorgensen 2005).

Table 5: Overall comparison of isolates.

Isolate	**1**	**2**	**3**	**4**	**5**
Sample site	**2**	**2**	**1**	**3**	**2**
Identity	*Idiomarina spp.*	*Bacillus aryabhattai*	*Thalassobacills hwandionensis*	*Idiomarina spp.*	*unknown*
Gram stain	negative	positive	positive	negative	positive
Morphology	Rod	rod	rod	rod	short rod
Arrangement	palisade	strepto	strepto	strepto	palisade
Endospore	negative	positive (terminal)	positive (terminal)	negative	negative
Mannitol fermentation	negative	negative	positive	negative	negative
Citrate permease	negative	negative	positive	negative	positive
Triple Sugar Iron Slant	K/A	K/A	K/A	K/K	K/K
Nitrate reductase	positive	negative	negative	negative	negative
Catalase	positive	positive	positive	positive	positive
Fermentation of sugars	Yes	yes	yes	no	no
Oxidase	negative	negative	negative	negative (expected +)	negative (expected +)
Motility	motile	non-motile	non-motile	motile	non-motile
Indole	negative	negative	negative	negative	negative
Hydrogen sulfide production	negative	negative	negative	negative	negative
Gentamicin	resistant	resistant	resistant	resistant	intermediate
Erythromycin	susceptible	susceptible	susceptible	susceptible	susceptible
Doxycycline	susceptible	susceptible	susceptible	resistant	resistant
Ampicillin	susceptible	susceptible	resistant	intermediate	resistant
Sulfisoxazole	resistant	susceptible	susceptible	susceptible	susceptible
Ciprofloxacin	resistant	intermediate	intermediate	susceptible	susceptible

Isolate 1 displayed resistance to sulfasoxazole while all other isolates displayed susceptibility. The sulfa group of antibiotics are not produced by a microorganism but can be found in soils. Sulfa antibiotics were first used to treat bacterial infections after sulfanilamide, a compound found the dye Prontosil, was found to effectively kill bacteria.

The previously characterized mechanism of resistance to sulfa drugs involves a mutated *folP* gene that produces a modified dihydropteroate synthetase that renders the organism unaffected by sulfa antibiotics (Jorgensen 2005).

Isolate 1 displayed resistance to ciprofloxacin, isolates 2 and 3 were intermediately susceptible and isolates 4 and 5 were susceptible to the antibiotic. Because ciprofloxacin belongs to the synthetic quinolone group of antibiotics, Isolate 1 is a prime example of resistance to synthetic antibiotics found in nature. The antibacterial properties of quinolones were first discovered when nalidixic acid, an impurity of quinine synthesis, was found to kill bacteria. Ciprofloxacin is one of the many quinolone derivatives to have been developed since then. A previously characterized mechanism of bacterial resistance to Cipro drugs involves the gyrA gene which encodes subunit A of DNA gyrase (Jorgensen 2005).

Isolates 1, 2, 3, and 4 were resistant to gentamicin while isolate 5 was intermediately susceptible. Gentamicin is a natural antibiotic that was isolated from a fungus called *Micromonospora purpura.* Three main mechanisms of bacterial resistance to aminoglycosides include decreased cell permeability or uptake of the drug, alterations of the ribosomal binding site for gentamicin, or the production of aminoglycoside modifying enzymes that inactivate the drug by transferring amino and hydroxyl groups to the drug (Dowding 1977).

All isolates displayed susceptibility to erythromycin. Erythromycin is a natural antibiotic that was first isolated from a bacterium called *Streptomyces erythreus.* Microbial resistance to macrolides includes active efflux of the drug and methylase enzymes such as *ermB* that modify the ribosomal subunit that binds the drug

(Mouy 2001).

Isolates 4 and 5 were resistant to doxycycline while isolates 1, 2, and 3 were susceptible to this antibiotic. Doxycycline is a natural antibiotic that was first isolated from the bacterium *Streptomyces aureofaciens*. Two previously characterized mechanisms of this antibiotic include active efflux of the drug due to the acquisition of the *tetL* and *tetK* genes and the acquisition of the *tetO* and *tetM* genes mediating protection of the ribosomes to allow effective protein synthesis of the organism (Schmitz 2001).

The antibiotic resistance patterns observed in these isolates illustrate the presence of widespread antibiotic resistance genes and mechanisms found in nature. Because these organisms were isolated from the environment and have likely never encountered synthetic antibiotics before, these genes probably evolved over time to serve other purposes of bacterial defense. The resistance of Isolate 1 to ciprofloxacin is especially significant in demonstrating the resistance to synthetic antibiotics found in nature.

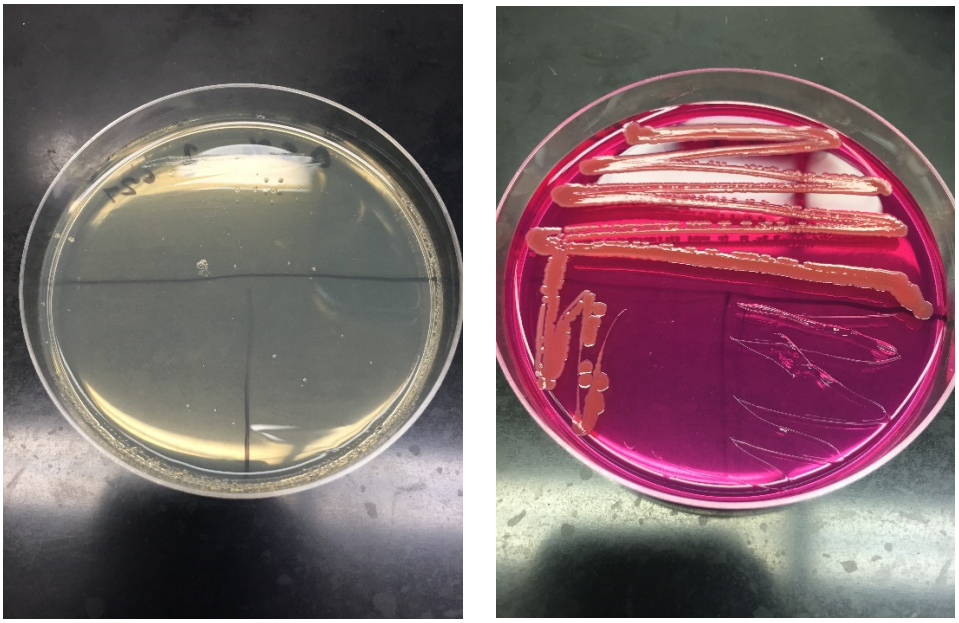
Confirmation of the modes of antibiotic resistance and the presence of known antibiotic resistance genes utilized by bacteria isolated from the environment along with genetic and biochemical profiling to identify the organisms can serve to better uncover how these genes have functioned and changed over time. Taxonomic tree information can be used to trace the presence and function of the gene over time.

Further antibiotic resistance studies conducted on environmentally isolated bacteria should be conducted to continue to develop the scientific community's knowledge and understanding of antibiotic resistance genes found in nature. The results

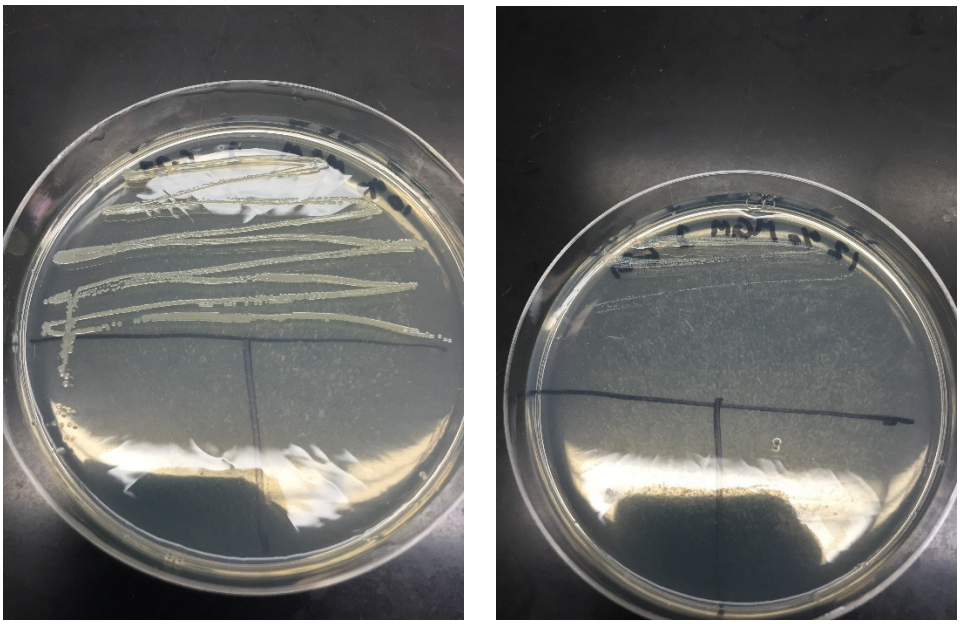
found in this project demonstrate that the overuse of antibiotics is only one factor in the growing problem of microbial antibiotic resistance in common of human pathogens.

APPENDIX

i) ii)

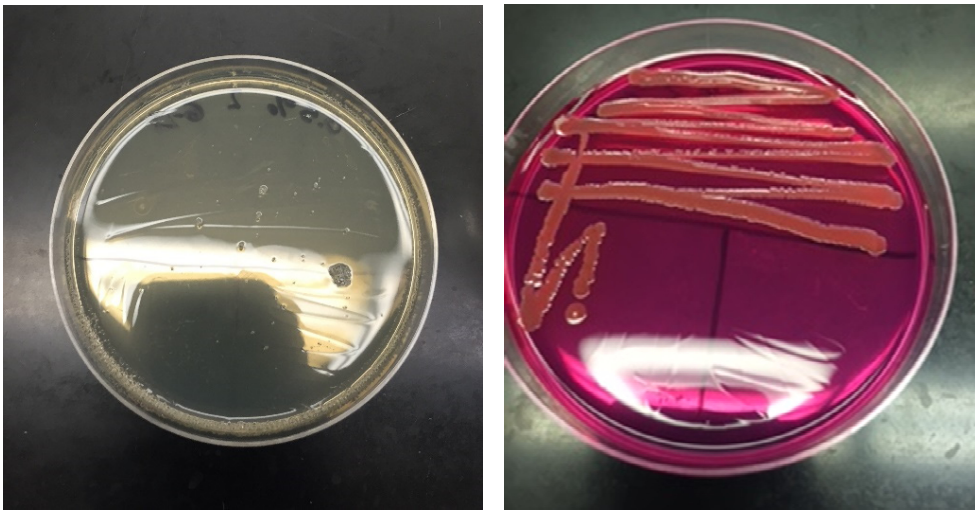


iii) iv)

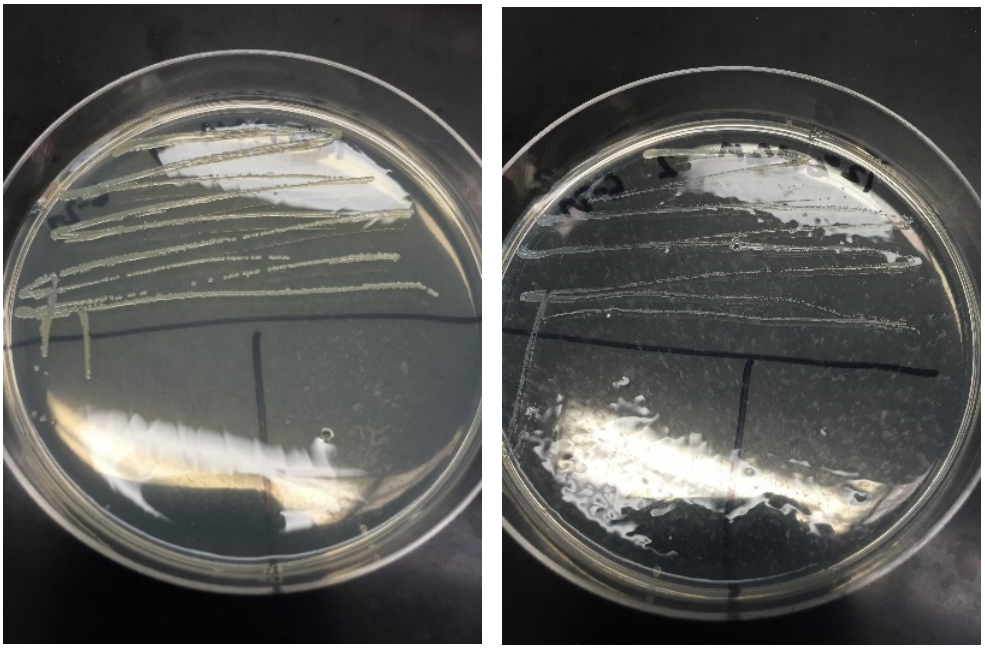


A1: Isolate 1 streak plated on tryptic soy agar (0.5% sodium chloride), mannitol salt agar (7.5% sodium chloride), 10% MGM, and 12% MGM for optimum salt concentration analysis. The isolate displays no growth on TSA (i) or 12% MGM (iv), but grows heavily with an orange pigment on MSA (ii) and grows lightly on 10% MGM.

i) ii)

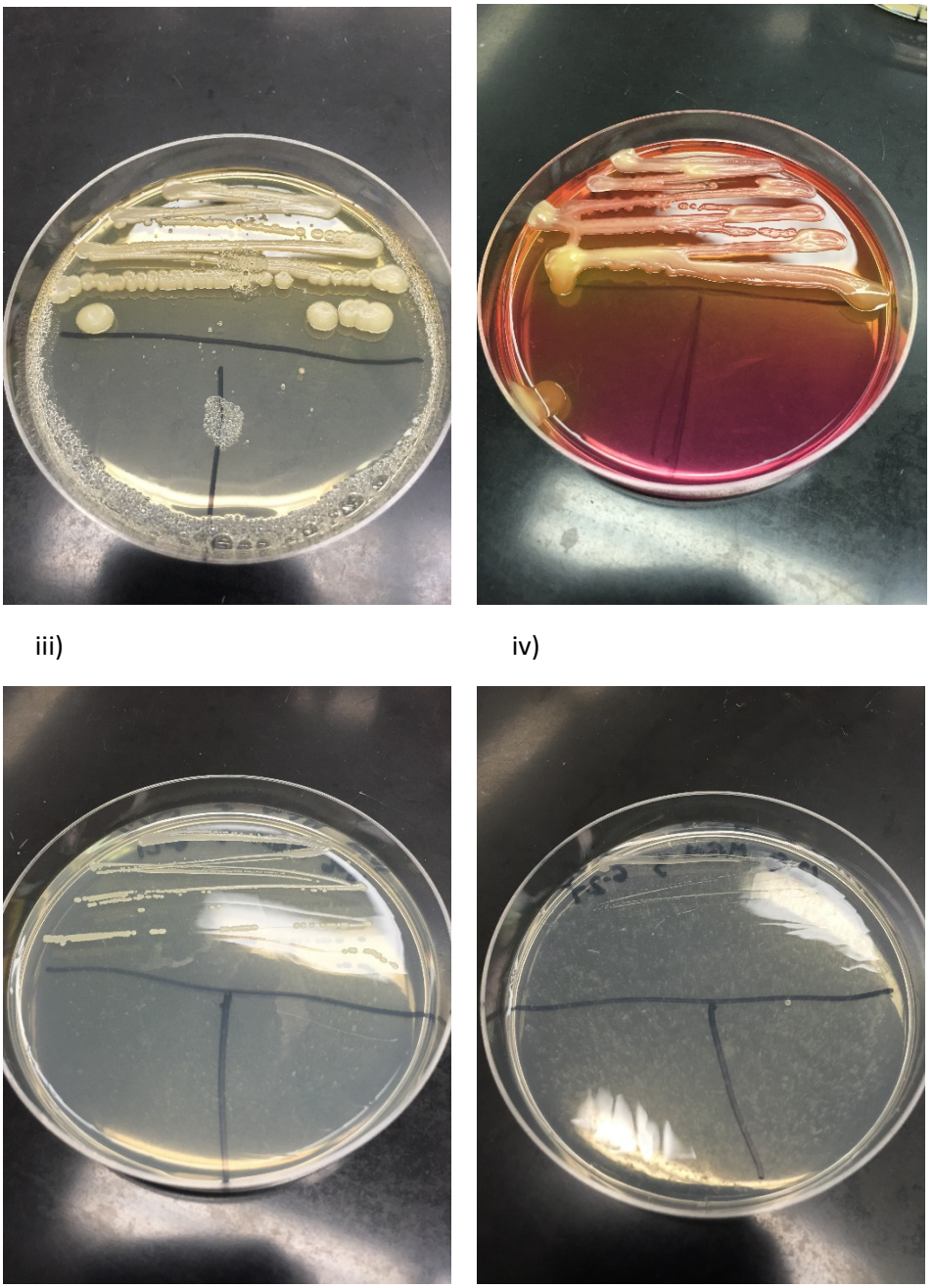


iii) iv)



A2: Isolate 2 on tryptic soy agar (0.5% salt), mannitol salt agar (7.5% salt), 10% MGM and 12% MGM.

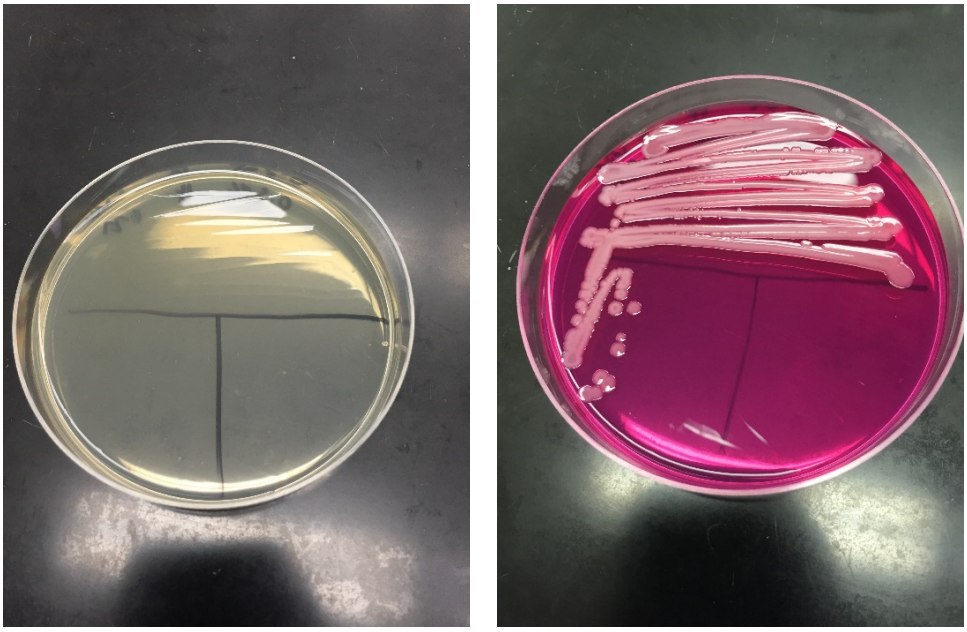
i) ii)



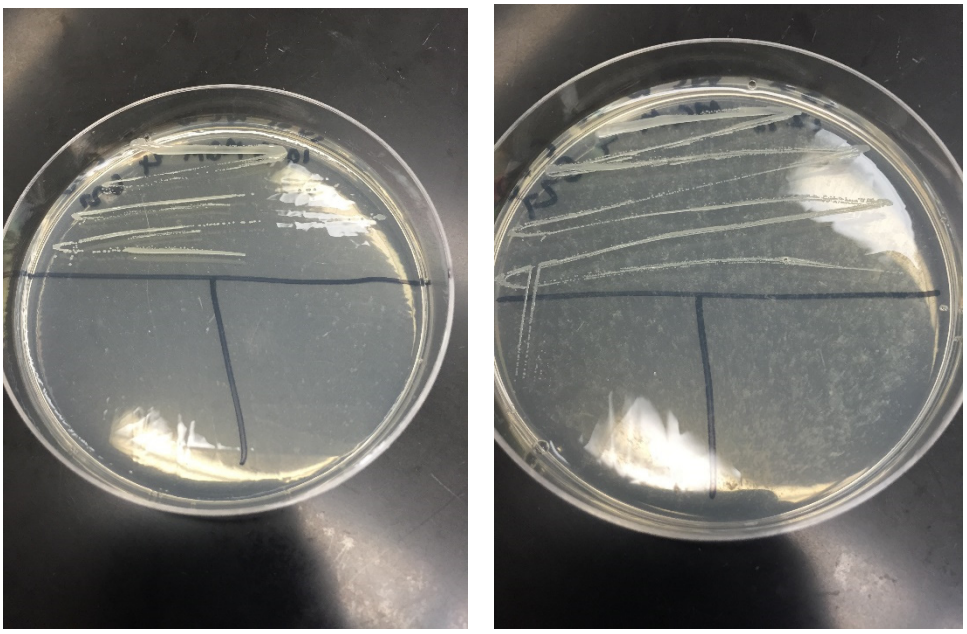
iii) iv)

A3: Isolate 3 displaying marked growth with a matte consistency on tryptic soy agar (0.5 % salt) (i), heavy, mucoid growth on mannitol salt agar (7.5% salt) (ii) with mannitol fermentation, very light growth on 10% MGM (iii) and no growth on 12% MGM (iv).

i) ii)

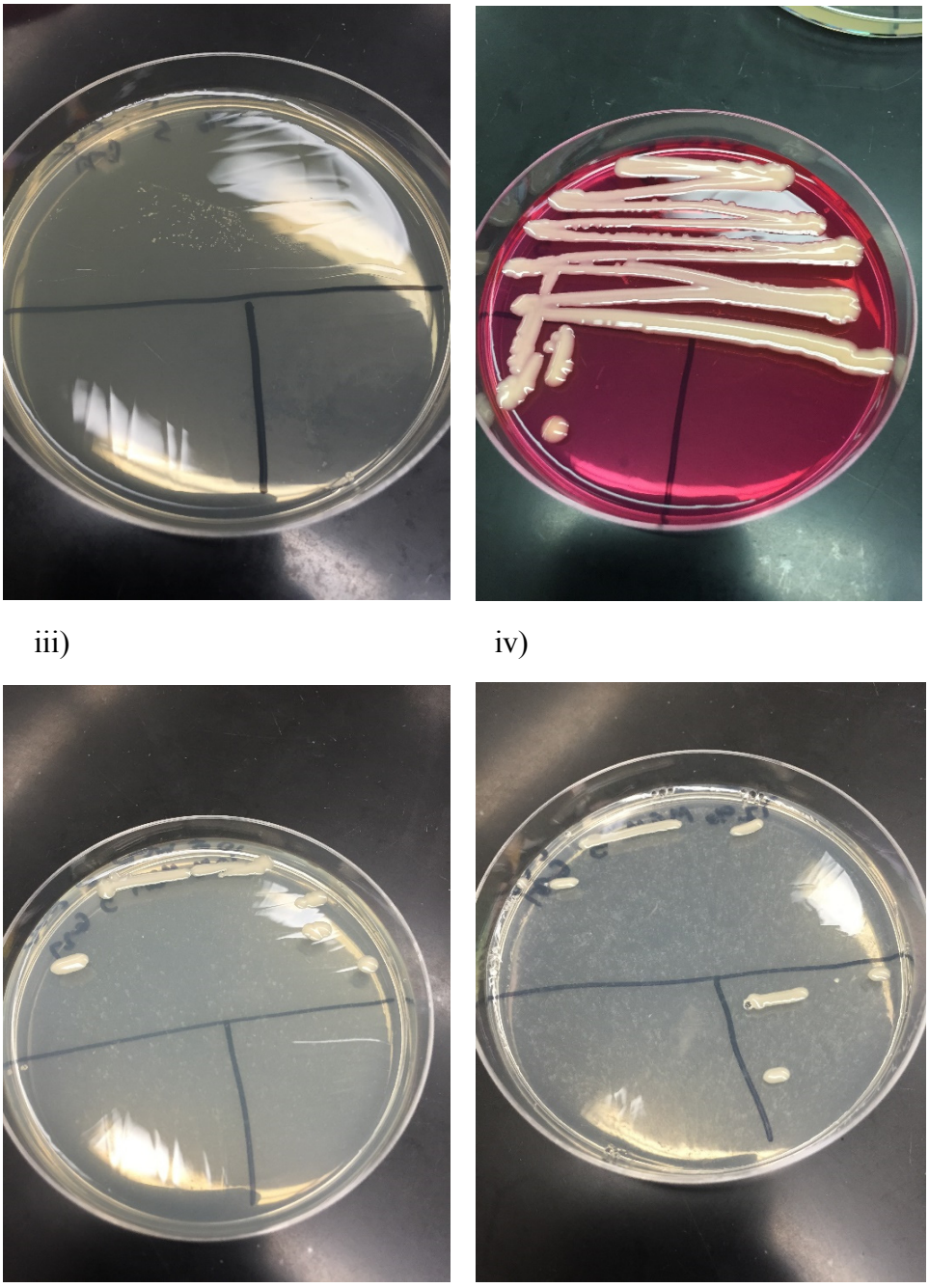


iii) iv)



A4. Isolate 4 does not grow on tryptic soy agar (0.5% salt) (i), displays heavy, mucoid growth on mannitol salt agar (7.5% salt) (ii) and light, sparse growth on 10% MGM (iii) and 12% MGM (iv).

i) ii)



iii) iv)

A5: Isolate 5 showing no growth on tryptic soy agar (0.5% salt) (i), heavy growth on mannitol salt agar (7.5% salt) (ii), and somewhat light growth on 10% MGM (iii) and 12% MGM (iv).

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