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pH dependent antibiotic resistance of an alkaliphilic, halotolerant bacterium isolated from Soap Lake, Washington

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pH DEPENDENT ANTIBIOTIC RESISTANCE OF AN ALKALIPHILIC, HALOTOLERANT BACTERIUM ISOLATED FROM SOAP LAKE, WASHINGTON

by

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This thesis has been prepared in the style of four journals. The first, a review has been prepared for submission to *The Science of the Total Environment*. A second manuscript has been prepared for submission to the *International Journal of Systematic and Evolutionary Microbiology*. A third manuscript has been prepared for submission to *Extremophiles*. A forth manuscript has been prepared for submission to *Letters in Applied Microbiology*. Pages 4 to 29 are prepared for submission as a review to *The Science of The Total Environment*. Pages 30 to 49 are prepared for submission as a journal article to the *International Journal of Systematic and Evolutionary Microbiology*. Pages 50 to 77 are prepared for submission as a journal article for submission in *Extremophiles*. Pages 78 to 103 are prepared for submission as a journal article in *Letters in Applied Microbiology*. Pages 105 to 110 are an introduction that will be submitted for publication to either *PLoS One* or *Frontiers* as part of a joint research project with Dr. Matthias Hess. Pages 1 to 3 and 111 to 129 are included as the standard these preparation.

ABSTRACT

Soap Lake, located in Washington State, is a meromictic, soda lake. Many bacterial isolates retrieved from Soap Lake have been noted to possess resistance to multiple antibiotics. A likely explanation for the wide range of antibacterial resistance exhibited by these strains is due to the impact of high alkalinity on the antibiotics themselves and not due to the presence of antibiotic resistance genes. The aim of our study was to determine if select antibiotics are effective against *Halomonas eurialkalitoleranis,* a bacterium capable of growth over a wide range of neutral to alkaline pH values, to investigate the influence of alkalinity on antibiotic activity. Five strains of *Halomonas eurialkalitoleranis* were isolated from Soap Lake sediment. *Halomonas eurialkalitoleranis* Isolate 9 was inoculated into media buffered over a range of pH values, 7-11. Select antibiotics; tetracycline, ampicillin, vancomycin, neomycin trisulfate salt, demeclocycline, sulfamethizole, kanamycin, chloramphenicol, streptomycin, roxithromycin, erythromycin, and sulfamethaxole, were suspended in inoculated media, to determine their minimum inhibitory concentration (MIC) over the range of pH values tested. Tetracycline, ampicillin, kanamycin, neomycin, roxithromycin, and streptomycin, were found to become ineffective against Isolate 9 at pH values above 8. Vancomycin did not produce statistical differences in MIC values at any pH tested. Erythromycin and sulfamethizole were found to be more effective against Isolate 9 at pH 11 than in neutral media. In addition, polymerase chain reactions were performed to determine if Isolate 9 possessed known genes for antibiotic resistance against the twelve antibiotics tested. Isolate 9 was found to possess resistance genes for all antibiotics tested, except kanamycin and streptomycin.

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1. INTRODUCTION

1.1. BACKGROUND

Soap Lake is a unique haloalkaline, soda lake that is the terminal lake in the chain of lakes formed in the Lower Grand Coulee, a basalt canyon, in central Washington State. Soap Lake is unique even among soda lakes because it is meromictic and it is speculated that the lake has remained in a stable meromictic state for at least 2,000 years (Pinkart et al., 2006). Soap Lake has a nearly constant pH of 9.8, extremely high sulfide concentrations in the monimolimnion, and has a salinity of around 14% (Pinkart et al., 2006). Soap Lake has no surface inlets or outlets and this lack of outlets is speculated to be among the reasons for Soap Lake's salinity (Anderson, 1958). Water levels in this lake are maintained via evaporation and water is supplied by water runoff from cliffs and plateaus that surround the lake and from groundwater seepage (Anderson, 1958).

The alkaline, saline, meromictic waters are known to harbor a number of unique organisms, including *Halanerobium hydrogeniformans, Halomonas campisalis,* and *Nitroncola lacisaponensis* (Begemann et al., 2012; Mormile et al., 1999; Dimitriu et al., 2005). Interestingly, the bacteria isolated from this lake carry a large number of antibiotic resistance to a wide variety of antibiotics including erythromycin, bacitracin, kanamycin, novobiocin, polymyxin B, neomycin, gentamicin, streptomycin, carbenicillin, rifampicin and tetracycline (Dimitriu et al., 2005; Mormile et al., 1999). The use of antibiotics from the surrounding agriculture might have contributed to the antibiotic resistances in the bacteria isolated from Soap Lake. Grant County, where Soap Lake is located has 50,260 acres of land designated for use in agriculture, mainly apple orchards (USDA, 2002).

Plant based agriculture accounts for 36 metric tons of antibiotics in the United States (McManus, 2014). In plant based agriculture, five antibiotics are approved for usage; streptomycin, oxytetracycline, gentamycin, oxolinic acid, and kasugamycin. Despite the limited usage of these antibiotics in plant-based agriculture, antibiotic resistant plant pathogens have emerged. Two examples are streptomycin resistant *Erwinia amylovora* and *Xanthomonas campestris pv. Vesicatoria,* which are two serious infectious agents of plants (McManus, 2001 and Stall, 1962). These streptomycin

resistant plant pathogens have seriously devastated crops such as fruit trees, tomatoes, and pepper crops and these infections have become a major problem in plant-based agriculture. Due to the method of application, a fine mist spray of the antibiotic over the crop, there is concern over the impacts that such antibiotic usage might have on human health and antibiotic resistance. These fine mist sprays settle onto the soil, can be washed away from the application site, and may contribute to the increase in antibiotic resistance conferring genes at the site or downstream of the impacted areas. Due to agricultural runoff from the areas surrounding Soap Lake it is possible that this contributes to the antibiotic resistome of Soap Lake.

Soap Lake's extreme environment makes it an ideal location to study the antibiotic resistances of novel halophilic, alkaliphilc bacteria. This lake was chosen due to its proximity to agricultural antibiotic usage and the highly alkaline, saline conditions. A likely explanation for the wide range of antibacterial resistance exhibited by these strains is due to the impact of high alkalinity on the antibiotics themselves and not necessarily due to the presence of antibiotic resistance-conferring genes. Antibiotics are known to be susceptible to environmental conditions such as temperature, light exposure, and pH (Falagas et al., 1997). Falagas et al. (1997) effectively demonstrated that acidic pH can negatively impact the effectiveness of several antibiotics. However, no work has been done to test the effect of alkaline conditions on the effectiveness of antibiotics.

1.2. OBJECTIVES AND GOALS

1.2.1. Isolate and Characterize Novel Isolates from Soap Lake Capable of Tolerating pH Range of 7-11. The first objective was to isolate novel organisms from the sediment samples taken from Soap Lake, Washington. The isolated organisms were selected for their ability to grow over a wide range of pH values from pH 7 to pH 11. Five organisms were successfully isolated and these organisms were characterized, by using traditional microbiology techniques, and identified as novel organisms of the genus *Halomonas* on the basis of the sequences of their 16S rRNA genes.

1.2.2. Determine the Impact of Alkaline Media on the Antibiotic Susceptibility of Isolate 9 from Soap Lake Washington. The second objective was to determine the impact of alkaline conditions on the antibiotic resistances of the five isolates. All isolates were screened with fifteen antibiotics, by using the Kirby-Bauer disc diffusion assay. Isolate 9 was selected for further testing. The minimum inhibitory concentration (MIC) was determined for twelve antibiotics across a range of concentrations from 0 µm to 160 µm and across a pH range of pH 7 to 11. The MIC values were compared and the amount of growth in each condition was statically analyzed by using Minitab software.

1.2.3. Identify Antibiotic Resistance Genes in Five Novel Isolates From Soap Lake Using PCR Amplification and Known Antibiotic Resistance Gene Primers. The third objective was to determine if the antibiotic resistance conferring genes were present in the five isolates for the twelve antibiotics. Polymerase chain reactions (PCR) were run to determine the presence of antibiotic resistance conferring genes using known antibiotic resistance gene primer pairs.

PAPER

I. ANTIBIOTIC RESISTANCE, AGRICULTURE, EXTREMOPHILES, AND THE RESISTOME: A REVIEW

To be submitted for publication in *The Science of the Total Environment*. This paper serves as the review of literature.

ABSTRACT

Microbial "warfare" has been waged between competing bacteria throughout time. Recently, antibiotics have been identified as signaling molecules between bacteria that happen to have antimicrobial properties in high concentrations (Clardy et al., 2009). Despite the innate purposes for antibiotics, they have become an integral part of modern society and are utilized for the treatment of various illnesses, animal feeding operations, the protection of crops, and in veterinary practices. However, with the overuse and misuse of these antibiotics, microbial resistance have become a major issue. Antibiotics are able to enter into the environment via point and non-point pollution sources and low concentrations of antibiotics, from various classes of antibiotics, can be found in surface water, groundwater, and the soil. The presence of antibiotics in the environment has been linked to an increase in antibiotic resistances among human, animal, and plant associated pathogens, and this is leading to an increase in the antibiotic resistome in the natural environment. However, until recently, the impact of antibiotic contamination has not been investigated in extreme environments.

Keywords: Antibiotic Resistance, Agriculture, Extremophiles, Antibiotic Resistome,

1. Introduction

With few exceptions, natural antimicrobial compounds are a result of secondary microbial metabolism (Walsh, 2003). Microorganisms produce antibiotics as a mechanism for self-protection and to aid in competitive interactions with other microorganisms. There is great evolutionary advantage to producing an antibiotic compound because this enables more growth of the bacterium by providing access to more nutrients through inhibiting other microorganisms and by uptaking the nutrients from the surrounding bacteria that are impacted by the antimicrobial compound (Walsh, 2003). Microbial "warfare" has been waged between competing bacteria throughout the evolution of all bacteria. Recently there has been put forth the idea that antibiotics are in fact signaling molecules between bacteria (Clardy et al., 2009; Yim et al., 2007). Despite the origins and evolution of antibiotics, it is important to note that these compounds have existed for hundreds of millions of years and recently the overuse and misuse of these compounds has led to an increase in antibiotic resistances in both clinical and environmental settings.

With the focus of antibiotics primarily on medical impacts and influences, very little is known about antibiotic resistance in environmental microorganisms and especially in extremophilic microorganisms and extreme environments. Agricultural usage of antibiotics is currently considered a contributing factor to the increasing number of antibiotic resistances in the environment. However, the impacts of agriculture on extremophilic microorganisms have not been considered until recently.

2. Modes of antibiotic resistances

The mechanisms by which bacteria are able to evade the action of antibiotics are complex and diverse. There have been a number of articles reviewing these mechanisms in great detail, therefore these mechanisms will only be discussed briefly in this article. Bacteria can develop a resistance to antibiotics via vertical or horizontal evolution (Schmieder, 2012). In horizontal evolution, a gene is transferred from one bacterium to another via horizontal gene transfer. In vertical evolution, a mutation occurs in the

bacterial DNA and is passed onto any daughter cells. Bacteria utilize both horizontal and vertical evolution to gain resistances to antibiotics, yet the mechanisms bacteria use to evade the action of the antibiotics remain similar in all bacteria.

There are four general methods for antibiotic resistances used by all bacteria: 1) the inactivation of the antibiotic (or modification); 2) altering the target site of the antibiotic; 3) the modification of effected metabolic pathways to mitigate the effect of the antibiotic; and 4) decreasing permeability or the use of efflux pumps to reduce the antibiotic accumulation intracellularly (Schmieder, 2012).

Inactivation or modification of drug molecules is a mechanism that is commonly utilized by a variety of bacteria. The most common example of this type of antibiotic resistance is demonstrated by the β-lactamase producing bacteria. The β-lactamases are enzymes that hydrolyze the β-lactam ring in penicillin and its derivatives (Walsh, 2003). By hydrolyzing the β-lactam ring, the bacteria effectively inactivate the antibiotic and it is no longer effective. It should be noted however, that the inactivation or modification of antibiotics as a mechanism of antibiotic resistance is only seen in naturally occurring antibiotics and has not yet been seen as a method to avoid synthetic antibiotics (Walsh, 2003).

The altering of the antibiotic target site is the second method utilized by bacteria to avoid the antimicrobial activities of antibiotics. In this case, the bacterium modifies the target of the drug to maintain cellular function, while rendering the antibiotic inactive. One such example of this type of resistance can be seen in *Methicillin-resistant Staphylococcus aureus (MRSA).* MRSA is able to evade the activity of methicillin by modifying a protein, penicillin binding protein (PBP). These organisms possess a modified gene product is known as PBP2A (Walsh, 2003). This modification to PBP enables MRSA to evade the action of methicillin while the PBP2A still retains the original function of PBP. Another example is resistance to the quinolone class of antibiotics that work by inhibiting the activity of the topoisomerase enzymes. Resistance to the quinolones occurs by either modifying the target enzyme or by limiting the permeability of the drug (Byarugaba, 2010). Similar modifications occur in metabolic pathways that are affected by antibiotics. Resistance to the sulfonamides is conferred by the sul1 and sul2 genes that encode for a modified form of dihydropteroate synthase that

is not inhibited by the sulphanamides. In some cases, overproduction of the effected target or mutations in the structure of the target also enable bacteria to avoid the action of antibiotics (Byarugaba, 2010).

Cellular efflux pumps are among the most common and universal methods for avoiding the action of antibiotics. The cellular efflux pump works to pump the antibiotic directly out of the cell before the antibiotic can damage the cell. These efflux pumps are most commonly utilized to avoid the actions of β-lactams, macrolides, pristinamycin peptides, flouroquinolones, and tetracyclines (Walsh, 2003). Interestingly, most efflux pumps have a narrow range of specificity and as such, several types are required by various bacteria.

3. Antibiotic usage in agriculture

Antibiotic use in agricultural systems has become a commonplace practice in both animal and plant based systems. The exact quantity of antibiotics that are used for agricultural purposes is not currently known (McManus, 2014). However, according to the US Food and Drug Administration website, more than 13,542 metric tons of antibiotics were sold for use in food animal production and 36 metric tons were used in crop based agriculture (US FDA, 2014). The use of antibiotics in agriculture is a globally accepted practice and is not exclusively limited to the treatment of infected animals or crops. In the 1950s, it was found that animal growth could be increased by the addition of antibiotics to the animal feed (CDDEP, 2011). This practice is common because it enables the production of larger meat animals. This use of antibiotics for animal feed has been identified as one of the leading causes for the increase of antibiotic resistance among disease causing bacteria (CDDEP, 2011). The European Union has banned the use of feed based antibiotics in order to slow the spread of antibiotic resistance among these bacteria.

In terms of crop based antibiotic application, antibiotics are applied to crops in order to prevent the infection of crops from plant-based pathogens. The question of whether this use of antibiotics has an impact on the antibiotic resistome is currently being studied. However, a recent review article by McManus (2014) claims that the relatively

small amount of antibiotics used in plant based agriculture does not greatly contribute to the increase in resistant bacteria. This is not to say that this use of antibiotics does not contribute to the antibiotic resistome, it is merely much less of a contributor than animal based agriculture or human consumption of antibiotics.

3.1. Plant Based Agriculture

Antibiotics are applied to high value fruit and ornamental crops to protect them from a variety of infections by spraying antibiotics over crops in a suspension that contains between 50 to 300 ppm (McManus, 2002). Due to the application method, there have been concerns presented that the antibiotics may settle onto soil particles and be washed downstream of the application site (McManus, 2014). Additionally, the antibiotics that are applied to these crops may cause resistance to develop in soil bacterial communities, previously identified as important reservoirs of antibiotic resistance (Walsh and Duffy, 2013; Wright, 2010). Furthermore, Wright (2010) identified the soil microbiome as a possible link to clinical laboratories. Additionally, crops protected by these antibiotics and consumed by humans, may contribute to a direct exchange of antibiotic resistance within the human host (Abriouel et al, 2008).

Thirty-nine different antibiotics are used in animal based agriculture and only five are used in plant based agriculture (Durso, 2014; McManus, 2014). Streptomycin, oxytetracycline, gentamycin, oxolinic acid, and kasugamycin are used to treat or prevent infections of valuable crops and ornamental plants from infections. In most cases, serious infections such as apple blight, pear blight and, more recently, citrus Hauanglongbling (HLB) have been treated or prevented by using these antibiotics (McManus, 2002 and Zhang, 2014). However, only high value crops such as fruit, vegetable, and ornamental plants are dusted with these antibiotics due to the high cost and environmental regulations that have become more stringent in recent years. Despite the limited usage of these antibiotics, antibiotic resistant plant pathogens have begun to emerge. Two examples are streptomycin-resistant *Erwina amylovora* and *Xanthomonas campestris pv. Vesicatoria*, serious infectious agents of plants (McManus, 2001 and Stall, 1962). Streptomycin-resistant plant pathogens have seriously devastated crops such as

fruit trees, tomatoes, and pepper crops. However, according to McManus (2002, 2014), the emergence of streptomycin-resistant plant pathogens has been less frequent than would be expected and is very sporadic in its occurrence.

Three classes of antibiotics are used to treat infections in plant agriculture, the aminoglycosides, the tetracyclines, and the quinolones. Streptomycin, kasugamycin and gentamycin are aminoglycosides and are characterized by the glycosidic bond between aminocyclitol and a series of amino sugars (Thiele-Bruhn, 2006). Streptomycin is most commonly used to treat infections of apple and pear blight caused by *E. amylovora*, and other infectious agents such as *Pectobacterium spp., Pseudomonas spp.,* and *Agrobacterium tumefaciens* (McManus, 2002). Gentamycin is used to treat similar infections as well infections caused by *Ralstonia*, and *Xanthomonas* (McManus, 2002). Kasugamycin is used to treat infections causing rice blast disease (McManus, 2014). Oxytetracylcine belongs to the tetracycline class of antibiotics and is characterized by a naphthacene ring structure and are polyketides (Thiele-Burhn, 2006). Tetracycline is used to treat infections from *E. amylovora*, *X. arboricola*, and *Pseudomonas spp*, on pear and peach trees and some vegetable crops. In some cases it can be used on apple trees, if the apple tree infection is from a streptomycin-resistant pathogen (McManus, 2014). Oxolinic acid is a quinolone and is characterized by the presence of aromatic fluorine at the c-6 position (Thiele-Bruhn, 2006). The oxolinc acid is used in Israel to treat streptomycin-resistant *E. amylovora* (McManus, 2002 and Shtienberg, 2001).

Due to the rising concerns of antibiotic usage and the heavier usage of these drugs, the impacts of these antibiotics has been assessed by a number of research teams. A number of studies have been done on plant pathogen resistance and the potential impact of these drugs on the soil bacterial community (Rodrigeuz et al, 2006; Tolba et al, 2002; Donato et al, 2010; and Popowska et al, 2012). Of particular interest in plant production is the causative agent of the fire blight, *E. amylovora*. Several resistant strains have popped up sporadically in the last fifty years of antibiotic application to plants. These resistant strains have been found all over the world in locations such as Israel, Lebanon, New Zealand, California, Oregon, Washington, Michigan, Missouri, New York, Florida, and many more according to McManus (2001). Despite the usage of

antibiotics at these sites, the occurrence of resistant strains has not yet been recognized as a serious problem.

Antibiotics are applied as a fine mist on agricultural plants. Frequent application of antibiotics to these plants and the cropland soil applies a selective pressure to soil bacteria and result in increased antibiotic resistance (Shade et al, 2013). However, application of streptomycin to apple trees had no effect on the bacterial soil community (Shade et al, 2013). Walsh et al (2014) also found that a restricted usage of streptomycin did not alter the soil community (Walsh, 2014). It has been hypothesized that the concentrations of antibiotics that reach the soil are not great enough to cause a selective pressure large enough to cause a shift in the soil bacterial community (Shade et al, 2013). In contrast, Walsh et al. (2014), found that up to 12 mg/ml of streptomycin could be found in the top 10 cm of soil after application of streptomycin during fire blight conditions. McManus (2014) states that even though the 12 mg/ml concentration is high enough to cause a selective pressure that other factors such as photodegradation, dilution, adsorption, and biodegradation may influence the pressures applied to the bacterial communities. Though plant pathogens sporadically develop resistance to the antibiotics used to treat them, the impacts and potential risk to human health are much lower than that of animal based agriculture or human medical uses of these drugs. However, these compounds are not entirely benign and their effects need to be studied further.

4. Transport of antibiotics into extreme environments

Antibiotics are able to enter into the natural environment via both point and non-point sources. Point sources are defined as a source of pollution that has an obvious source of entry into the water body from the contamination site, for example the discharge of wastewater directly into a stream (EPA, 2012). In contrast, non-point sources are those sources of pollution that do not have a direct route of pollution into the receiving water body and as such, these sources are much harder to trace (EPA, 2012). Non-point source pollution arises from any number of human activities without any obvious source of pollution. Both point and non-point sources of antibiotic introduction into the environment can lead to increased levels of antibiotic resistance.

Human applied antibiotics are able to reach the natural environment via a number of point and non-point sources including wastewater, agriculture, and medical usage. Human use of antibiotics can often lead to contamination of the environment due to their improper disposal. In addition, antibiotics and their metabolites are excreted by animals and humans (Wellington et at, 2013). In animal-based agriculture, antibiotics are heavily used to treat infections and to increase meat production in meat animals. These antibiotics are able to enter into the environment via both direct and indirect routes. Direct routes of release include the use of antibiotics in aquaculture and the use of fine mist sprays in plant based agriculture. Antibiotics can enter into the environment indirectly through the use of animal manure and wastewater slurry as fertilizer (Wellington et al, 2013). The routes of agriculture exposure are complex and as such are often considered non-point source pollution events (EPA, 2012).

Once the antibiotics have entered into the environment, they are easily disseminated into the soil and carried away from the source of pollution via surface water or groundwater. Antibiotics are eventually released into streams and rivers. Furthermore, antibiotics can be released into the soil, where they can remain in high concentrations for long periods of time. According to Wellington et al. (2013), several antibiotics are not inherently biodegradable and these antibiotics are able to persist in the soil long periods of time. Several classes of antibiotics have been found in the environment including flouroquinolones, sulphonamides, tetracyclines, and macrolides in concentrations at around 1 mg/ml in surface waters (Wellington et al., 2013). Furthermore, reports of concentrations as high as 2-5 mg/L have been reported in India downstream of a wastewater treatment plant (Fick et al., 2009).

Despite what can arguably be considered low concentrations of antibiotics being detected in the environment, their presence is still of great concern. According to Andersson and Hughes (2012) antibiotic resistance can evolve at either lethal concentrations or at non-lethal concentrations of antibiotics in the environment. When lethal concentrations of antibiotics are applied to a system obvious evolutionary selective pressure is applied to the system, if antibiotic resistant genes are present, they will presist. However, in some cases, low levels of antibiotics that are not lethal will also lead to an evolutionary selective pressure. These non-lethal concentrations of antibiotics result in

small differences in bacterial fitness that eventually lead to increased rates of mutation and promotes the development of antibiotic resistances in the microbial population over time (Andersson and Hughes, 2012). Therefore, even though relatively low levels of antibiotics can be detected in natural environments, these antibiotics can cause a shift towards higher levels of antibiotic resistance.

Often antibiotics in the environment are considered in regards to surface and groundwater as well as the soil. However, environmental contamination of extreme environments is not considered. In the case of Soap Lake, a meromictic, haloalkaline soda lake, the source of possible antibiotic input is due to non-point source contamination from the surrounding plant based agriculture. Grant County, where Soap Lake is located, has the second largest number of acres designated for apple orchards in Washington States (USDA, 2002). The surround agriculture is protected via the application of antibiotics in a fine mist spray that contains concentrations of up to 300 ppm of the antibiotic (McManuus, 2002 and McManus, 2014). This fine mist spray of antibiotics could serve as non-point source pollution of Soap Lake. The antibiotics could possibly enter into the lake through irrigation runoff. If present, low concentrations of antibiotics in Soap Lake would provide the same evolutionary pressures as other water bodies.

Another example of an extreme environment that is subject to notable antibiotic pollution is the Salton Sea, California. The Salton Sea is a hypersaline lake located in southeastern California (LeBlanc and Kuivila, 2008). In recent years, the water quality of the Salton Sea has become a great concern because migratory waterfowl use this location as a resting point during migration. Unfortunately, these waterfowl have been dying due to poor water quality and as such several studies have been done to investigate the sources of pollution into this environment (Patten and McCaskie, 2003). One study done by LeBlanc and Kuivila (2008) determined that the Slaton Sea was subject to pollution from agricultural pesticides that were utilized in the areas around the Salton Sea. According to De Vlaming et al. (2000) approximately 1.4 million kg of the active ingredients in pesticides (including antibiotics) are applied to the fields in the Salton Sea watershed. The use of these agriculture pesticides are linked to a decreased water quality in the Salton Sea. Other hypersaline lakes such as the Aral Sea in Uzbekistan are subject

to similar contamination and as such the water quality of these lakes should be of concern (LeBlanc and Kuivila, 2008).

It is evident that even extreme environments are subject to contamination with antibiotics from a variety of point and non-point sources. This contamination of extreme environments, even in low concentrations, can lead to a shift in the microbial populations towards those with antibiotic resistance. However, it should be noted that extreme conditions, such as highly acidic or alkaline pH, can impact the effectiveness of the antibiotics themselves and reduce the need for antibiotic resistance mechanisms in extremophiles. However, many extremophilic microorganisms do possess antibiotic resistance.

5. Antibiotic resistance and extremophilic microorganisms

Extremophilic microorganisms are capable of surviving in extreme conditions all over the world such as deep-sea hydrothermal vents, acid saline lakes, and the Atacama Desert. Extreme conditions encompass acidic conditions (acidophiles), alkaline conditions (alkaliphiles), high salinity (halophiles), extremely cold temperatures, below 15°C (psychrophiles), extremely high temperatures, in excess of 45°C (thermophiles), high pressure (piezophiles), and desiccation (xerophiles) (Rothschild, 2001). A new class of extremophiles, antibiotic resistant extremophiles (AREs), have recently been described (Gabani, 2012). These microorganisms are capable of thriving under conditions with excessive concentrations of antibiotics.

5.1. Acidophiles – Low pH Loving Bacteria

Acidophiles are microorganisms that are capable of thriving in conditions of extremely low pH values. pH values below 4.0 are considered acidic, while pH values between pH 4.0 to 6.0 are considered moderately acidic. In some cases, pH values below 0 have been reported in acid mine-drainage sites and microorganisms capable of thriving in these environments are present (Nordstrom, 2003). Acidophilic microorganisms have been used to bioremediate acid mine drainages (Dopson, 2004), produce novel enzymes

(Mohanram et al., 2013), and as a source of antimicrobial agents, such as isosulfazecin (Haibara, 1981). Though there are a number of characterized acidophilic bacteria, the publications on these microorganisms do not frequently provide data on the presence of antibiotic resistance.

However, a few articles have presented results on the antibiotic susceptibilities of acidophilic microorganisms. *Ferroplasma acidarmanus* and *Ferroplasma acidiphilum* strains were isolated from an acid mine drainage site with a pH value of pH 1.2. The antibiotic susceptibilities of these organisms were tested against ampicillin, chloramphenicol, kanamycin, rifampin, tetracycline, and gentamicin. *F. acidaramanus* was found to be resistant to gentamicin and was only partially susceptible to ampicillin, chloramphenicol, and kanamycin. *F. acidiphilium* was found to be more susceptible to antibiotics than *F. acidaramanus* (Dopson, 2004). *F. acidiphilum* was found to be resistant to ampicillin, chloramphenicol, kanamycin, rifampicin, and streptomycin and to be sensitive to tetracycline and gentamycin (Golyshina, 2000). *Acidothermus cellulolyticus* was found to be resistant to penicillin G, and was slightly susceptible to erythromycin and kanamycin (Moltagheghi et al., 1986).

Of particular concern to the medical community is antibiotic resistant *Helicobacter pylori*, due to being the causative agent of stomach ulcers and can be found in bacterially-contaminated drinking water supplies (Giao, 2008). Antibiotic resistance *H. pylori* has become a serious problem in recent years and treatment of these serious infections has become more challenging. A systematic review provides the prevalence of antibiotic resistances in more than 11,000 patients. It was determined that 17.2% of cases were resistant to treatment with clarithromycin, 26.7% were resistant to metronidazole, 11.2% amoxicillin, 16.2% to levofloxacin, 5.9% to tetracycline, 1.4% to rifabutin, and 9.6% of cases were found to be resistant to more than one antibiotic (De Francesco, 2010). Nearly ten percent of all treated cases are resistant to more than one antibiotic.

Acidic conditions may impact the antibiotic susceptibility microorganisms. Acidic conditions were found to lower the effectiveness of a number of antibiotics from the aminoglycoside family (Falagas et al, 1996). The antibiotic susceptibilities of *Bacteroides fragilis* were determined at pH values of 7.1, 6.3, and 5.8. It was found that of all the antibiotics tested, trovafloxacin, ciprofloxacin, clindamycin, ampicillin-

sulbactam, piperacillin-tazobactam, imipenem, and meropenem were more effective in near neutral conditions of pH 7.1, than the acidic pH 5.8 conditions (Falagas, 1996). The results of this study demonstrated acidic conditions could negatively impact the effects of antibiotics. A similar study done by Venglarcik et. al. (1986) determined that the antibiotic susceptibility of *Staphylococcus aureus* displayed similar pH dependency. These studies highlight the importance of understanding the effects of acidic conditions on antibiotics. Under acidic conditions, several bacteria were found to be resistant to multiple antibiotics but it is unknown if these resistances are due to the environment or to the presence of antibiotic resistance conferring genes.

5.2. Alkaliphiles – High pH Loving Bacteria

Alkaliphilic microorganisms are organisms capable of tolerating conditions in excess of pH 8.0. Naturally occurring alkaline environments include alkaline soda lakes, some alkaline glaciers, and alkaline groundwater (Grant, 2006). Several papers published on isolated alkaliphilic organisms, from a variety of sources, provide data on the antibiotic resistance and susceptibilities of these novel organisms. For example, *Alcaligenes latus* was isolated from alkaline industrial waste effluents and found to be resistant to carbenicillin, furazolidone, penicillin, and zinacef (Ali et al., 2009). Another bacterium, *Anoxvabacillus pushchinensis* was found to be resistant to multiple antibiotics including penicillin, vancomycin, ampicillin, streptomycin, and chloramphenicol (Pikuta et al., 2000). *Chimaereicella boritolerans* was found to be resistant to kanamycin, gentamycin, and penicillin and *Chimaereicella alkaliphilia* was found to be resistant to kanamycin, cephalothin and chloramphenicol (Ahmed et al., 2007).

Alkaliphilic organisms have been investigated for their ability to produce novel antibiotics that are stable in alkaline conditions. These organisms, such as the *Streptomycetes*, were found to produce a number of unique antimicrobial compounds. *Streptomycetes sannanensis* strain RST-1 produces an antibiotic that is particularly effective against gram-positive organisms. Other members of the *Streptomycetes* genus have been found to produce fattivaracin, chinikomycin, and lajollamycin (Vasavada et

al., 2006). Organisms such as *actinomycetes* and *Nocariopsis* strains have been found to produce novel antibiotics in conditions above pH 9.0 (Ulukanli and Digrak, 2002).

Alkaline conditions are known to negatively impact the effectiveness of several common antibiotics including ampicillin, streptomycin, and tetracycline (Florey, 1972; Regna et al., 1946; Loftin et al, 2004). However, in some cases antibiotics such as kanamycin and neomycin have been reported to be somewhat stable in alkaline media with a pH up to 9.0 (Finegold, 1959; Simone and Popino, 1995). More often than not, the stability of these antibiotics is unknown in alkaline media.

5.3. Halophiles – Salt-Loving Bacteria

Halophilic microorganisms thrive in salty conditions such as salt brines, the ocean, soda lakes, and other saline environments. These organisms have long been used in processes such as the salting and preserving of foods, such as puffer fish ovaries and Swedish fermented herring (Ma et al., 2009). Halophilic organisms are useful in industrial applications, chemical fermentations, and microbial enhanced oil recovery. A number of novel halophilic organisms have been isolated and characterized. Of interest, these organisms contain resistance genes to a number of antibiotics. For example, *Methanohalophilus zhilinae*, a halophilic and alkaliphilic bacterium, was found to be resistant to penicillin, ampicillin, carbenicillin, and cycloserine (Mathrani, 1988). *Marinobacter aquaeolei*, isolated from a Vietnamese oil producing well, was found to be resistant to ampicillin, anisomycin, novobiocin, oleandomycin, and tetracycline (Huu, 1999). *Halanaerobium alcaliphilum* was isolated from the Great Salt Lake sediments in Utah found to be resistant to penicillin G, D-cycloserine, tetracycline, and streptomycin (Tasi, 1995). *Halomonas hydrothermalis* was found to be resistant to ampicillin, methicillin, and vancomycin (Jayaraman, 2012). These reported antibiotic resistances were derived from the Kirby-Bauer disk diffusion assays. It is unknown if antibiotic resistance conferring genes are present in these organisms.

An investigation into the antibiotic resistance profile of several halophilic organisms isolated from a tannery effluent confirmed that they were resistant to antibiotics such as ampicillin, kanamycin, and neomycin (Ghosh, 2007). Additionally, it

was determined that the antibiotic susceptibilities of these organisms depended on the salinity of the environment. Moderate halophiles tended to demonstrate more resistances to drugs such as nalidixic acid, spectinomycin, and tetracycline and were highly sensitive to other drugs such as rifampicin and trimethoprim (Ghosh, 2007). Further studies indicated that the antibiotic resistance genes were found on plasmids. This infers that halophilic microorganisms possess the ability to the transfer of antibiotic resistance genes to other bacteria.

5.4. Psychrophiles – Low Temperature Loving Bacteria

Psychrophilic microorganisms thrive in extremely cold conditions. These organisms are found in locations such as Antarctica, glaciers, permafrost soils, the deep ocean, and at high latitudes. They are able to thrive in conditions as low as 0°C, and some have been isolated from soils and sediments that have not been subject to human activity in thousands of years (D'Costa et al. 2011). The antibiotic resistances of such psychrophilic organisms are of interest because locations such as Antarctica have not been subject to extensive human contamination and can provide opportunities for sampling in areas that have not been exposed to antibiotics released due to human activities.

Several unusual psychrophilic species have been isolated and characterized. Of these psychrophilic organisms, several have been shown to have antibiotic resistance to several antibiotics. *Flavobacterium frigidarium*, isolated from Antarctica, is resistant to ampicillin, neomycin, kanamycin, gentamycin, and streptomycin (Humphry, 2001). *Polaromonas vacuolata*, also isolated from Antarctica, was found to be resistant to gentamycin and streptomycin (Irgens, 1996). *Halomonas glaciei*, isolated from sea ice attached to the Antarctica coast line, is resistant to nalidixix acid, nitrofurantoin, rifampicin, streptomycin, polymoxin B, erythromycin, and novobiocin (Reddy, 2002). The antibiotic resistances of these novel bacteria were identified using the standard Kirby-Bauer disc diffusion assay.

One study performed by D'Costa et al. (2011) determined that antibiotic resistance is an ancient phenomenon. 30,000-year-old samples from the Beringian

permafrost were analyzed to determine if antibiotic resistances could be found in ancient microorganisms. The samples were dated at 30,000 years old through the use of radiocarbon dating and the samples were considered uncontaminated by modern activities due to the preserved relict permafrost indicating that the site had not thawed since the permafrost had been deposited and the researchers did not find any fluid leaching at the site. DNA sequences retrieved from uncontaminated cores were analyzed to determine the presence of such resistances. Resistances to β-lactams, tetracyclines, and glycopeptide antibiotics were found. It was concluded that ancient psychrophilic organisms harbored antibiotic resistances similar to those seen in modern organisms.

Another study compared these psychrophilic organisms to mesophilic organisms accidentally introduced into the environment by human presence in Antarctica (Miller et al, 2009). Antibiotic resistance genes to tetracycline, kanamycin, ampicillin, nalidixic acid, and streptomycin were found in psychrophilic organisms. When considering resistance to one or more antibiotics, 13 psychrophilic bacteria were found to have a resistance to one or more antibiotics where 117 mesophilic bacteria were found with resistances to one or more antibiotics from the four field stations tested (Miller et al, 2009). Overall, the resistances in psychrophilic organisms were found in a significantly smaller population than the resistances in the mesophilic organisms.

A study done by Kobori et al. (1984) determined that 48 isolated bacteria from Antarctica contained plasmids. Seven of these retrieved plasmids were found to contain antibiotic resistance genes to ampicillin. Of the bacteria that did not contain plasmids, only two out of 107 bacteria were able to grow equally as well on medium both unamended and media amended with ampicillin indicating a resistance to ampicillin indicating that among psychrophilic microorganisms, antibiotic resistance is present and located on easily transferable elements like plasmids.

5.5. Thermophiles – High Temperature Loving Bacteria

Thermophilic microorganisms thrive in high temperatures and found in thermal hot springs and deep-sea hydrothermal vents. They are of particular interest in terms of microbiology due to their potential uses in commercial industry. *Thermus aquaticus* was

isolated from a thermal hot spring in Yellowstone National Park. This bacterium is the source of the thermal stable Taq polymerase, used in polymerase chain reactions, and has given rise to molecular biology as a fast and relatively simple technique. Since the discovery of Taq polymerase, thermophilic microorganisms have been investigated for any number of potential applications such as the L-aminoacylases which was isolated from *Thermococcus litoralis* and the γ-lactamase that was isolated *Sulfolobus solfataricus*. Both of these enzymes are commercially useful because they enable research to obtain a higher purity amino acid from various bacteria (Littlechild, 2011).

Several isolated thermophilic organisms have resistances to a number of antibiotics when tested with the Kirby-Bauer disc diffusion assay. One bacterium, *Rhodothermus marinus* is a thermophilic, halophilic bacterium isolated from a submarine hot spring in Iceland. This bacterium was found to be resistant to aminoglycosides such streptomycin, kanamycin, and gentamycin (Alfredsson, 1987). *Lebetimonas acidiphilia* an acidophilic, thermophilic bacterium, was isolated from a deep-sea hydrothermal vent, and was found to be resistant to chloramphenicol, streptomycin, kanamycin, ampicillin, and rifampicin (Takai, 2005).

Many antibiotics are degraded at high temperatures. Traub and Leonhard (1995) investigated the heat-stability and heat-liability of 62 antimicrobial compounds by putting them under heat stress at 56°C for 30 min and 121°C for 15 min. After the heat stress had been applied the minimum inhibitory concentration (MIC) for each antibiotic was tested against *B. subtilis*. The β-lactams, erythromycin, doxycycline, tetracycline, nitrofurantoin, polymyxin B, rifampicin, and teicoplanin were partially heat-labile while azlocillin, aztreonam, mezlocillin, oxacillin, aminoglycosides, all quinolones, chloramphenicol, clindamycin, coumermycin, fosfomycin, josamycin, mupirocin, novobiocin, co-trimoxazole, trimethoprim, and vancomycin proved heat-stable.

6. Can the resistome be impacted by antibiotic resistant extremophilic bacteria?

Antibiotics have long been utilized by microorganisms engaged in microbial "warfare" and as a mechanism for signaling molecules (Clardy et al, 2009 and Yim et al, 2007). More recently human activity has utilized antibiotics to treat and prevent illness

caused by microorganisms. However, in recent years the expansion of antibiotic resistance in disease causing organisms has led to a global health crisis. Due to this crisis, vast amounts of research has been conducted to better understand the mechanisms of transmission of antibiotic resistance via horizontal gene transfer, as well as the selective pressures that cause shifts in increased antibiotic resistance. A major selective pressure being applied to bacteria is from the use of antibiotics in agriculture. Animal agriculture accounts for more than 13,000 metric tons of antibiotics in the United States alone (US FDA, 2014). Plant based agriculture accounts for 36 metric tons of antibiotics in the United States (McManus, 2014). Both animal and plant based agriculture use antibiotics as a means to prevent infections and to treat infections that have already occurred. However, in animal based agriculture, antibiotics are also used to increase growth. The use of antibiotics in agriculture has been linked to an increase in antibiotic resistant bacteria and in some cases these antibiotic resistant bacteria have caused infections in human hosts (Durso, 2014). MRSA infections have been linked to swine manure being used as a fertilizer (Durso, 2014). Furthermore, the waste lagoons of concentrated animal feed operations (CAFOs) have been linked to harboring a number of antibiotic resistance genes and these genes have been shown to leach into the surface water and groundwater tables due to the spread of animal manure as a fertilizer source and through the use of lagoons (Sanford et al., 2009). However, the link between plant based agriculture and infections in human hosts is less defined. Such usage of antibiotics should not be overlooked. It is evident that agriculture based usage of antibiotics has a serious impact on the antibiotic resistome however; the interplay between the environmental parameters, increased resistance, and antibiotic useage is not fully understood and is surprisingly complex. It is evident that these impacts need to be studied more extensively.

The antibiotic resistome is a highly dynamic and expansive collection of the antibiotic resistance genes within the environment. Since the discovery of antibiotics and their uses in modern medicine, animal agriculture, plant agriculture, and industry bacteria have developed or gained antibiotic resistance genes due to the selective pressures that are applied to natural systems. This is the case regardless of the origins of the antibiotics, be it medical, agricultural, or industrial (Allen et al., 2010). It should be noted that

selective pressures can also be from natural sources such as competing bacteria or fungi, although the concentrations of these natural antibiotics have not been well studied and the impact of these selective pressures remains unknown (Allen et al., 2010).

When considering the antibiotic resistome of extremeophilic bacteria it is important to consider the routes by which antibiotics can enter into these sites and the possible influences of antibiotic resistant extremophiles. Briefly, antibiotic resistant genes can be transferred through physical forces such as wind and watershed, interactions with animals in the environment, and human influences (Allen et al., 2010). Physical forces, such as watersheds, enable antibiotic resistance genes to be transferred from one site to another easily. In cases such as the Salton Sea, California the lake has several watersheds that feed the lake and large rivers that exit from the lake both of which can be influential in the transport of antibiotic resistance genes. Animals can also be major influence in the dissemination of antibiotic resistance genes; for example bacteria associated with wild birds are known to harbor a number of antibiotic resistance genes. Due to birds ability to fly, antibiotic resistance genes can be transferred long distances during migration periods, and passed into the environment by means such as fecal matter (Allen et al., 2010). Lastly human activity should be considered. It has been noted that the closer a site is to human activity the larger the number of antibiotic resistances are present and our activities such as disposal of wastewater allow for the transport of antibiotic resistances into the environment.

It is known that antibiotic resistance among extremophilic microorganisms is present. Bacteria such as *H. pylori*, an acidiophilic bacterium, *Methanohalophilus zhilinae*, a halophilic bacterium, *Lebetimonas acidiphilia*, a thermophilic bacterium, and *Anoxvabacillus pushchinensis*, an alkaliphilic bacterium, are just a few extremophilic bacteria that have been reported to be resistant to common antibiotics. Extreme environments, such as Soap Lake and the Salton Sea, are subject to environmental contamination from agricultural sources. It is of note that several isolates retrieved from Soap Lake have possessed resistance to a number of antibiotics. The link between agricultural usage of antibiotics and the number of resistances seen in Soap Lake is unclear because the amount of antibiotics that enter the lake is unknown and once in the

lake, many antibiotics are degraded in alkaline waters. However, it is clear that more research needs to be done in order to determine the link between antibiotic resistance, agriculture, extremophiles, and the resistome.

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II. HALOMONAS EURIALKALITOLERANIS SP. NOV., A NOVEL HALOTOLERANT, ALKALIPHILIC BACTERIUM ISOLATED FROM SOAP LAKE, WASHINGTON

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Abstract

Five strains of *Halomonas eurialkalitoleranis* were specifically isolated and selected for the ability to tolerate a pH range from pH 7 to 11 to determine if antibiotic sensitivity was altered by the pH of the media. The bacteria were isolated from aerobic sediment samples, retrieved from Soap Lake, Washington. The strains designated Isolate 6, Isolate 7, Isolate 8, Isolate 9, and Isolate 10 are all gram negative rods 1 µm to 2 µm long and 0.5 µm wide. Electron acceptors include oxygen and nitrate. The organisms can grow on NaCl concentrations ranging from no added NaCl to 250 g/L, with an optimum growth occurring at 50 g/L for Isolate 6, 100 g/L for Isolate 7 and Isolate 10, 75 g/L for Isolate 9, and 120 g/L for Isolate 8. Growth occurred at pH 7 to pH 11 for all isolates, with optimal pH occurring at pH 8 for Isolate 6 and pH 9 for Isolate 7, Isolate 8, Isolate 9, and Isolate 10. The temperature range of growth occurs from 4°C to 40°C, with optima temperature of 30°C for all isolates. Phylogenetic studies indicate that all isolates belong to the genus *Halomonas* and represent new members of this genus. We propose the name *Halomonas eurialkalitoleranis* for these species and Isolate 7 as the type strain.

Introduction

The genus of *Halomonas* belongs to the family of *Halomonadacea* and the type strain of the *Halomonas* genus is *Halomonas elongate*, first described by Vereland et al (1980). At the time of this writing, more than 80 species belonging to genus *Halomonas* have been characterized (Amouric et al, 2013; Jiang et al, 2014). The member of the genus *Halmonas* are halophilic or halotolerant, many of them are considered extremophiles on the basis of their salt tolerances (Arahal et al, 2006; Ventosa et al, 1998). Because these genera represent a number of extremely halophilic species, they

have largely been isolated from extremely halophilic environments such as salt water brines, saline lakes, saline soils, marine environments and solar salt facilities (Kim et al, 2010). Furthermore, a number of the member of the *Halomonas* genus are capable of tolerating a wide range of pH values including *H. campisalis* (pH range 6-12), *H. alkaliantarctica* (pH range 7.4-9.6), and *H. olivaria* (pH range 5-11) to name a few (Mormile et al, 1999; Poli et al, 2007; Amouri et al, 2013).

The species within the *Halomonas* are quite heterogeneous in regards to their metabolic properties and their physiological nature. In addition, members of this genus usually have G+C DNA contents that range from 51.4 to 74.3 mol% (Kim et al, 2010 and Amouric et al, 2013). Due to differences in the 16S and 23S rRNA, the *Halmonas* genus has been split into two distinct groups; group 1 (*Halomonas sensu stricto*) and group 2 (Arahal et al., 2002; Haba et al., 2010). However, despite these two groups being described on the bases of the 16S rRNA and 23S rRNA gene sequences, there have been no clear phenotypic or chemotaxonomic features seen to support this division (Haba et al., 2012).

Soap Lake was chosen for this study because it is a highly productive, meromictic, soda lake in central Washington State (Anderson, 1958). The alkaline, saline, meromictic waters are known to harbor a number of unique organisms, including *Halomonas campisalis*, and *Nitroncola lacisaponensis* (Mormile et al., 1999; Dimitriu et al, 2005). Interestingly, the bacteria isolated from this lake carry a number of antibiotic resistances to a wide variety of antibiotics including erythromycin, bacitracin, kanamycin, novobiocin, polymyxin B, neomycin, gentamicin, streptomycin, carbenicillin, rifampicin and tetracycline (Dimitriu et al., 2005; Mormile et al., 1999). It is likely that the range of antibacterial resistance exhibited by these bacteria is due to the impact of high alkalinity on the antibiotics and not necessarily due to the presence of antibiotic resistance genes.

This study aimed to isolate and characterize new bacterial species that were capable of tolerating a wide range of pH values so antibiotic resistances of these species could be determined across a wide range of pH values from 7 to 11. Five strains of a new bacterial species belonging to the genus *Halomonas* are described on the basis of phylogenetic and phenotypic studies. The five bacterial isolates capable of growth in a

pH range of 7 to 11 were isolated from sediment samples taken from Soap Lake, Washington. On the basis of phylogenetic similarity, G+C content of the DNA, and DNA-DNA hybridization studies *Halomonas eurialkalitoleranis* is presented as a new species and Isolate 7 is presented as the type strain.

Materials and methods

Isolate Origin.

The strains in this study were isolated from sediment samples retrieved from Soap Lake, Washington, a unique meromictic, haloalkaline, soda lake. The pH of the lake remains at a nearly constant 9.8 and the anaerobic sediments of the lake contain upwards of 140 g/L NaCl (Mormile et al, 1999).The sample site was chosen for three reasons; 1) the lake is subject to agricultural runoff from surrounding orchards 2) a number of bacteria previously isolated from Soap Lake have demonstrated antibiotic resistances and 3) the lake is an extreme soda lake with high alkalinity and salinity.

Soap Lake is the terminal lake in a series of lakes with increasingly saline and alkaline conditions located in the arid Grant County of Washington State (Anderson, 1958). In 2002, the United States Department of Agriculture (USDA) reported that a total of 50,260 acres were utilized for fruit crop production in orchards (USDA, 2002). The usage of antibiotics in agriculture has been linked to causing an increasing number of antibiotic resistant bacteria to emerge. However, this increase in antibiotic resistance has not been studied in regard to extremophilic bacteria. The usage of land for agricultural purposes in Grant County, combined with the extreme conditions of this lake made this site the ideal location to determine the antibiotic resistances of halotolerant, alkaliphilic bacteria across a range of pH values.

Isolation of Bacteria from Sediment Samples.

Medium for enrichment of bacteria contained 8 g/L Difco (Becton Dickinson, New Jersey) nutrient broth (NB) and 120 g/L NaCl. All media was adjusted to a final pH of 6, 7, 8, 9, or 10 by using 1M NaOH and 1M HCl. All media was supplemented with a final concentration of 1% total volume glucose as a carbon source. Sediment samples were pasteurized for 90 seconds in a boiling water bath to select for *Bacillus*-like

bacteria. Cultures were incubated at 30°C until turbid. Cultures were then transferred to solid medium adjusted to a pH of 6, 7, 8, 9, or 10, and supplemented with 1% (w/v) glucose. Plates were incubated and individual colonies were selected and streaked for isolation on fresh media. Bacteria underwent several rounds of isolation until pure cultures were obtained. After pure cultures were obtained, bacteria were transferred to NB and subcultured as required. All cultures were subsequently maintained on NB amended with 75 g/L NaCl and supplemented with 1% (w/v) glucose. Unless otherwise stated all tested were performed with nutrient broth at pH 9.0 and 75 g/L NaCl. The growth of the strains was determined by measuring the optical density of the cultures at 600 nm.

Morphological and physiological characteristics.

The gram stain reaction and endospore stains were performed by using the methods described by Murray et. al., 1994. Cellular morphology was observed by using a Leica DML phase contrast microscope with cells from a culture in the exponential growth phase after applying a simple stain using crystal violet. Motility was determined examining cells for any movement, and inoculation into used semisolid NB agar tubes, adjusted to pH 9 and 7.5% NaCl with a 1% glucose final concentration. Colony morphology and pigmentation were determined by viewing growth on solid medium. The production of exopolysaccharides (EPS) was determined by using the method described by Mata et al. (2002).

The salinity range and optimum was determined with NB media adjusted to pH 9 and NaCl concentrations ranged from no added NaCl to 350 g/L. pH range and optimum were determined by using NB media adjusted to 7.5% NaCl and tested over a range of pH 5 – 12 using 1M NaOH and 1M HCl to adjust the pH. All media was buffered according to the recommendations of Breznak and Costilow, 1994. The temperature range (4 - 50˚C) and optimal was determined using NB amended to 75 g/L NaCl and pH 9.

Nutritional Tests.

The ability of the strains to grow aerobically was determined by using a NB agar tube adjusted to pH 9 and 7.5% NaCl. The tubes were inoculated heavily with an inoculating needle and incubated for 7 days. The tubes were examined for growth throughout the depth of the tube. Anaerobic growth was also examined during the glucose fermentation tests in which one tube was overlaid with sterile mineral oil and the other was aerobic. The ability of the strains to utilize a variety of substrates were tested by preparing a media that was recommended by Arahal et al. (2007), and contained the following in grams per liter; NaCl, 75; KCl, 2; $MgSO_4$, 0.2; KNO_3, 1, $(NH_4)_2HPO_4$, 1; and KH_2PO_4, 0.5; and the media was adjusted to pH 9 by using 1M NaOH. The following substrates were tested; carbohydrates (aesculin, L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, D-melezitose, D-raffinose, L-rhamnose, ribose, D-salicin, starch, trehalose, D-xylose), organic acids (acetate, citrate, formate, fumerate, gluconate, malonate, propionate, succinate), alcohols (adinitol, ethanol, glycerol, myo-inistol, D-mannitol, sorbitiol), and amino acids (L-alanine, L-cysteine, L-histidine, L-isoleucine, L-lysine, L-methionine, L-serine, L-valine). All substrates were filter sterilized and a final volume of 1 g/L was used for all substrates except for the carbohydrates, where 2 g/L was added as was described by Arahal et al. (2007). The ability of the isolates to produce acid from a range of carbon sources was determined by using the media described by Arahal et al., (2007) and thymol blue was chosen for a pH indicator. Only carbon sources that the bacteria were able to utilize were tested for acid production. The effects of specific ions on the growth of the isolates was determined by using the salt nitrate medium (SNM) (Mormile et al., 1999) amended to contain the following in g per liter; KH_2PO_4, 0.5; NH_4Cl, 1.0; yeast extract 0.25; $NaNO_3$, 3; NaCl, 75; and adjusted to pH 9 using 1M NaOH. In place of the NaCl the medium was tested using other salts, LiCl, $MgCl_2$, KCl, $CaCl_2$, $NaSO_4$, NaBr, $NaNO_3$, and $NaC_2H_3O_2$ with 75g/L. To determine specific ionic requirements all sodium was removed from the base media and substituted with either K, Ca, or Mg. To determine if chloride was required for growth all chloride was removed from the medium and replaced with either SO_4, $C_2H_3O_2$, NO_3, or Br.

Biochemical Tests.

To determine the presence of oxidase and catalase activities, the method described by Smibert and Krieg (1994) was followed. The following tests were performed by using select test media (Difco); indole, phenylalanine deaminase, lysine and ornithine decarboxylases, urease activity, Voges-Proskauer test, methyl red test, growth on MacConkey agar, and casein hydrolysis. The methods described by Smibert and Krieg (1994) were used when performing the following tests; hydrolysis or gelatin, hydrolysis of starch, H_2S production from L-cysteine, oxidation/fermentation of D-glucose, and reduction of nitrate to nitrite. Gas production from nitrate reduction was tested on nitrate broth (Difco) by using a Durham tube to capture gas bubbles. Production of poly-β-hydroxyalkanoate (PHA) was determined by using the methods described by McCool and Cannon (1998). Hydrolysis of Tween 20 and Tween 80 was done using the methods described by Mata et al. (2002). Hydrolysis of DNA was determined by using the methods described by Smibert and Krieg (1994). The o-Nitrophenyl-B-D-galactopyranosidase (ONPG) activity was determined by using the methods described by Phillips (1994). Respiration on nitrate, nitrite was determined by using the methods described by Mata et al (2002).

Sensitivity to Antimicrobial Agents.

The sensitivity to antimicrobial agents was determined with the Kirby-Bauer Disc Diffusion method. The isolates were spread on NB plates, as described above, and antibiotic impregnated paper Seni Discs were placed on top of the agar. Zones of inhibition were measured to determine the sensitivity of the bacterial isolates to the following antibiotics; amoxicillin (20 µg), ampicillin (10 µg), bacitracin (10 units), carbenicillin (100 µg), cefoxitin (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), kanamycin (30 µg), nalidixic acid (30 µg), polymyxin (300 IU/IE/UI), rifampicin (5 µg), streptomycin (10 µg), sulfamethoxazole (1.25 µg), tetracycline (30 µg), and tobramycin (10 µg).

Scanning Electron Microscopy.

Actively growing broth cultures were used and the bacteria were heat fixed to a microscope cover slip. Samples were fixed by using a 3% glutaraldehyde for 1 hour. Cells were dehydrated using a series of increasing concentrations of ethanol for 15 minute periods, starting with 30% and working up to 100% in 10% increments. The 100% dehydration step was repeated. Samples were dried by using an HMDS and ethanol mixture in a series with a 1:2 ration of HMDS to ethanol, a 2:1 HMDS to ethanol ratio, and pure HMDS for 20 minutes at each step. Samples were mounted onto pin stubs with carbon paint and the grounded with copper tape. Samples were coated Au/Pd using the Hummer VI sputter coater. An automatic cycle of 1 minute was used. Samples were imaged using the coated, mounted samples in the S-4700 scanning electron microscope.

Nucleic Acid Characterization.

The extraction of genomic DNA was performed using the GeneJET Genomic DNA Purification kit from ThermoScientific. The 16S rRNA sequence was determined by PCR amplifying the 16S region using universal primers 27F (AGA GTT TGA TCM TGG CTC AG) and 1492R (CGG TTA CCT TGT TAC GAC TT) as described by Lane (1991). The PCR products were run on 0.8% agarose gel to determine the size of the product and the PCR products were purified by using the Nucleospin Gel and PCR Clean Up kit (Macherey-Nagel, Bethlehem, PA). Purified PCR products were inserted into T-easy Vector systems (Promega, Madison, WI). Plasmids were isolated using the Plasmid Mini Kit (Qiagen, Valencia, CA). Samples were prepared for sequencing with three reactions for each sample. The reactions contained 25 pmol of one primer t7 (TAA TAC GAC TCA CTA TAG GG), sp6 (ATT TAG GTG ACA CTA TAG), or 1492R (CGG TTA CCT TGT TAC GAC TT), at least 200 ng of DNA, and were filled to a final volume of 16uL. Sequencing was performed at the DNA core Facility at the University of Missouri, Columbia. Raw sequence data was edited by using FinchTV (http://finchtv.software.informer.com/1.4/) (Geospica INC. 2013). Sequences were assembled with the CAP3 Sequence Assembler Program (http://doua.prabi.fr/software/cap3) (Haung et al., 2013). The most closely related species were determined by using EZTaxon (Altschul et al., 1994). The obtained sequences were

aligned with the type strain of the most closely related species using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/) (Edgar, 2004). Aligned sequences were edited with JalView software (http://www.jalview.org/) (Waterhouse et al., 2009). A phylogenetic tree was assembled using the MEGA software (http://www.megasoftware.net/) (Tamura et al., 2013; Goujon et al., 2010; Li et al., 2015; McWilliam et al., 2013).

Results

Isolation.

Five isolates were obtained that were capable of growth over a pH range of 7 to 11. Due to their abilities to grow over the desired pH range these organisms were selected for further testing of the antibiotic susceptibilities.

Cellular Properties.

The colonies formed by Isolates 6, 7, 8, 9, and 10 are cream colored, circular, and have smooth edges. Gram negative, non-motile rods were observed. Cells were typically 1 to 2 µM long and 0.5 µM wide (Figure 1a to 1e). Cells did not form endospores. Scanning electron microphotographs did not reveal the presence of inclusion bodies. Cells grown in liquid medium developed a white to cream colored exopolyshacaride layer that caused the cells to clump heavily in culture.

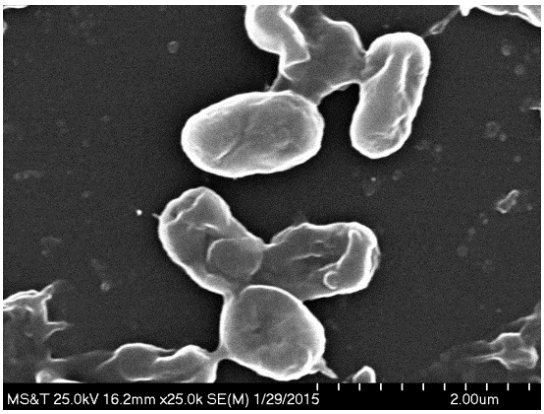

Fig. 1a. Isolate 6

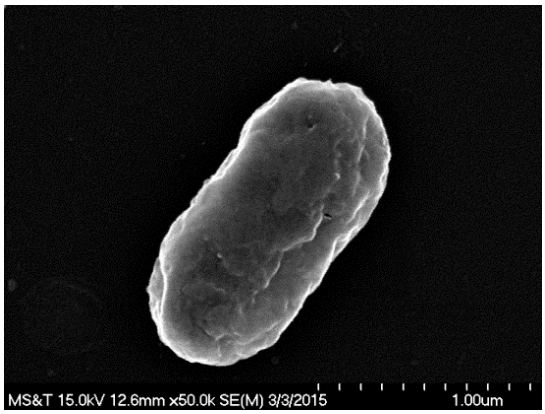

Fig. 1b. Isolate 7

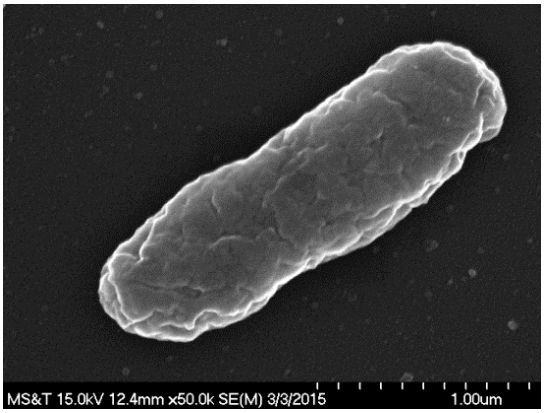

Fig. 1c. Isolate 8

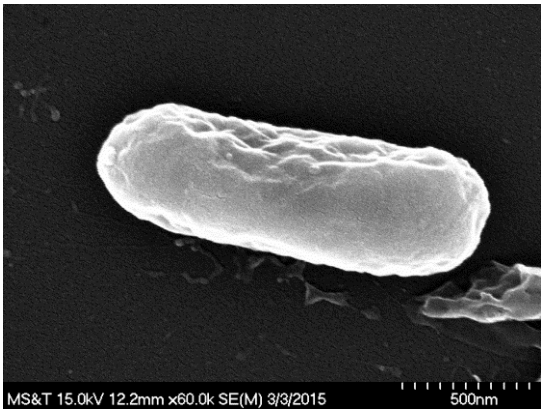

Fig. 1d. Isolate 9

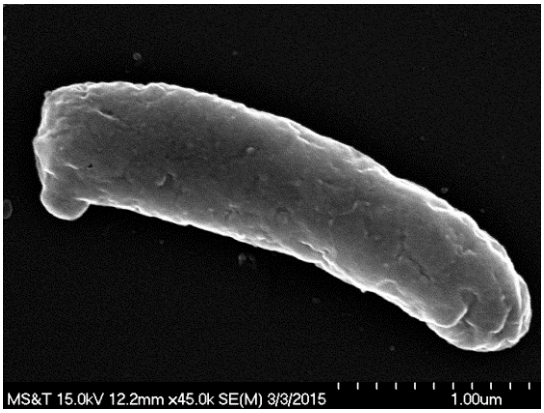

Fig. 1e. Isolate 10

Fig. 1a-e. Scanning electron micrographs of *Halomonas eurialkalitoleranis* Isolates 6-10

Growth and Metabolic Properties.

The optimum temperature for the growth of Isolates 6, 7, 8, 9, and 10 was 30°C with growth occurring at temperatures between 4°C to 40°C (Figure 2). The organisms can grow on NaCl concentrations ranging from no additional NaCl added to the NB to 250 g/L, with an optimum growth occurring at 50 g/L for Isolate 6, 100 g/L for Isolate 7 and Isolate 10, 75 g/L for Isolate 9, and 120 g/L for Isolate 8 (Figure 3). Growth occurred at pH 7 to pH 11 for all isolates, with optimal pH occurring at pH 8 for Isolate 6 and pH 9 for Isolate 7, Isolate 8, Isolate 9, and Isolate 10 (Figure 4). Isolates were able to grow in medium with minimal Na^+, however, when Na^+ was removed from the SNM media no growth was seen. These results indicated that Na^+ is required for growth. In NB and SNM

media supplemented with $CaCl_2$ at 75 g/L, no growth occurred indicating that high concentrations of Ca^{+2} are inhibitory to the isolates. Of the salts tested Isolates 6, 7, 8, 9, and 10 were able to grow in NB media supplemented with 7.5% $NaC_2H_3O_2$, $NaSO_4$, $MgCl_2$, NaBr, $NaNO_3$, KCl, and nutrient broth media without added salts. In the SNM media without Na^+ no growth was seen.

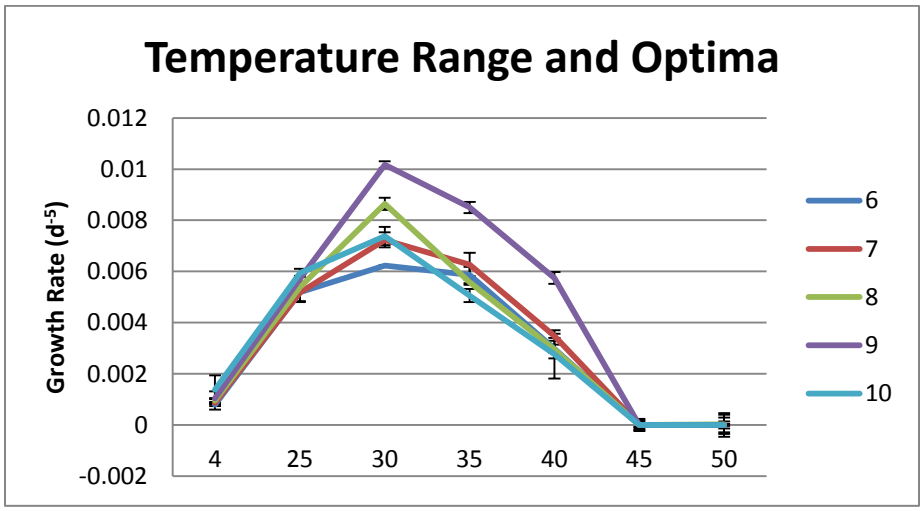

Fig. 2. Temperature Range and Optimization for *Halomonas eurialkalitoleranis* Strains 6-10

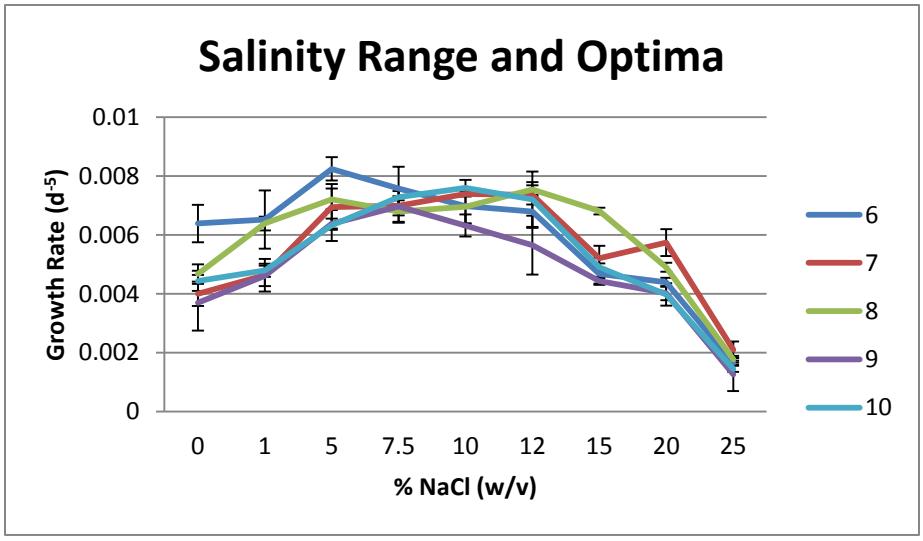

Fig. 3. Salinity Range and Optimization for *Halomonas eurialkalitoleranis* Isolate 6-10

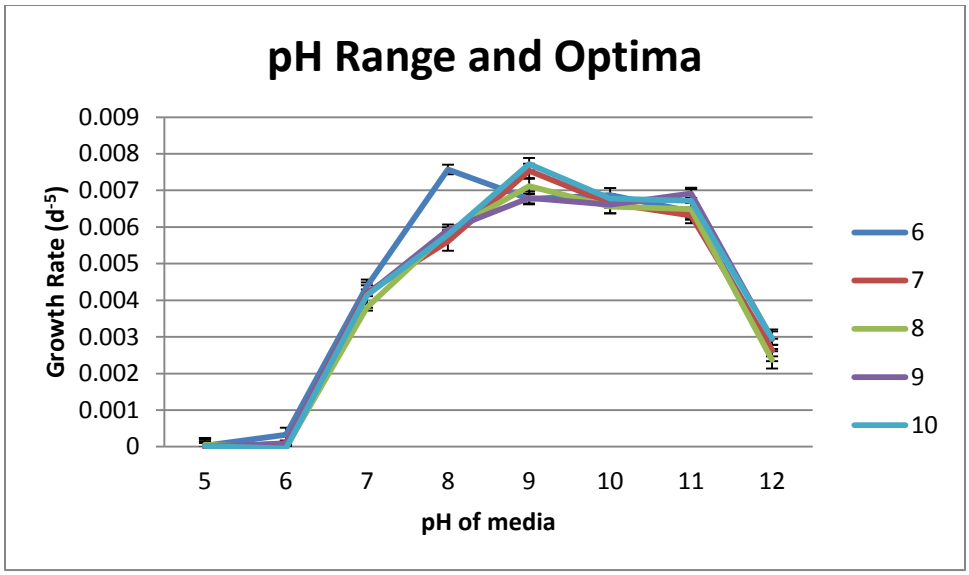

Fig. 4. pH Range and Optimization for *Halomonas eurialkalitoleranis* Isolate 6-10

Isolates were able to grow on alanine, cellobiose, ethanol, fructose, gluconate, glucose, glycerol, histidine, inositol, isoleucine, lactose, lysine, serine, sorbitol, starch, succinate, and starch. Isolate 8 was also able to grow on acetate, citrate, and melezitose. The ability of the isolates to produce acid was tested for a variety of carbon sources. The isolates were able to produce acid from alanine, cellobiose, ethanol, fructose, gluconate, glucose, glycerol, histidine, inositol, lysine, serine, sorbitol, and starch. In some cases, the amount of acid production was minimal, although the effect was enough to be detected via a color change in thymol blue containing media.

Physiological characteristics.

Isolates 6, 7, 8, 9, and 10 are positive for nitrate reduction, nitrite reduction, casein hydrolysis, oxidase activity, catalase activity, DNA hydrolysis, citrate utilization, and were able to grow on MacConkey agar. Isolate 6, 7, 8, 9, and 10 were weakly positive for H_2S production, lysine decarboxylase, and gelatinase activity. Isolates 6, 7, 8, 9, and 10 were negative for phenylalanine deaminase, starch hydrolysis, PHA inclusion bodies, urease activity, methyl red, and Voges-Proskauer tests. They were also unable to grow on triple sugar iron agar and were negative for facultative anaerobic growth. Isolates 6, 7, 8, 8, and 10 did not show any ONPG activity.

Antibiotic Sensitivity.

All strains were resistant to amoxicillin, ampicillin, bactracian, erythromycin, kanamycin, rifampicin, streptomycin, tetracycline, and tobramycin. The results (Table 1) showed that all strains appeared to be inhibited by carbenicillin and polymyxin. All other antibiotics had susceptibilities that varied between the bacterial strains. Isolate 6 was resistant to cefoxitin and sulfamethoxazole while Isolates 7, 8, 9, and 10 were inhibited by these antibiotics. Only Isolate 7 was inhibited by chloramphenicol. Isolates 8, 9, and 10 were inhibited by nalidixic acid while Isolates 6 and 7 were resistant.

Table 1. Antibiotic Resistances of *Halomonas eurialkalitoleranis* strains 6 to 10

Antibiotic	**6**	**7**	**8**	**9**	**10**
Amoxicillin	R	R	R	R	R
Ampicillin	R	R	R	R	R
Bacitracin	R	R	R	R	R
Carbenicillin	S	S	S	S	S
Cefoxitin	R	S	S	S	S
Chloramphenicol	R	S	R	R	R
Erythromycin	R	R	R	R	R
Kanamycin	R	R	R	R	R
Nalidixic Acid	R	R	S	S	S
Polymyxin	S	S	S	S	S
Rifampicin	R	R	R	R	R
Streptomycin	R	R	R	R	R
Sulfamethoxazole	R	S	S	S	S
Tetracycline	R	R	R	R	R
Tobramycin	R	R	R	R	R

Comparison of the phylogenetic and phenotypic data.

The phylogenetic analysis of Isolates 6, 7, 8, 9, and 10 indicated they are members of the *Proteobacteria* and belong to the genus *Halomonas*. The phylogenetic data are in agreement with the phenotypic features of the organisms found in this genus. Figure 5 shows the phylogenetic tree of the five isolates with the most closely related organisms. According to Vreeland (1991) all *Halomonas* demonstrate including gram negative reactions, rod shaped cells, yellow to white colony color, oxidase and catalase positive, and the utilization of a broad range of carbon sources. The phenotypic characteristics of the most closely related organisms are given in Table 2.

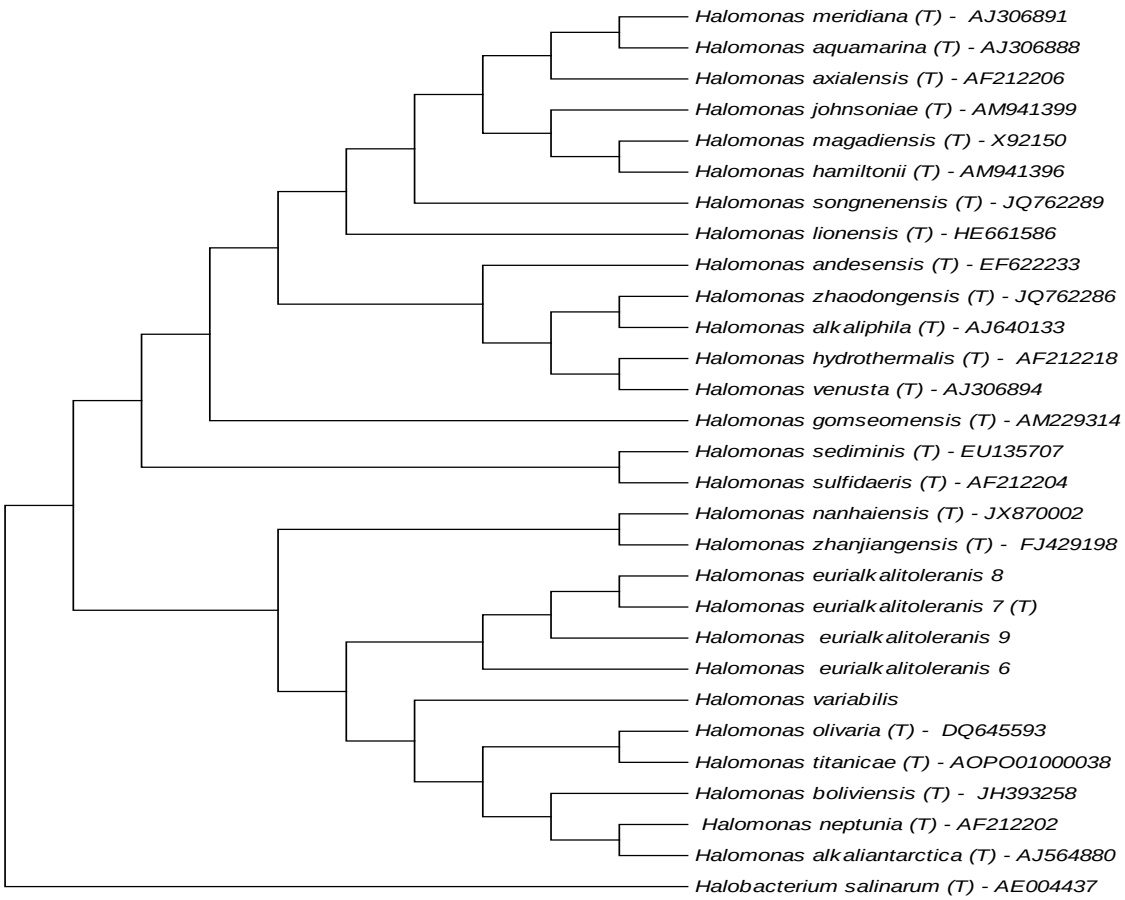

Fig. 5. Phylogenetic Tree of *Halomonas eurialkalitoleranis*

The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (2 categories (+*G*, parameter = 0.4529)). The analysis involved 29 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1243 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Table 2. Phenotypic Comparisons

Experiment	6	7	8	9	10	H. alkaliantarctica	H. neptunia	H. boliviensis	H. olivaria	H. variabilis
Growth Characteristics										
Salt (%)	0.5 to 25	0.5 to 25	0.5 to 25	0.5 to 25	0.5 to 25	2.2 to 22.2	0.5 to 27	0 to 25	0 to 25	8.73 to 29.25
Optimum salt (%)	5	10	12	7.5	10	10	2 to 3	5	7	11.7
pH range	7 to 11	7 to 11	7 to 11	7 to 11	7 to 11	7.4 to 9.6	5 to 12	6 to 11	5 to 11	6.5–8.4
Optimum pH	8	9	9	9	9	9	7–8	7.5–8.0	7	7.5
Temperature (°C)	4 to 40	4 to 40	4 to 40	4 to 40	4 to 40	10 to 37	-1 to 35	0 to 45	4 to 50	15 to 37
Optimum temperature (°C)	30	30	30	30	30	30	30	25 to 30	35	33
Enzymology										
Nitrate reduction	+	+	+	+	+	+	+	+	+	-
Nitrite reduction	-	-	-	-	-	-	-	ND	-	+
Casein hydrolysis	+	+	+	+	+	-	-	ND	-	+
Gelatinase	W+	W+	W+	W+	W+	-	-	-	-	-
Indole production	-	-	-	-	-	+	-	-	ND	-
Oxidase	+	+	+	+	+	+	+	ND	+	+
Phenylalanine deaminase	-	-	-	-	-	-	-	ND	-	-
Starch hydrolysis	W+	W+	W+	W+	W+	-	-	ND	-	-
PHB production	-	-	-	-	-	-	ND	ND	+	+
Carbon Source Utilization										
Glycerol	+	+	+	+	+	+	+	-	+	+
Glucose	+	+	+	+	+	+	+	+	+	+
Trehalose	-	-	-	-	-	+	+	+	+	+
d-arabinose	-	-	-	-	-	-	+	-	ND	+
Lactose	+	+	+	+	+	+	+	-	ND	-
Cellobiose	+	+	+	+	+	+	+	-	+	-
Xylose	-	-	-	-	-	-	+	+	+	-
Maltose	-	-	-	-	-	+	+	+	ND	+
Fructose	+	+	+	+	+	+	+	+	+	+
Sucrose	-	-	-	-	-	+	+	+	+	-

Several member of the *Halomonas* genus are capable tolerating a wide range of pH values including *H. alkaliantarctica* (pH 7.4-9.6), *H. neptuni* (pH 5-12), *H. boliviensis* (pH 6-11), *H. andesensis* (pH 6-11), *H. olivaria* (pH 5-11), and *H. variabilis* (pH 6.5-8.4) to name just a few. The pH ranges and optimums of these representative species are shown in table 2. However, on the basis of phenotypic and genotypic results, *Halomonas eurialkalitoleranis* is considered a distinct species. Phylogenetically, it is closely related to *H. alkaliantarctica*, *H. neptuni*, *H. boliviensis*, *H. olivaria*, and *H. variabilis.*

Description of Halomonas eurialkalitoleranis sp. Nov.:

Halomonas eurialkalitoleranis Isolates 6, 7, 8, 9, and 10 are 1 to 2 µm long by 0.5 µm wide. They are gram negative and unable to form spores. They are able to use oxygen and nitrate as final electron acceptors. They are oxidase and catalase positive. The optimal growth conditions are; 30°C for all Isolates (range of 4 to 40°C), pH 9.0 for Isolates 7, 8, 9, and 10 (pH range of 7 to 11), pH 8.0 for Isolate 6 (pH range of 7 to 11). All isolates were able to grow in nutrient broth with no added NaCl to 250 g/L NaCl with optimal concentrations at 50 g/L NaCl for isolate 6, 100 g/L NaCl for isolate 7 and 10, 120 g/L NaCl for isolate 8, and 75 g/L for isolate 9. All isolates were able to utilize alanine, cellobiose, ethanol, fructose, gluconate, glucose, glycerol, histidine, inositol, isoleucine, lactose, lysine, serine, sorbitol, starch, and succinate as carbon sources. Isolate 8 was also able to grow on acetate, citrate, and melazitose as carbon sources. Acid production occurred on alanine, cellubiose, ethanol, fructose, gluconate, glucose, glycerol, histidine, inistol, lysine, serine, sorbitol, and starch.

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III. pH DEPENDENT ANTIBIOTIC RESISTANCE OF AN ALKALIPHILIC HALOTOLERANT BACTERIUM ISOLATED FROM SOAP LAKE, WASHINGTON

To be submitted to the journal *Extremophiles*.

Abstract.

Soap Lake is a meromictic, soda lake and several bacterial isolates from this lake possess resistance to multiple antibiotics. It is possible that the antibiotic resistance identified in these organisms is due to selective pressure from antibiotics entering the lake. However, a more likely explanation for the wide range of antibacterial resistance exhibited by these strains is due to the impact of high alkalinity on the antibiotics and not necessarily due to the presence of antibiotic resistance genes in the microbial community. The aim of our study was to determine the effectiveness of antibiotics against bacteria that are capable of growth over a wide range of pH values and investigate the influence of alkalinity on antibiotic activity. Select antibiotics were tested to determine the minimum inhibitory concentration of each antibiotic across the range of pH values, from 7 to 11. The minimum inhibitory concentration of several antibiotics, including tetracycline, was found to vary across the different pH values. As expected several antibiotics were found to become ineffective against *Halomonas eurialkalitolerans* (Isolate 9) at pH values above 8 including tetracycline, ampicillin, kanamycin, neomycin, roxithromycin, and streptomycin. Vancomycin was ineffective at all pH values tested due to its known degradation under alkaline conditions. Surprisingly, erythromycin and sulfamethizole were more effective against *Halomonas eurialkalitolerans* Isolate 9 at pH 11 than in neutral media. The change in the observable MIC under alkaline conditions seems to indicate that the antibiotic resistance seen in Soap Lake bacteria is the effect of alkalinity on the tested antibiotics and not necessarily the activity of antibiotic resistance gene products.

Keywords: Antibiotic resistance, Soap Lake, Extremophiles, Minimum Inhibitory Concentration (MIC)

Introduction

Soap Lake is the terminal lake in a chain of lakes that lie in a basaltic canyon in Washington State. The pH of Soap Lake is maintained at a pH of 9.9 due to the high concentrations of $NaHCO_3$ and Na_2CO_3 in the water (Anderson, 1958). It is hypersaline, containing nearly 12% dissolved salts (Peyton and Yonge, 2002). Due to its salinity gradient and basin shape, Soap Lake is meromictic. The mixolimnion is aerobic and it extends to a depth of 15 m. The anaerobic monimolimnion lies between 20 and 28 m in depth and possesses the highest natural concentrations of sulfide currently known to exist in any lake at approximately 200 mM (Sorkin et al, 2007). The chemocline lies between 15 and 20 meters in depth. The mixolimnion and the monimolimnion waters have not mixed, despite the spring and autumn circulations of the mixolimnion, in over 2000 years (Peyton and Yonge, 2002).

Grant County, where Soap Lake is located, has 50,260 total acres devoted to orchards (USDA, 2002). In agriculture such as this, antibiotics including streptomycin, oxytetracycline, gentamicin, and oxolinic acid are often used. However, antibiotic use in plant-based agriculture is expensive and limited to high value crops such as fruits and vegetable crops or ornamental plants (McManus, 2014). Most often fruit tree crops are protected by using fine mist sprays of streptomycin and oxytetracycline against numerous plant pathogens including *Pectobacterium spp., Agrobacterium tumefaciens, Peronospora tabacina, and Erwina amylovora.* Soap Lake is subject to agricultural runoff that likely introduces antibiotics into this environment thus creating a selective pressure for antibiotic resistance.

A number of isolates from Soap Lake possess antibiotic resistances to a number of different antimicrobial agents from varying classes of antibiotics. One such example, *Nitrincola lacisaponensis*, is reported to be resistant to several antibiotics including erythromycin, bacitracin, novobiocin, polymyxin B, neomycin, gentamicin, streptomycin, carbenicillin, rifampicin and tetracycline, and susceptible to nalidixic acid, chloramphenicol, ampicillin and penicillin (Dimitriu et al., 2005). One explanation for the wide range of antibacterial resistance exhibited by these strains is due to the impact of

high alkalinity on the antibiotics themselves and not necessarily due to the presence of antibiotic resistance-conferring genes.

Antibiotics are known to be susceptible to environmental conditions such as temperature, light exposure, and pH (Falagas et al., 1997). A study done in 1997 by Falagas et al. (1997) effectively demonstrated that acidic pH can negatively impact the effectiveness of several antibiotics. The impact of the acidity on the antibiotics was not discussed, however the impacts of the shift in antibiotic susceptibility was considered. Falagas et al. (1997) presented three possible explanations; the permeability of the bacterial membranes are impacted by the pH, the bacterial enzyme stability and kinetics are influenced by the pH, and lastly, the stability and kinetics of the antibiotics themselves are influenced by the pH. To our knowledge, no work has been done to test the effect of alkaline conditions on the effectiveness of antibiotics.

The aim of this study is to determine the effectiveness of antibiotics on bacterial cultures grown over a wide range pH values from neutral to highly alkaline. Determining the range of antibiotic activity through this range of pH values will help to better understand the impact that alkalinity has on this phenotypic characteristic of alkalotolerent and alkaliphilic organisms. Little is known about the effects of alkaline conditions on antibiotics. The optimal pH range for the antibiotics used to determine the minimum inhibitory concentration (MIC) experiment for Isolate 9 is given in Table 1. In most cases, alkaline media decreases the effectiveness of the antibiotic; however, a few antibiotics are known to be stable in alkaline media (pH 9). The work presented here demonstrates that the pH of the medium greatly impacts most antibiotics tested. Vancomycin had no effect on the bacteria tested at any pH value. Erythromycin and sulfamethizole appeared to have an increased effectiveness in more alkaline media. All other antibiotics that were tested had a decrease in effectiveness as the pH of the media was increased. The results of this research indicate that antibiotic susceptibility or resistance is most likely due to the effect of alkalinity on the antibiotic and not due to the presence of antibiotic resistance genes.

Table 1 Known Antibiotic Effectiveness Ranges

Antibiotic	Class of Antibiotic	Effective Range	References
Kanamycin	Aminoglycoside	pH 2 to 11	Finegold, 1959
Neomycin	Aminoglycoside	pH 2 to 9	Simone, 1995
Streptomycin	Aminoglycoside	pH 3 to 7	Regena, 1946
Chloramphenicol	Amphenicol	pH 2-7	James and Leach, 1970
Vancomycin	Glycopeptide	pH 3 to 5	Mathew, 1995
Erythromycin	Macrolide	pH 7 to 8 (optimal) stable in neutral to alkaline conditions	Sultana, 2013; Brisaert, 1996
Roxithromycin	Macrolide	pH 3 -7.5	Wahba, 2012
Ampicillin	Penicillin	Not stable above pH 7	Florey, 1972
Sulfamethoxazole	Sulfonamide	No known effective range	
Sulfamethizole	Sulfonamide	No known effective range	
Demeclocycline	Tetracycline	No Known effective range	
Tetracycline	Tetracycline	pH 2 to 7.5	Loftin, 2004

Materials and methods

Enrichments and isolation

Sediment samples were aseptically retrieved from Soap Lake, Washington, and shipped on ice to Missouri S&T. To enrich for *Bacillus*-type bacteria, the sample was pasteurized by placing it into a boiling water bath for 90 seconds. Suspensions of treated sediment were added to sterile nutrient broth media (NB) (Fisher Scientific, Waltham, MA) amended with 120 g/L NaCl and poised at a pH of 9.0 by using 1N NaOH. All media was supplemented with glucose at a final concentration of 1% (w/v) glucose. Solid media was made with the addition of 15 g/L agar powder. After subculturing for further enrichment, cultures were plated onto NB plates poised at pH values from 6 to 10, and incubated for 72 hours until distinct colonies were formed.

Isolated colonies were streaked out on fresh solid medium until pure cultures were obtained. After multiple rounds of isolation, five pure cultures were obtained.

pH Tolerance and Optimum of Cultures

The pH tolerance of each of the five strains of *Halomonas eurialkalitolerans* Isolates 6-10 was tested by using liquid nutrient broth poised at pH values of 5 to 11. All nutrient broth was buffered with an appropriate buffering system as described by Breznak and Costilow (1994). A citrate phosphate buffer system was used for pH 5. A phosphate buffer system was used for pH 6, 7, and 8. A tris-hydrochloride buffer system was used for pH 9 and 10. A borate buffer system was used for pH 11. The ability of each strain to grow across the pH range 5-11 was tested by measuring the increase in absorbance 600 nm.

Antibiotic Disc Diffusion Assay

Kirby Bower disc diffusion assays were performed to determine if the strains possessed resistances against select antibiotics. The following antibiotics were tested: amoxicillin, ampicillin, bacitracin, carbenicillin, cefoxitin, chloramphenicol, erythromycin, kanamycin, nalidixic acid, polymyxin, rifampicin, streptomycin, sulfatrimethoxazole, tetracycline, and tobramycin. Disc diffusion assays were conducted by using nutrient broth plates that were amended with 7.5% (w/v) NaCl concentration and a pH at 9.0. The medium was supplemented with glucose at 1% final concentration (w/v). The zone of inhibition was read for each disc in millimeters from the edge of the disc to the edge of the no growth zone. Replicates were run in triplicate. The recommendations for the BD-BBL-Sensi Discs were followed to determine resistance or susceptibility of the strains to the antibiotics.

Minimum Inhibitory Concentration (MIC) Test Assay

Isolate 9 was selected for further testing. The MIC of Isolate 9 was determined for twelve antibiotics: tetracycline, ampicillin, vancomycin, neomycin trisulfate salt, demeclocycline, sulfamethizole, kanamycin, chloramphenicol, streptomycin, roxithromycin, erythromycin, and sulfamethaxole. NB media amended with 7.5% (w/v) NaCl was used and adjusted to either pH 7, 8, 9, 10, or 11 with 5M NaOH. The media was buffered as described above. The media was supplemented with glucose for a final 1% (w/v) concentration. Concentrated filter sterilized antibiotic solutions were added to provide concentrations of 0 µM, 20 µM, 40 µM, 80 µM, 100 µM, or 160 µM of each antibiotic being tested. Absorbance was measured at 600nm over time to determine the growth of Isolate 9 in the media containing antibiotics. All tests were run in triplicate and MIC_{50} value was determined for each antibiotic.

Chemical Modeling of Antibiotic Structures in Marvin Sketch

The computer program Marvin Sketch was utilized to model the dominate microspecies present at each pH tested, the isoelectric point of each antibiotic, and the pKa value for each antibiotic at each pH tested. Marvin Sketch is available from Chem Axon at https://www.chemaxon.com/products/marvin/marvinsketch/.

Results

Enrichments and isolations

The heat-treated sediment samples yielded dense growth in liquid media. The resulting cultures were transferred to solid medium and underwent several rounds of streaking to yield pure cultures. The isolated colonies presented as pale cream in color and circular with smooth edges. Five strains of *Halomonas eurialkalitolerans* (Edwards, in prep) were obtained that were capable of growth across a pH range of 7 to 11 and these strains were used for further testing in this experiment.

pH range and optimum

All five strains were screened for their ability to grow over a wide range of pH values from pH 5 to pH 11. All the strains were able to grow in media with a pH of 7 to 11. Of the strains obtained, Isolate 6 has a pH optima at pH 8 and strains 7, 8, 9, and 10 have optima at pH 9 as determined by the amount of growth in liquid NB media over a period of five days.

Antibiotic Disc Diffusion Assays

Kirby Bower disc diffusion assays were done for all five strains by using paper Sensi discs impregnated separately with several common antibiotics. Fifteen antibiotics were chosen for this primary study: amoxicillin, ampicillin, bacitracin, carbenicillin, cefoxitin, chloramphenicol, erythromycin, kanamycin, nalidixic acid, polymyxin, rifampicin, streptomycin, sulfatrimethoxazole, tetracycline, and tobramycin. All strains appeared to have a resistance to amoxicillin, ampicillin, bactracian, erythromycin, kanamycin, rifampicin, streptomycin, tetracycline, and tobramycin. The results (Table 2) showed that all strains appeared to be inhibited by carbenicillin and polymyxin. All other antibiotics had susceptibilities that varied between the bacterial strains. Isolate 6 was resistant to cefoxitin and sulfamethoxazole while Stains 7, 8, 9, and 10 were inhibited by these antibiotics. Only Isolate 7 was inhibited by chloramphenicol. Strains 8, 9, and 10 were inhibited by nalidixic acid while Isolate 6 and 7 were resistant.

Table 2 Antibiotic Resistances of *Halomonas eurialkalitoleranis* strains 6 to 10

Antibiotic	**6**	**7**	**8**	**9**	**10**
Amoxicillin	R	R	R	R	R
Ampicillin	R	R	R	R	R
Bacitracin	R	R	R	R	R

Table 2 Antibiotic Resistances of *Halomonas eurialkalitoleranis* strains 6 to 10 (cont.)

Carbenicillin	S	S	S	S	S
Cefoxitin	R	S	S	S	S
Chloramphenicol	R	S	R	R	R
Erythromycin	R	R	R	R	R
Kanamycin	R	R	R	R	R
Nalidixic acid	R	R	S	S	S
Polymyxin	S	S	S	S	S
Rifampicin	R	R	R	R	R
Streptomycin	R	R	R	R	R
Sulfamethoxazole	R	S	S	S	S
Tetracycline	R	R	R	R	R
Tobramycin	R	R	R	R	R

MIC_{50} Data

The minimum inhibitory concentration (MIC_{50}) was determined to be the lowest concentration of antibiotic required to cause a reduction of fifty percent in the growth rate of Isolate 9 when compared to the control as described by Falagas et al. (1997). The MIC_{50} value for all antibiotics tested are presented in Table 3 and Figures 1 through 12 are graphical representations of growth compared to controls without antibiotics for each of the antibiotics.

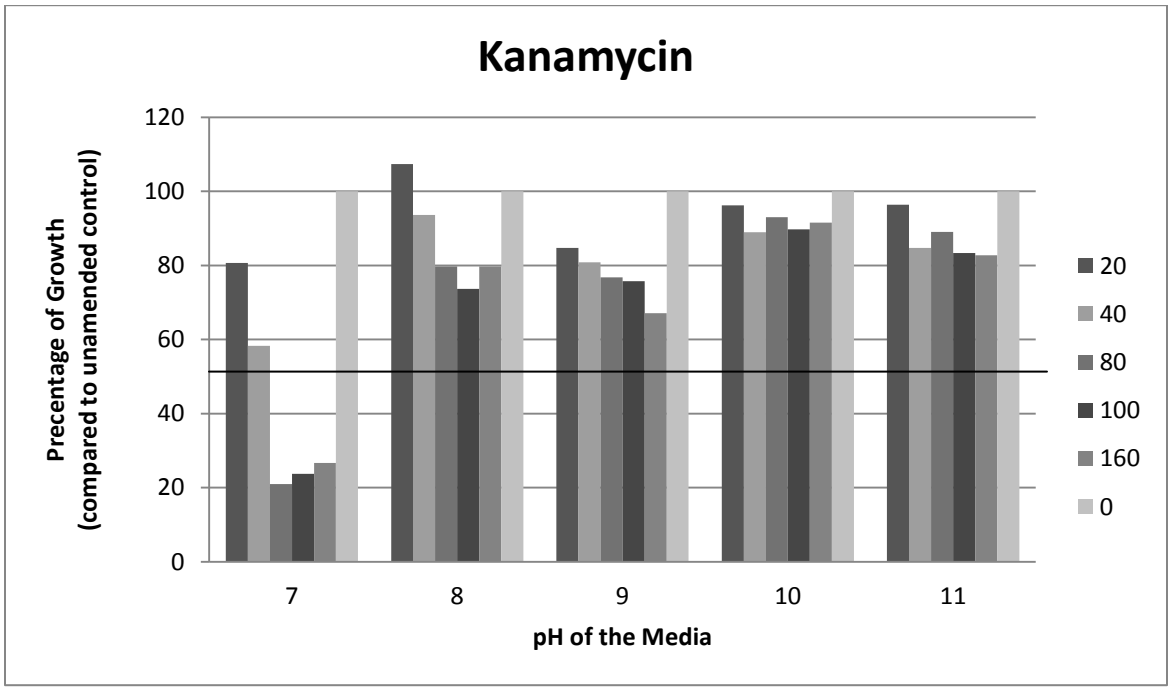


Fig. 1. The MICs of kanamycin against Isolate 9 in media poised at pH 7 though pH 11.

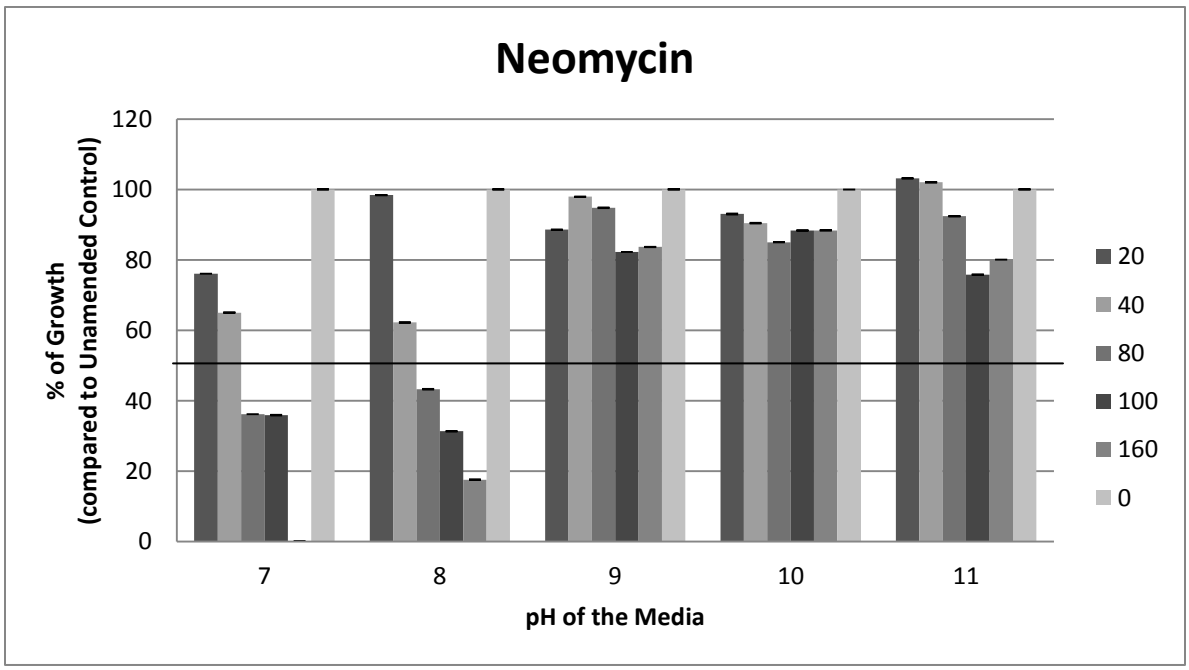


Fig. 2. The MICs of neomycin against Isolate 9 in media poised at pH 7 though pH 11.

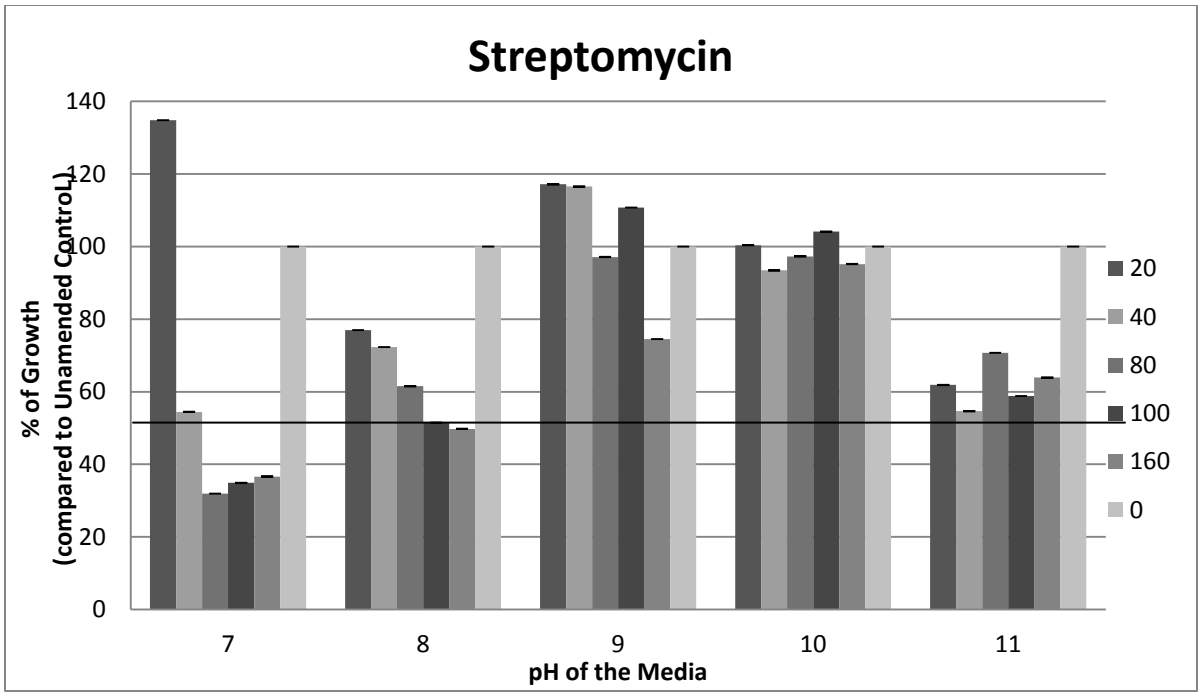


Fig. 3. The MICs of streptomycin against Isolate 9 in media poised at pH 7 though pH 11.

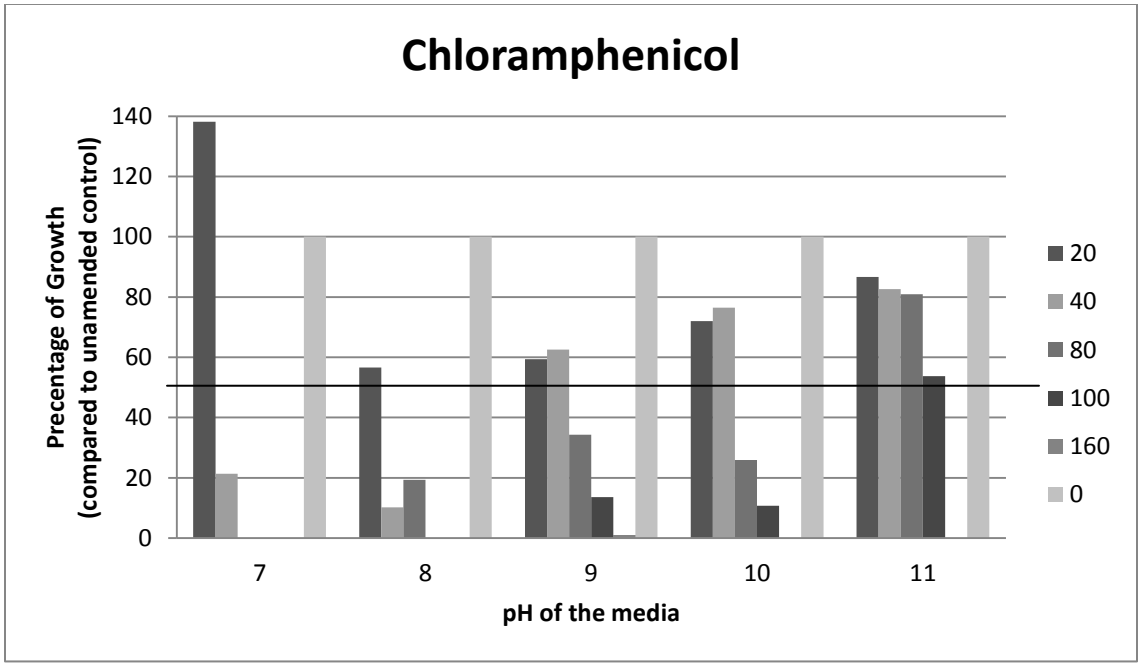


Fig. 4. The MICs of chloramphenicol against Isolate 9 in media poised at pH 7 though pH 11.

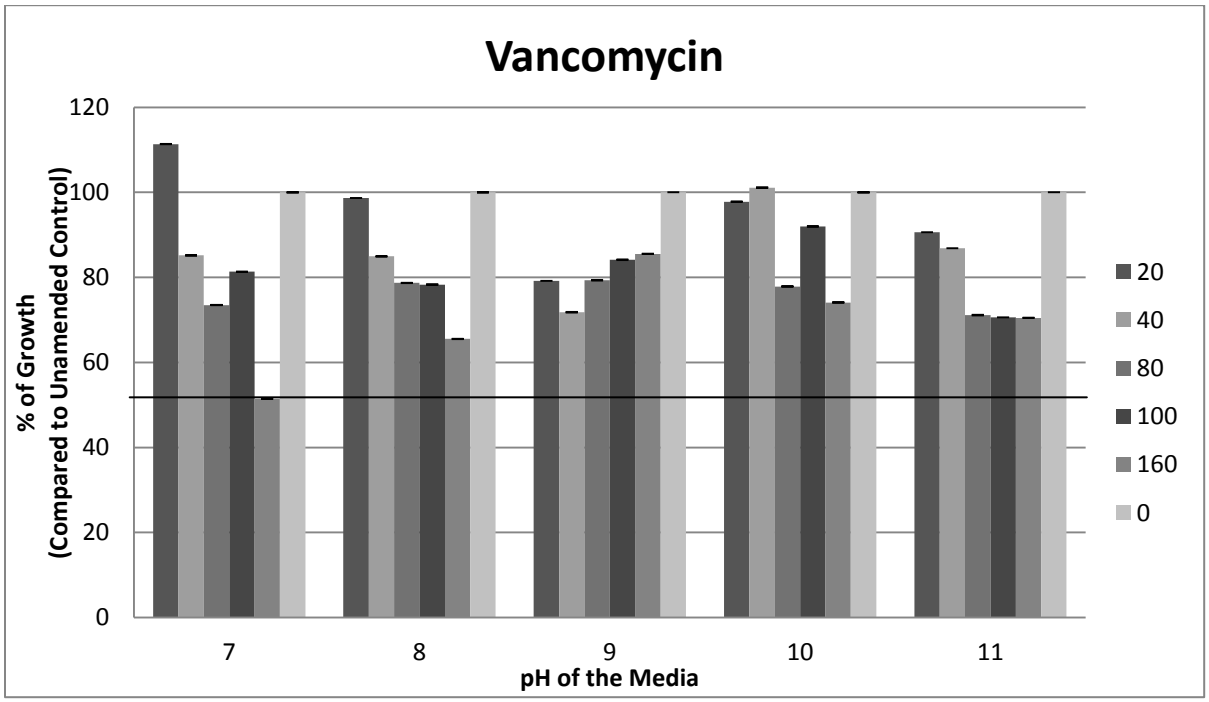


Fig. 5. The MICs of vancomycin against Isolate 9 in media poised at pH 7 though pH 11

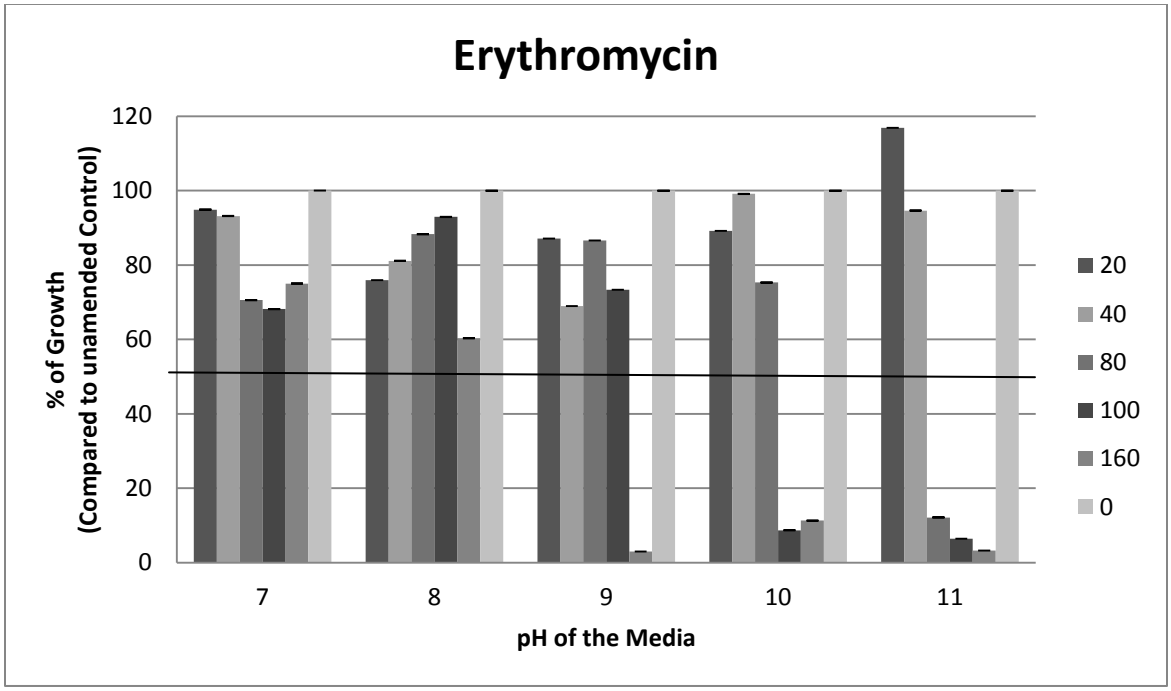


Fig. 6. The MICs of erythromycin against Isolate 9 in media poised at pH 7 though pH 11.

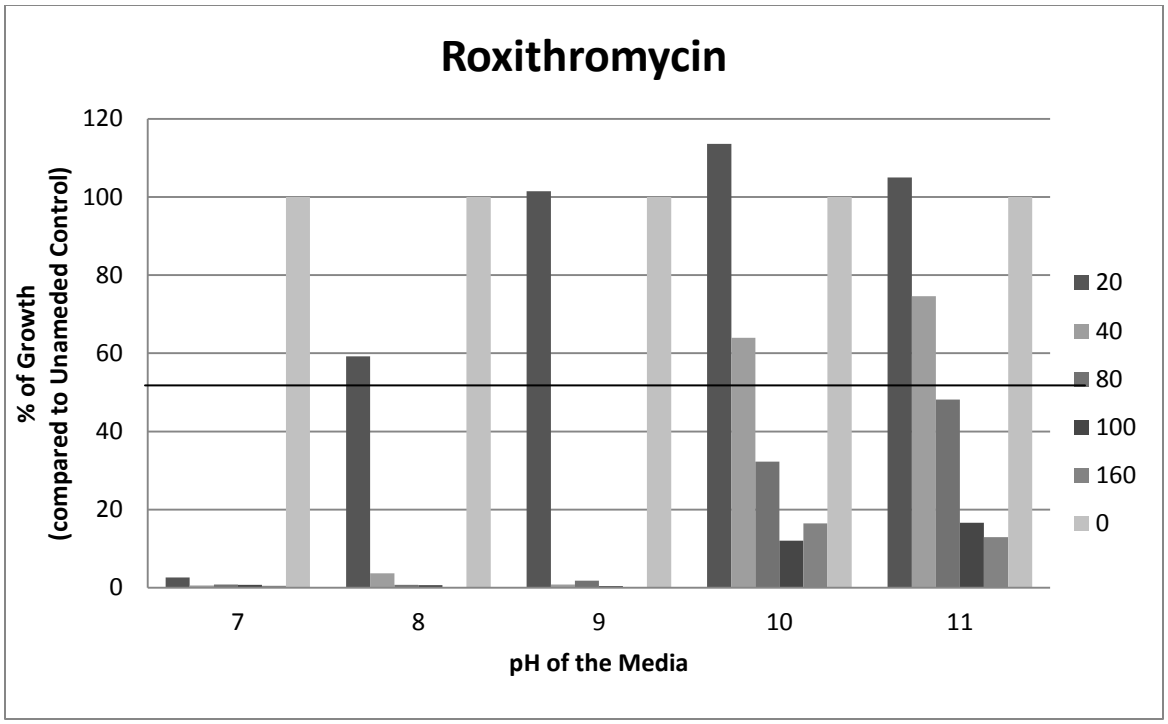


Fig. 7. The MICs of roxithromycin against Isolate 9 in media poised at pH 7 though pH 11.

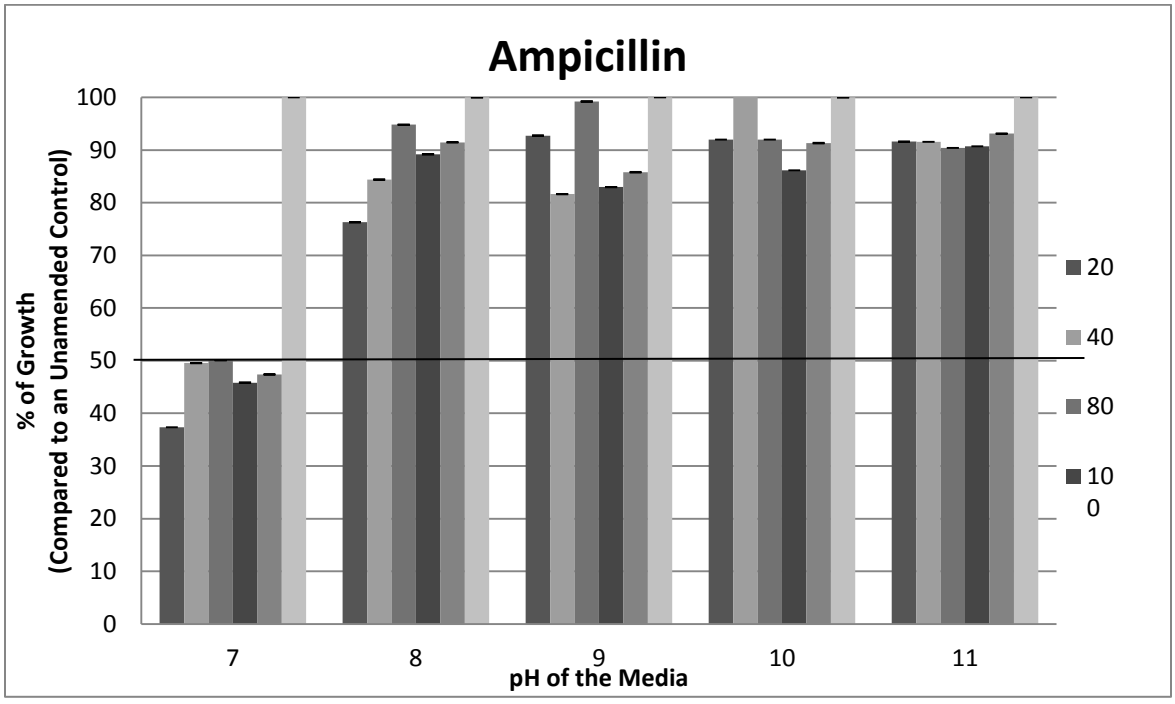


Fig. 8. The MICs of ampicillin against Isolate 9 in media poised at pH 7 though pH 11.

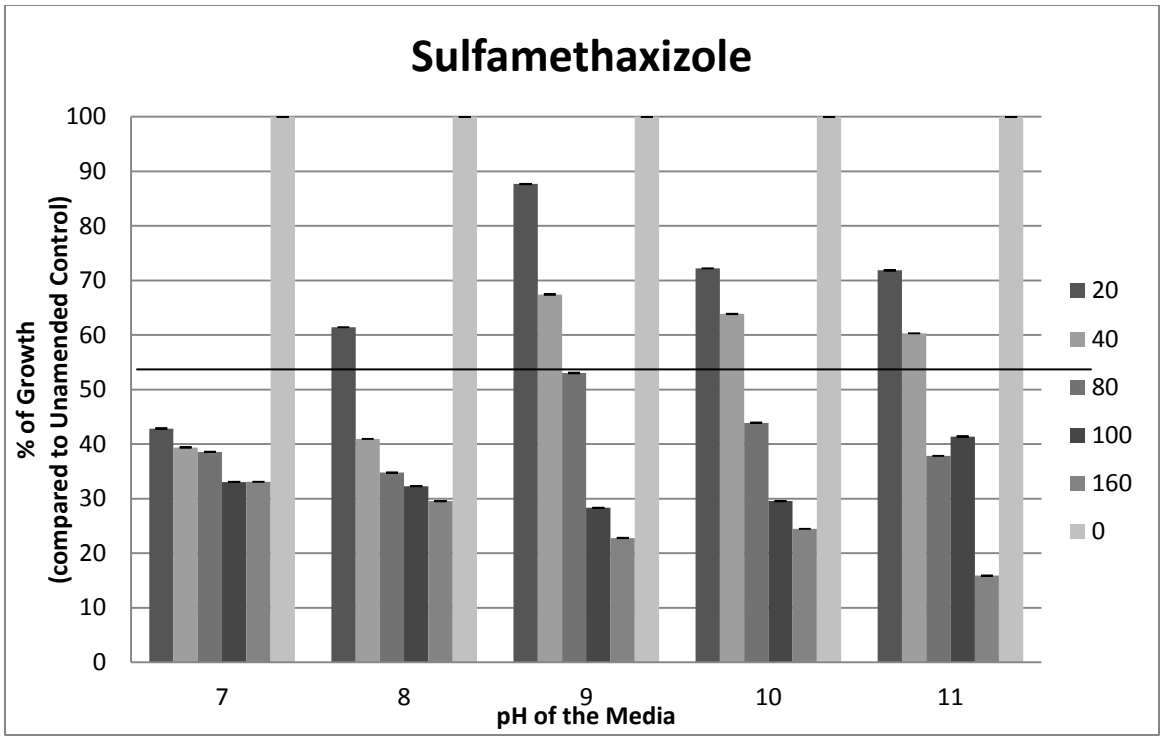


Fig. 9. The MICs of sulfamethaxizole against Isolate 9 in media poised at pH 7 though pH 11.

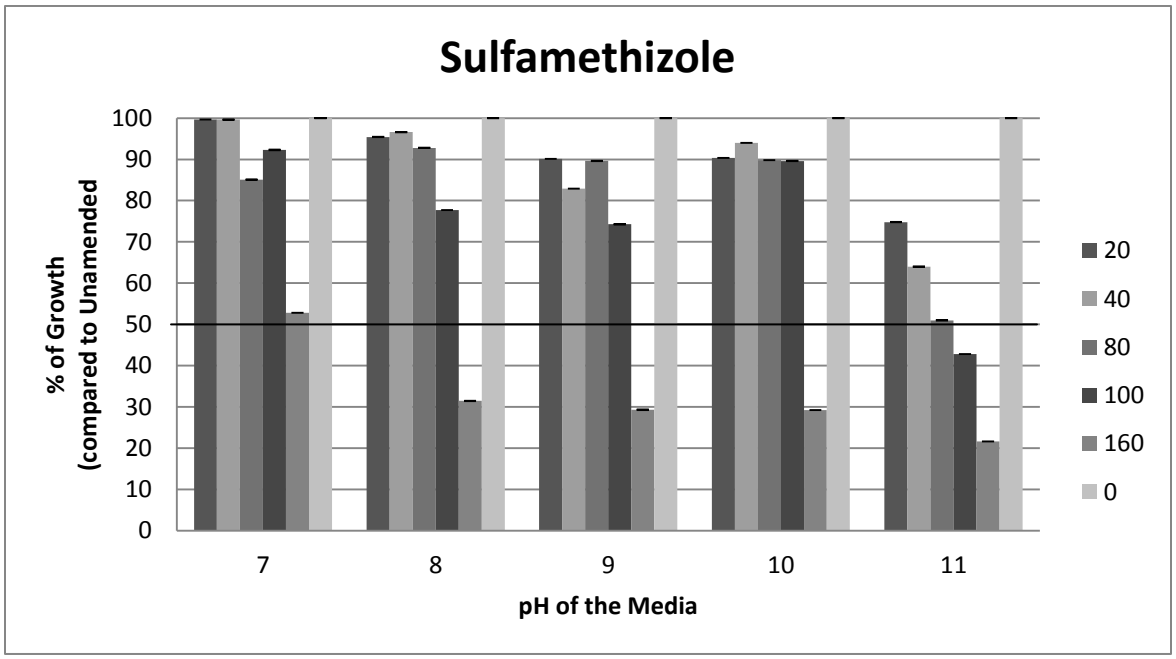


Fig. 10. The MICs of sulfamethizole against Isolate 9 in media poised at pH 7 though pH 11.

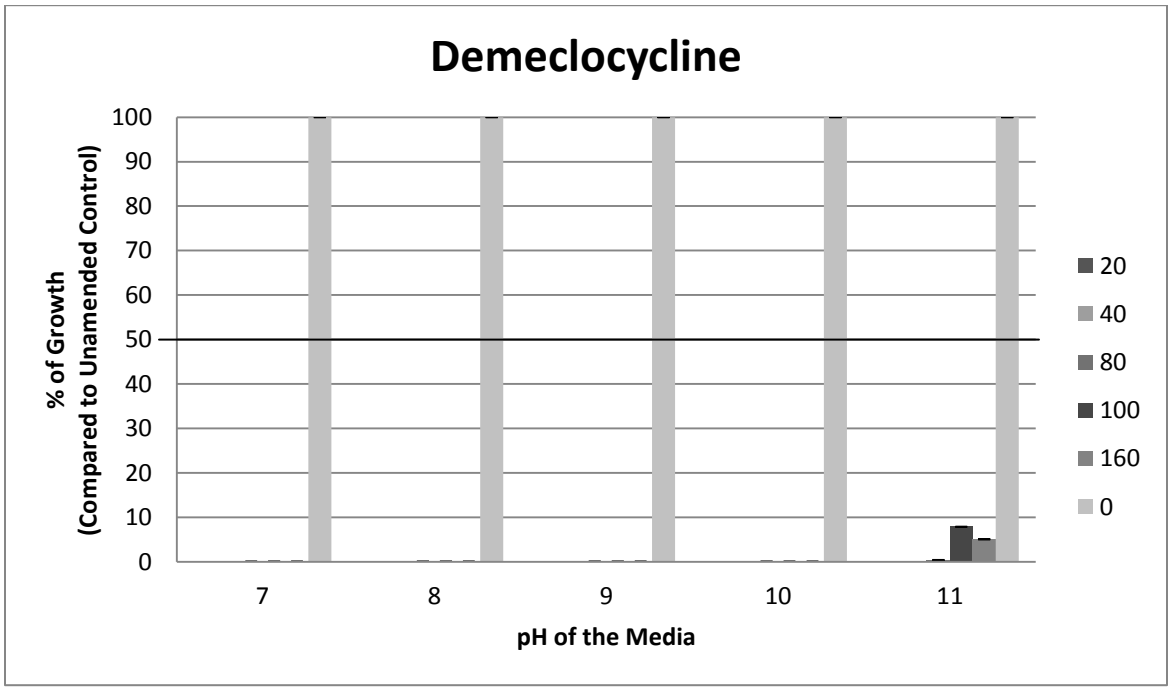


Fig. 11. The MICs of demeclocycline against Isolate 9 in media poised at pH 7 though pH 11.

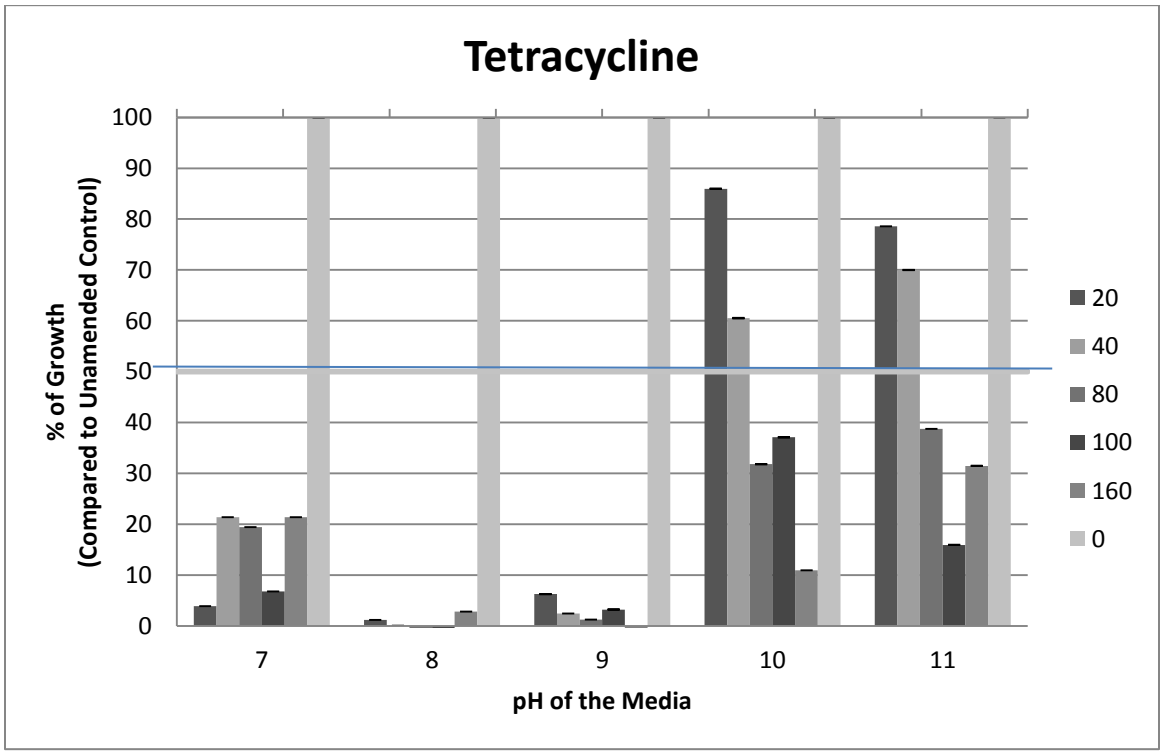


Fig. 12. The MICs of tetracycline against Isolate 9 in media poised at pH 7 though pH 11.

Table 3 Concentration of Antibiotic Required to Induce an MIC_{50} Values In Isolate 9

Antibiotic	**pH 7**	**pH 8**	**pH 9**	**pH 10**	**pH 11**
Kanamycin	40	NA	NA	NA	NA
Neomycin	80	80	NA	NA	NA
Streptomycin	80	160	NA	NA	NA
Chloramphenicol	40	40	80	80	160
Vancomycin	NA	NA	NA	NA	NA
Erythromycin	NA	NA	160	100	80
Roxithromycin	20	40	40	80	80
Ampicillin	20	NA	NA	NA	NA
Sulfamethaxizole	20	40	100	80	80
Sulfamethizole	NA	160	160	160	100
Demeclocycline	20	20	20	20	20
Tetracycline	20	20	20	80	80

Of the antibiotics tested, ampicillin, chloramphenicol, kanamycin, neomycin, roxithromycin, streptomycin, sulfamethaxizole, and tetracycline were more effective in neutral media than pH values above 10. Ampicillin became inactive at pH 8 and above, indicating that it is highly susceptible to alkaline conditions. Chloramphenicol was effective at pH 7 and pH 8. Kanamycin was effective only at pH 7. Neomycin became inactive at pH 9 and above indicating that it was likely to be affected by alkaline media.

Roxithromycin was more effective at pH 7 than higher pH values. Streptomycin was inactive at pH 9 and above. The MIC_{50} for tetracycline was 20 µM at pH values 7 to 9 and 80 µM at pH values 10 and 11. Vancomycin did not inhibit growth at any pH tested. Demeclocycline amended media was effective at inhibiting growth at all pH values tested, with an MIC_{50} value of 20 µM. However at pH 11 some growth was observed. Sulfamethaxizole was most effective at pH 7 with an MIC_{50} at 20 µM. Surprisingly, erythromycin and sulfamethizole were found to be more effective in alkaline conditions than neutral conditions.

Chemical Modeling By Using Marvin Sketch

The isoelectric point, pKa values, and major micro-species were determined for each of the antibiotics tested. Of the antibiotics tested, the dominant microspecies present in the media at the optimal pH for effectiveness of the antibiotic was different when compared to the dominant microspecies present at pH 10 for ampicillin, demecolcycline, erythromycin, kanamycin, neomycin, roxithromycin, streptomycin, tetracycline, and vancomycin. Chloramphenicol, sulfamethoxizole, and sulfamethizole had the same dominant microspecies present at the optimal pH and pH 10. The isoelectric point of each antibiotic was determined and the results are shown in Table 4. In addition, the chemical structure of each dominate micro-species is shown at optimal conditions and at pH 10 are provided in Table 4.

Table 4 Microspecies of Antibiotics at Optimal Effective Conditions and pH 10. Arrows indicate changes in the structure.

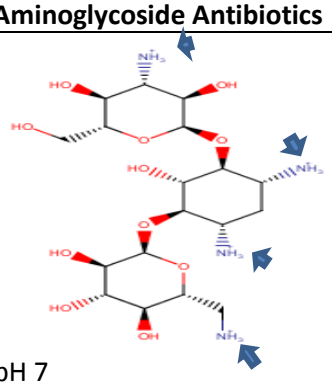
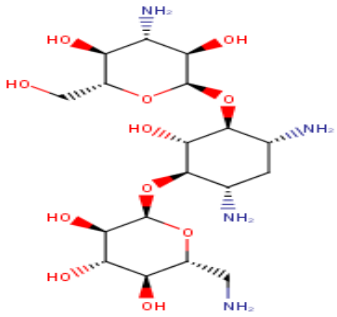
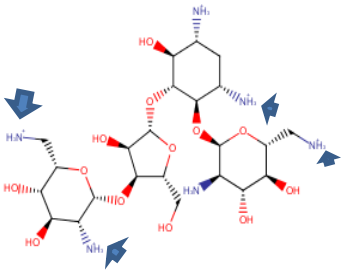
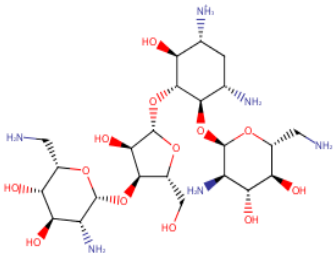
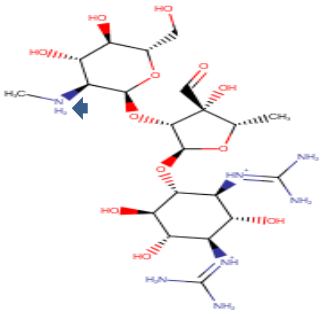
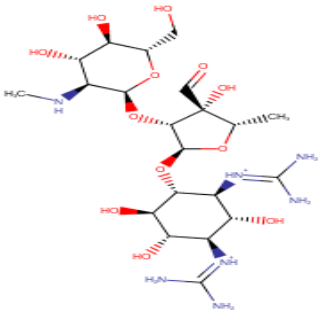
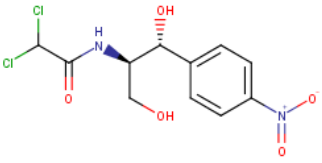
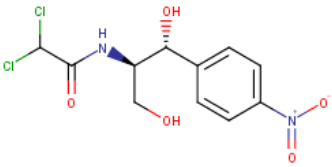
Antibiotic	Isoelectric Point	Optimal Conditions	pH 10
Aminoglycoside Antibiotics			
Kanamycin	pH 10.81	 pH 7	 pH 10
Neomycin	pH 10.98	 pH 7	 pH 10
Streptomycin	pH 10.72	 pH 7	 pH 10
Amphenicol Antibiotics			
Chloramphenicol	pH 3.84	 pH 7	 pH 10

Table 4 Microspecies of Antibiotics at Optimal Effective Conditions and pH 10. Arrows indicate changes in the structure. (cont.)

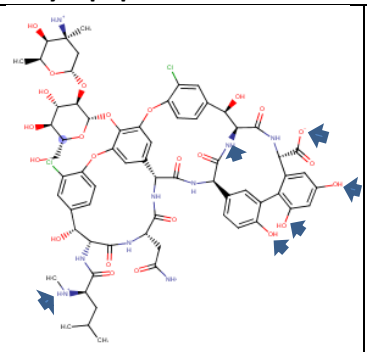
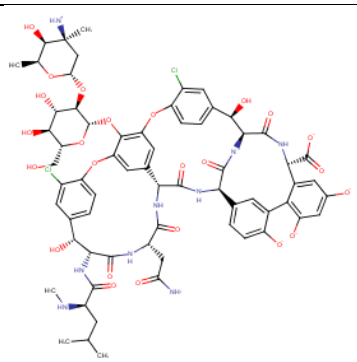
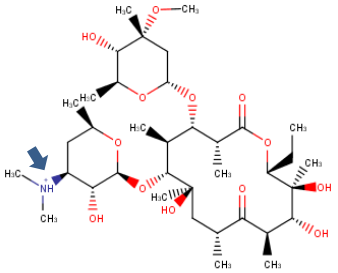
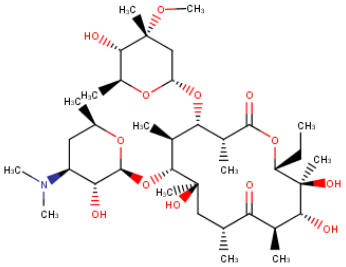
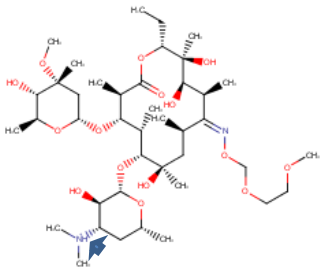
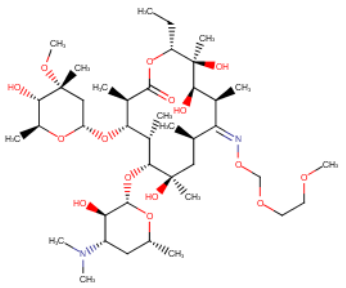
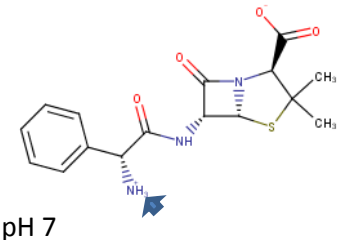
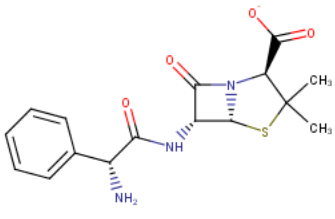
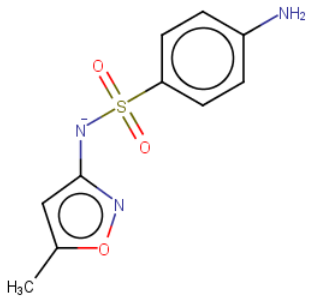
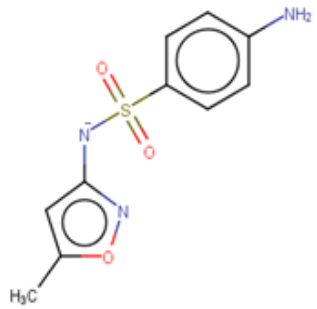
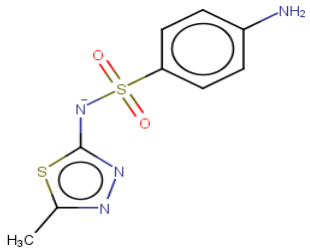
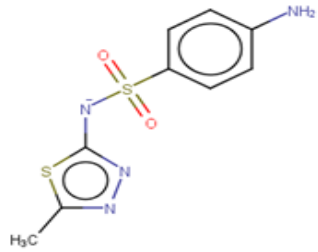
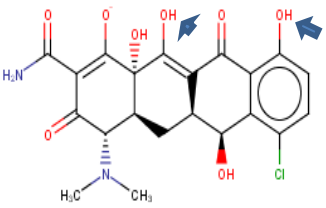
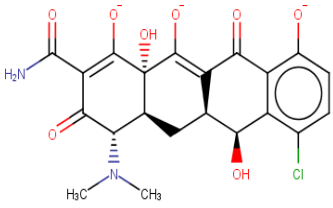
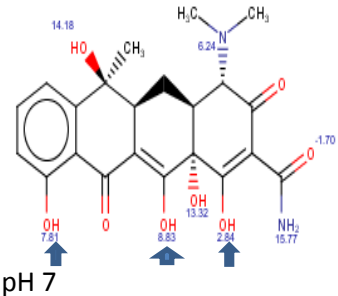
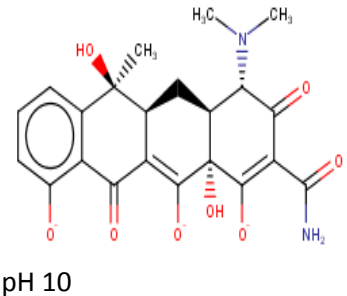
Glycopeptide Antibiotics			
Vancomycin	pH 8.41	 pH 4	 pH 10
Macrolide Antibiotics			
Erythromycin	pH 10.29	 pH 7	 pH 10
Roxithromycin	pH 10.64	 pH 7	 pH 10
Penicillin Antibiotics			
Ampicillin	pH 5.39	 pH 7	 pH 10

Table 4 Microspecies of Antibiotics at Optimal Effective Conditions and pH 10. Arrows indicate changes in the structure. (cont.)

Sulfonamide Antibiotics			
Sulfamethaxizole	pH 4.06	 pH 7	 pH 10
Sulfamethizole	pH 4.33	 pH 7	 pH 10
Tetracycline Antibiotics			
Demeclocycline	pH 5.0	 pH 7	 pH 10
Tetracycline	pH 5.0	 pH 7	 pH 10

Discussion

There have been a few studies that have investigated the effectiveness of antibiotics in acidic conditions and to our knowledge, no studies have investigated the impacts of alkaline conditions on the effectiveness of antibiotics. It is known that acidic pH can affect the effectiveness of antibiotics, rendering many of them ineffective (Falagas et al, 1997; Venglarcik et al, 1982; and Baudoux et al, 2007). *Staphylococcus aureus*, a common pathogenic bacterium is capable of tolerating a wide range of pH conditions from acidic to neutral. Thus, it has been the focus of a number of studies relating to antibiotic resistance and the impact of acidic conditions. One of these studies demonstrated that strains of *S. aureus* had different tolerances to oxacillin depending upon the pH of the media (Venglarcik et al, 1982). Further studies demonstrated that a decrease in pH markedly increased the MICs of macrolide and aminoglycosides groups of antibiotics causing the MIC to drastically increase when the pH of the media was acidic (Baudoux et al, 2007). It was also demonstrated that the acidic pH of the media had the opposite effect on the B-lactam group of antibiotics, with the MIC decreasing as the pH became more acidic (Baudoux et al, 2007).

Falagas et al. (1997) sought to determine the effect of the acidic medium on the antibiotic susceptibilities of another common pathogen, *Bacteroide fragilis*. Antibiotics including trovafloxacin, ciprofloxacin, clindamycin, ampicillin-sulbactam, piperacillin-tazobactam, imipenem, and meropenem, were tested for effectiveness in a pH range from 7.1 to 5.8 (Falagas et al., 1997). This study demonstrated that in all cases the more acidic media caused the MICs to increase, although the exact magnitude of the effect varied between the antibiotics.

Very little information on the stability of antibiotics in alkaline media is available. The optimal pH ranges of the 12 antibiotics tested in this experiment are shown in Table 1. Of the 12 antibiotics that were tested, only two of the antibiotics were reported to be stable in alkaline media (kanamycin and neomycin) (Finegold, 1959; Simone and Popino, 1995). Erythromycin was reported to be somewhat stable in alkaline media with an optimal effective pH of 7.0 to 8.0 (Sultana et al, 2013; Brisaert et al, 1996). Four of the antibiotics were known to be stable in media with a neutral pH including ampicillin,

chloramphenicol, roxithromycin, streptomycin, and tetracycline (Florey, 1972; James and Leach, 1970; Wahba, 2012; Regena et al, 1946; Loftin et al, 2004). Vancomycin was the only antibiotic reported to be stable only in acidic conditions with a stable pH range of pH 3 to pH 5 (Mathew and Gupta, 1995). We were unable to find information on the stability in alkaline media for demeclocycline, sulfamethaxizole, and sulfamethizole.

In this work, a pH range from pH 7 to pH 11 was tested to determine if the effectiveness of the antibiotics varied with the pH of the media. As expected, vancomycin did not have any effect on the growth rates of Isolate 9 at any pH tested due to its instability at neutral or higher pH values. On the other hand, erythromycin was found to be more effective in media that was more alkaline. Sulfamethizole was also found to be more effective against Isolate 9 at higher pH values. In all other cases the antibiotics were found to be more effective in neutral media when compared to alkaline media. However, the magnitude of this effect varied depending upon the antibiotic tested.

The mechanisms of action of several antibiotics are affected by high alkalinity. Ampicillin, for example, acts via a mechanism that weakens the peptidoglycan layer (PG) of bacterial cells undergoing proliferation. The PG layer weakening is due to the blocked transpeptidation reactions preventing formation of cross-linking. Serine hydrolase enzyme catalyzes this reaction (Florey, 1972). The structure of the β-lactams, like ampicillin, closely resembles the potential cross-linking structure of the PG chains. Serine hydrolase enzyme is capable of adding to the four member β-lactam ring, forcing it to arrest in mid catalytic cycle. This process continues trapping more and more transpeptidases in the forming complexes, eventually causing the cell to lyse. The process decreases at a pH above 8 because the β-lactam is degraded to penicilloic acid. The rate of ampicillin degradation increases with the increasing pH (Florey, 1972). At pH 7, Isolate 9s growth was inhibited at 20 μM but was not inhibited at any concentration tested at pH 8 and above.

Erythromycin acts by binding to the bacterial rRNA complex and subsequently inhibiting the transfer of the tRNA strands (Wolfe and Hahn, 1964). Erythromycin is relatively stable in neutral or alkaline pHs, but not in acidic conditions (Sultana et al, 2013). However, the optimal pH range of erythromycin is between pH 7 to 8 (Brisaert et al, 1996). Isolate 9 was not inhibited at any concentration of erythromycin tested at pH 7

or 8. However, at higher pH values, erythromycin did inhibit growth (Table 3 and Figure 5). Interestingly, erythromycin was reported to be most effective at optimal conditions between pH 7 to pH 8 by Brisaert et al (1996); however according to Sultana et al, erythromycin was reported to be stable in media with an alkaline pH (2013).

Streptomycin is known to be effective from pH 3 to 7, water soluble, and remains relatively stable in solutions over time (Regena et al., 1946). It blocks the biosynthesis of bacterial proteins by binding to the 30s subunit at the A site on the 16S rRNA, causeing early release of the developing protein, rendering the resulting protein nonfunctional (Regena et al., 1946).

Tetracyclines block protein synthesis by binding to the A site of the 30s subunit of the 16S rRNA (Chopra and Roberts, 2001). This creates early termination of protein synthesis resulting in nonfunctional proteins (Loftin et al, 2004). At pH values above 7, the rate of hydrolysis increases. Demeclocyline is a semisynthetic tetracycline derivative that inhibits bacterial ribosomal synthesis by binding to the 30S subunit of the bacterial ribosome (Smythies et al., 1972). Mojica et al. (2014) investigated the change in absorbance and emission spectras for various tetracycline derivatives including demeclocyline. They noted a marked shift in both absorbance and emission spectra for demeclocyline across a pH range of 2-12. However, this work did not determine if changes in the pH would cause a change in effectiveness of the drugs.

Chloramphenicol prevents protein synthesis by binding to the ribosomes, blocking polysome formation and protein biosynthesis (Allison et al, 1962). Chloramphenicol is stable in media with a pH between pH 2 to 7. Above pH 7 the effectiveness of the antibiotic decreases due to hydrolysis of the amine group, above pH 7 (James and Leach, 1970).

Kanamycin is an aminglycoside known to be stable between pH 2 to 11 (Finegold, 1959). It binds the 30S ribosomal subunit and inhibits the translation of proteins (Faraji et al, 2006). Neomycin is another aminoglycoside that inhibits bacterial protein synthesis. It is reported to be stable between pH 2 to 9 (Simone et al, 1995). Vancomycin is another common aminoglycoside that is stable at pH values 3 to 5 (Mathew et al, 1995). Vancomycin inhibits peptidoglycan synthesis in the cell wall formation (Reynolds, 1989).

To our knowledge, there is no information on the antibiotic stability in alkaline conditions of sulfamethaxizole, and sulafmethizole. Sulfa drugs inhibit bacterial growth by blocking a critical enzyme in the synthesis of folate (Walsh, 2003). Though the stability of both sulfamethoxizole and sulfamethizole is not known, a closely related antibiotic, sulfathiazole was found to be stable in media up to pH 9 (Loftin et al., 2004). When tested, sulfamethizole was found to be more effective in alkaline media than in neutral media. Sulfamethoxizole was found to be somewhat less effective in alkaline media, although this effect was not as significant as the effects seen by other antibiotics.

A pH dependent response was seen for all but one of the antibiotics. Erythromycin and sulfamethizole were found to more effective in alkaline media. All other antibiotics tested were found to be less effective in alkaline media, although there was a significant variance in the magnitude of this effect. Previously, similar effects were seen under acidic conditions for a number of antibiotics tested. Falagas et al. (1996) presented three possible reasons that the pH of the media may impact their susceptibility on bacteria. One possible explanation is that the stability of the antibiotics are negatively impacted by the pH of the medium. This is appears to be the case for tetracycline, streptomycin, and ampicillin in our study. This is the most likely explanation, because these three drugs were reported to undergo hydrolysis reactions in alkaline media and the effectiveness of these drugs decreased in alkaline media. It is also possible that bacterial enzymatic stability or activity may be influenced the pH of the medium. Several enzymes are known to be required in order to inactivate antibiotics enabling resistant organisms to avoid the antibiotic toxicity. Therefore, if these enzymes are affected by the pH of the medium, the antibiotic susceptibility of the isolates would be impacted as well. It is also possible that the pH of the medium might impact the membrane permeability of the isolate (Falagas et al, 1997).

A wide number of extremophilic bacteria have been isolated and classified and as is customary most of the work is performed under optimal conditions. However, the results of this work demonstrate that the antibiotics tested might be rendered ineffective at the optimum growth conditions tested, thus providing no useful information. The impact of the growth conditions should be taken into consideration when interpreting results obtained when attempting to determine antibiotic resistance.

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IV. ANTIBIOTIC RESISTANCE GENE PROFILE OF *HALOMONAS EURIALKALITOLERANIS*, A NOVEL BACTERIUM ISOLATED FROM SOAP LAKE, WASHINGTON

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Abstract

Many bacterial isolates from Soap Lake, Washington, have been found to possess resistance to multiple antibiotics. One possible explanation for the wide range of antibacterial resistance exhibited by these isolates is due to the impact of high alkalinity on the antibiotics themselves, and not necessarily due to the presence of antibiotic resistance conferring genes. Though selection pressure can be low due to the degration of antibiotics under alkaline conditions, antibiotic resistance genes can still be present in these haloalkaliphilic bacteria. Polymerase chain reactions (PCR) were performed to determine if antibiotic resistance conferring genes are present in five bacterial strains of *Halomonas eurialkalitoleranis*, isolated from Soap Lake, by using primer pairs to amplify antibiotic resistance encoding genes. The antibiotic resistance genes tested includes those that confer resistance against ampicillin, chloramphenicol, erythromycin, kanamycin, streptomycin, sulfamethoxazole, sulfamethizole, tetracycline, and vancomycin. *Halomonas eurialkalitoleranis* was found to possess antibiotic resistance conferring genes for all antibiotics tested except kanamycin and streptomycin.

Key Words: *Halomonas eurialkalitoleranis,* Antibiotic resistance, Polymerase chain reaction (PCR), Soap Lake

Introduction

The antibiotic resistome is a concept first presented by D'costa et al. (2006) to encompass the entirety of the antibiotic resistance genes in all bacteria, both pathogenic and environmental. The resistome consists of all antibiotic resistance genes, cryptic

resistance genes, specific and nonspecific resistance encoding genes, and precursor genes required to evade the action of antibiotics (Wright, 2007). The antibiotic resistome is highly expansive and dynamic and has been around since microorganisms have been producing antibiotics. It is critical to keep in mind that antibiotics are not just drugs to be utilized against microbial borne illness. Antibiotics are utilized by microorganisms in the environment for self-protection, as tools in competitive interactions with other microorganisms, and as signaling molecules (Clardy et al., 2009; Yim et al., 2007). The recent over usage and misusage of antibiotics in medical, agricultural, and industrial settings has caused a massive explosion in the antibiotic resistome in both clinical and environmental settings.

The antibiotic resistome can be impacted by the presence of antibiotics in the environment and by the concentrations of these antibiotics within these environments. Antibiotics can be present in natural environments due to the actions of antibiotic producing organisms, but they are present in low concentrations. The recent human associated usage of antibiotics has led to an overabundance of antibiotics being released into the environment. Antibiotics can enter into the environment through a myriad of human activities via both point and non-point sources. According to the EPA (2012), point sources are those sources that have an obvious source of entry into the environment, while non-point sources do not have an obvious source of entry. One of the most common sources of antibiotic contamination of the environment comes from agricultural based usage of antibiotics. In both animal and plant based agriculture antibiotics are used to prevent and treat infections, and to produce a higher yield product. Animal based agriculture routinely feeds animals low concentrations of antibiotics to increase feed efficacy in those animals (US FDA, 2014; CDDEP, 2011). These antibiotics enter into the environment indirectly through the use of animal fecal matter as fertilizer (Wellington et al., 2013).

There have been numerous studies done on the impacts of antibiotic usage on the resistome from clinical to environmental settings. However, very few studies have investigated the antibiotic resistances of extremophilic microorganisms. Extreme environments harbor microorganisms capable of thriving in the most extreme locations of Earth, from the Atacama Desert in Africa, to deep sea hydrothermal vents, to the hot

springs in Yellowstone National Park, USA. Extremophiles thrive in high temperatures, low temperatures, high salinity, acidic environments, alkaline environments, desiccated environments, and any combination of these conditions. Despite the abundance of these extreme environments and the widespread presence of these environments, very little information is known about the antibiotic resistances of these microorganisms or the impacts of potential antibiotic contamination on the antibiotic resistome within these environments.

This study investigated the antibiotic resistances of five bacterial isolates from Soap Lake, Washington. Soap Lake was chosen for this study because it is a unique haloalkaline soda lake located in central Washington State, USA. The pH of the lake lies at a nearly constant pH of 9.8, and has a salinity around 15g liter-1 in the mixolimnion and 140g liter-1 in the monimolimnion (Dimitriu et al, 2006 and Sorokin et al, 2007). Soap Lake is meromictic and the waters of the mixolimnion and the monimolimnion have not mixed despite the spring and autumn circulations of the mixolimnion in over 2000 years (Peyton and Yonge, 2002). In addition to the unique physical characteristics of this lake, it was chosen for this study because Grant County, where Soap Lake is located, has 50,260 total acres devoted to orchards (USDA, 2002). Traditionally, antibiotics such as streptomycin, oxytetracycline, gentamicin, and oxolinic acid are used to protect these high value fruit crops (Mcmanus, 2014). Therefore, due to the proximity of antibiotic use and the possibility that these antibiotics are entering into Soap Lake, this site was chosen for study. It is possible that these antibiotics enter into Soap Lake, causing a selective pressure to be applied to the antibiotic resistome of the Soap Lake microbial community.

Bacterial isolates from Soap Lake have been previously shown to be resistant to a wide variety of antibiotics including erythromycin, bacitracin, novobiocin, polymyxin B, neomycin, gentamicin, streptomycin, carbenicillin, rifampicin and tetracycline (Dimitriu et al, 2005; Mormile et al, 1999; and Mormile, 2014). This laboratory has isolated species such as *Nitrincola lacisaponensis* and *Halomonas campisalis*, from Soap Lake and these bacteria have shown antibiotic resistances to several common antibiotics (Dimitriu et al, 2005; Mormile et al, 1999).

Previously, *Halomonas eurialkalitoleranis* was found to be resistant to a number of antibiotics including amoxicillin, ampicillin, bacitracin, chloramphenicol, rifampicin,

streptomycin, tetracycline and tobramycin (Edwards et al., in prep). However, it was also noted by Edwards et al. (in prep) that these antibiotic susceptibilities were impacted by the alkalinity of the media, and the minimum inhibitory concentration (MIC) of the antibiotics was highly dependent upon the pH of the media. Therefore, one possible explanation for the wide range of antibacterial resistance exhibited by this organism is due to the impact of high alkalinity on the antibiotics themselves, and not necessarily due to the presence of antibiotic resistance conferring genes. On the other hand, the organisms can possess antibiotic resistance genes. Thus, the aim of this study is to determine if the strains of *Halomonas eurialkalitoleranis* possess antibiotic resistance genes. Known antibiotic resistance gene primer pairs were used to interrogate each of the strains. A number of antibiotic resistance genes were present in the strains and also potentially active against the antibiotics.

Materials and Methods

Isolation of Genomic DNA

Cultures were grown in nutrient broth (Difco) media amended with 75 g/L NaCl and adjusted to a pH of 9.0 with 1M NaOH until the cultures reached an optical density of approximately 0.600 at 600nm. Genomic DNA was isolated by using the GeneJET Genomic DNA Purification Kit from Thermo Scientific (Waltham, MA). Genomic DNA extract was obtained from each bacterial isolate and these products were measured for DNA content by using a Nanodrop. Products were utilized for PCR amplification if the genomic DNA concentration was 50 ng/µL or greater. The purity and concentration of the DNA was assessed using a NanoDrop 2000 (Thermo Scientific, Waltham, MA) (Johnson, 1994).

Polymerase Chain Reactions (PCR)

PCR primers were taken from primary literature papers for each of the antibiotics tested. Supplemental Table 1 shows the DNA sequence for each primer used and the

source of the primer sequences. All primers were synthesized by Intergraded DNA Technologies (IDT) (https://www.idtdna.com/site).

PCRs were run by using 30 µL volumes containing 15 µL of GoTaq Green master mix (Promega) (2X concentration), 5 µL of template DNA, 45 pmol of each primer, and filled final 30 µL volume with PCR water. All PCR products were visualized by running 5 µL aliquots on a 1.2% agarose gel stained with ethidium bromide. If the PCR reaction did not show a visible PCR product during gel electrophoresis, a second PCR reaction was run using 1 µL of the original reaction as template DNA as described by Macauley et al (2007).

Results and Discussion

Grant County, where Soap Lake is located, has 50,260 total acres devoted to orchards (USDA, 2002). In plant based agriculture, streptomycin, oxytetracycline, gentamycin, oxolinic acid, and kasugamycin are used to prevent infections from common bacterial pathogens including *Pectobacterium spp.*, *Agrobacterium tumefaciens*, *Peronospora tabacina*, *and E. amylovora* (McManus, 2014). These antibiotics are applied to the crops in a fine mist spray and the use of these antibiotics has been linked to an increased number of antibiotic resistances. Two examples are streptomycin resistant *E. amylovora* and *X. campestris pv. Vesicatoria*, two serious infectious agents of plants (McManus, 2001 and Stall, 1962). Streptomycin resistant plant pathogens have seriously devastated crops such as fruit trees, tomatoes, and peppers, and these infections have become a major problem in plant-based agriculture.

The results of these experiments determined that all five strains of *Halomonas eurialkalitoleranis* could produce PCR gene products with primers to all but two of the antibiotic resistance genes tested (Table 1).

Table 1 Polymerase Chain Reaction Results

Antibiotic	Primer pair	6	7	8	9	10	Antibiotic	Primer pair	6	7	8	9	10
Ampicillin	pbp5-F/pbp5-R	+	+	+	+	+	**Streptomycin**	SAF/SBR	-	-	-	-	-
	Ddlfm-F/Ddlfm-R	+	-	-	-	-		rpslF/rpslR	-	-	-	-	-
	val629-F/val629-R	-	+	+	-	-		rrsF/rrsR	-	-	-	-	-
	TEM(321)/TEM(846)	+	+	+	+	+		rrs-segF/rrs-segR	-	-	-	-	-
	ROB(419)/ROB(1110)	-	-	-	-	-		gidBF/GIdBR	-	-	-	-	-
	RWO1/DG74	-	-	-	-	-	**Sulfamethoxazole**	sul1F/sul1R	-	-	-	-	-
Chloramphenicol	Cmr1/Cmr2	-	-	-	-	-		sul2F/sul2R	+	+	+	-	+
	catA1F/catA1R	-	-	-	-	-		SUL2-F/SUL2-B	+	+	+	+	+
	catA2F/catA2R	-	-	-	-	-		sulAF/sulAR	+	+	+	-	+
	catA3F/catA3R	-	-	-	-	-	**Sulfamethizole**	sul1f*/sul1B*	+	+	+	+	+
	cmlAF/cmlAR	+	+	+	+	+		Sul 2-F*/sul 2-B*	+	+	-	+	+
	floRF/floRR	-	-	-	-	-		Sul3-F*/ Sul3-B*	+	+	+	-	+
	KM-F1/KM-R1	-	-	-	-	-	**Tetracycline**	Tet A-FW/Tet A-RV	-	-	-	-	-
	KM-F2/KM-R2	-	-	-	-	-		Tet B-FW/ Tet B- RV	+	+	+	+	-
	Kan_NheI/Kan_PacI	-	-	-	-	-		Tet C-FW / Tet C-RV	-	-	-	-	+
	Fpkan/Rpkan	-	-	-	-	-		Tet D-FW/ Tet D - RV	+	+	+	-	+
Erythromycin	erm(A)F/erm(A)R	-	-	-	-	-		Tet E-FW/Tet E-RV	-	-	-	-	-
	erm(B)F/erm(B)R	+	+	-	+	+		Tet G-FW/ Tet G-RV	+	+	+	+	-
	erm(C)F/erm(c)R	+	+	-	-	+		Tet H-FW /Tet H-RV	-	-	-	+	-
	III1/III2b	+	+	-	-	+		Tet J-FW/Tet J-RV	-	+	+	-	+
	III6/III2b	-	-	+	+	-		Tet Y-FW/ Tet Y-RV	-	+	-	-	+
	III7/III2b	+	+	+	+	-		Tet Z-FW/Tet Z- RV	-	+	-	-	-
	III8 b/III9	+	-	+	+	+		Tet 30-FW/Tet 30-RV	-	+	+	+	+
	III10/III8 b	-	-	+	-	+	**Vancomycin**	Van A1/Van A2		-	-	-	-
	III13 b/III7	+	+	+	+	+		vanA-F/vanA-R		-	-	-	-
	III14/III15	+	+	+	+	+		vanB-F/vanB-R		+	+		+
Kanamycin	KM-F1/KM-R1	-	-	-	-	-		vanC1-F/vanC1-R		-	-	-	-
	KM-F2/KM-R2	-	-	-	-	-		vanC23-F/vanC23-R		-	-	-	-
	Kan_NheI/Kan_PacI	-	-	-	-	-		VanS/VanS1		-	-	-	-
	Fpkan/Rpkan	-	-	-	-	-		p3967/p5786		-	-	-	-
Neomycin	neo-F/neo-R	-	-	-	-	+		p4649/p6969		-	-	-	-
	neo1F/neo1R	+	+	+	+	-		p6081/p8607		-	-	-	-
	neo2F/neo2R	-	-	-	-	-		p8061/p9944		-	-	-	-
	neo3F/neo3R	-	-	-	-	+		p9052/p10585		-	-	-	-
	neo4F/neo4R	-	-	+	-	-		vanZ-1/vanZ-2		-	-	-	-

Interestingly, no antibiotic resistance genes were found for streptomycin (Table 1). Streptomycin is one of the five commonly used antibiotics in plant based agriculture (McManus, 2002 and McManus, 2014). The frequent application of streptomycin to plants has been investigated for its ability to cause a shift in the soil microbial communities that have been subject to streptomycin application. Two independent studies investigated the impacts of streptomycin in soil bacterial communities, and both studies found that no shift in soil bacterial communities could be found (Walsh et al., 2014 and Shade et al., 2013). Walsh et al. (2014) applied a number of different statistical tools in order to ensure that untreated soil communities and treated soil communities had no significant differences. It is possible that the concentrations of antibiotics that reach the soil are not great enough to cause a selective pressure large enough to cause a shift in the bacterial community. It is also possible that because antibiotics such as oxytetracycline and streptomycin are photodegradable that they are not active long enough to cause a shift in soil bacteria communities (McManus, 2014).

Ampicillin is a commonly used derivative of penicillin. Ampicillin acts via a mechanism that weakens the peptidoglycan layer (PG) of bacterial cells that are undergoing proliferation and eventually causes the bacterial cells to lyse (Florey, 1972). Previously, *Halomonas eurialkalitoleranis* Isolate 9 was inhibited by 20 µM concentrations of ampicillin at pH 7, however at pH 8 and above, no inhibition could be found at any concentration tested (Edwards et al., in prep). Two critical antibiotic resistance genes were found in all five strains tested and three of the strains demonstrated a third antibiotic resistance gene (Table 1). All five strains displayed a positive PCR result for the PBP5 gene. The PBP5 gene is a penicillin binding protein that has been linked to penicillin resistance, however, modifications that result in a decreased binding affinity have been also linked to ampicillin resistance (Mohn et al, 2004). In addition, all five isolates displayed a positive result for blaTEM gene, a β-lactamase gene known to confer resistance to the penicillin family of antibiotics including ampicillin (Bailey et al, 2010). Isolate 6 produced PCR products for the ddlfm gene that is a D-Ala-D-Ala ligase gene known to confer for antibiotic resistance for ampicillin (Mohn et al, 2004). Lastly, Isolates 7 and 8 produced a positive result for the Val629 gene that codes for a glutamate

to valine substitution at the 629 position. This gene product is known to cause an increased MIC for ampicillin (Mohn et al, 2004).

Chloramphenicol is a bacteriostatic antibiotic that is commonly used in modern medicine due to its ability to treat both Gram positive and Gram negative bacteria, including anaerobic bacteria. Chloramphenicol binds to the bacterial ribosome blocking polysome formation and protein biosynthesis (Allison et al, 1962). Previously, Isolate 9 was inhibited by a 40 µM concentration of chloramphenicol in pH 7 media, and in pH 11 media a concentration of 160 µM was required to inhibit Isolate 9 (Edwards et al., in prep). All five isolates assayed produced a positive PCR band for the cmlA gene (Table 1). The cmlA gene is a multidrug resistance efflux pump that enables resistance for chloramphenicol (Bohn and Bouloc, 1998; Lu et al., 2014). It is interesting to note that in bacteria with an overexpression of the cmlA gene there is a heightened sensitivity to streptomycin (Bohn and Bouloc, 1998).

Erythromycin is a commonly used macrolide that works by binding to bacterial rRNA complexes and subsequently inhibiting the transfer of the tRNA strands (Wolfe and Hahn, 1964). Interestingly, it was previously found that Isolate 9 was not inhibited by erythromycin in pH 7 media but 80 µM concentrations of erythromycin were required to inhibit Isolate 9 in pH 11 media (Edwards et al., in prep). The results of the PCR studies found that all five strains produced gene products for the III13 and III14 gene combinations. Additionally, PCR products were found for the ermB, ermC, III1, III6, III7, III8, and III10 genes for some strains (Table 1). The primer pairs were designed to encode for various combinations of ermA, ermB, ermC, ermTR, and ermAM genes (Reig et al., 2001 and Duran et al., 2012). Erythromycin resistance is conferred through a methyltransferase that methylates the adenine residue at the 2058 position of the 23S rRNA.

Kanamycin is an aminoglycoside that binds the 30S ribosomal subunit and inhibits the translation of proteins (Finegold, 1959; Faraji et al., 2006). In pH 7 medium, Isolate 9 was found to have an MIC_{50} value at a concentration of 80 µM. Above pH 9, no inhibition by kanamycin occurred (Edwards et al, in prep). The results of the PCR studies found no products formed for any of the genes tested (Table 1). This result is surprising

because this antibiotic did not inhibit the growth of any of the isolates. Thus, it is likely that kanamycin is not active at the pH values tested.

Neomycin is another common aminoglycoside antibiotic that binds irreversibly to the 30S subunit of susceptible bacteria. In pH 7 media, Isolate 9 was inhibited by 80 µM concentrations of neomycin, but above pH 9, no inhibition occurred (Edwards et al, in prep). In this study, it was determined that all isolates produced a positive PCR result to at least one neomycin resistance gene primer set (Table 1). Neomycin resistance is conferred by 3'-phosphotransferase enzymes that inactivate aminoglycoside antibiotics through phosphorylation (Valera et al., 1994). None of the primer pairs for these genes produced products for all isolates assayed. However, the neo 1 primer pair did result in PCR products for Isolates 6, 7, 8, and 9; Isolate 10 produced a positive result for neo3 gene (Table 1).

Sulfa drugs are a fully synthetic class of drugs that inhibit bacterial growth by blocking the synthesis of folate (Walsh, 2003). Sulfamethoxizole was previously shown to inhibit Isolate 9 in pH 7 media at a concentration of 20µM; sulfamethizole was unable to inhibit this strain at pH 7 at any concentration tested (Edwards et al, in prep). All isolates tested produced a positive PCR result for the sul1 gene, Isolates 6, 7, 9, and 10 produced products for the sul2 primers; Isolates 6, 7, 8, and 10 produced products for the sul3 primer sets (Table 1). Resistance to the sulfa drugs is conferred by the sul genes through a modified dihydropteroate synthesis gene, which is required for folate synthesis (Wondwossen et al, 2002).

Tetracycline blocks protein synthesis in bacteria by binding to the A site of the 30s subunit of the 16SrRNA (Chopra and Roberts, 2001). This creates early termination of protein synthesis resulting in nonfunctional proteins (Loftin et al, 2004). Oxytetracyclines are often used to protect agricultural industries such as those that occur around Soap Lake, Washington (McManus, 2002 and McManus, 2014). Isolate 9 was inhibited by tetracycline at a concentration of 20 µM in pH 7 media and 80 µM in pH 11 (Edwards et al., in prep). PCR products were produced for a number of tetracycline resistance conferring efflux pumps including tetB, tetD, tetG, tetH, tetJ, tetY, and tetZ in at least one of the five strains (Table 1). All five bacterial isolates contained tet efflux genes to at least three of the eleven genes tested. It is interesting to note that Isolate 9 has

been shown to be inhibited by tetracycline at a concentration of 20 µM in pH 7 media yet it produced a number of PCR products from tetracycline primer sets. According to Seiler and Berendonk (2012), certain antibiotic resistance genes have been known to be linked to heavy metal resistance in soil as well as water bodies that have been impacted by agriculture. In Soap Lake, it is possible that the wide number of tetracycline resistance genes, demonstrated by the five strains, have been modified to encode for heavy metal resistance, but it is impossible to determine this without first sequencing the gene products.

Vancomycin is a common glycopeptide antibiotic that inhibits the cell wall synthesis by binding to the D-Ala-D-Ala terminus of the peptidoglycan layer in the cell wall (Hammes and Neuhaus, 1974). Vancomycin is primarily effective against Gram positive bacteria. Our previous studies demonstrated that vancomycin was ineffective against all the isolates at any pH tested (Edwards et al., in prep). Even though vancomycin is not effective against *Halomonas eurialkalitoleranis,* the VanB gene primer pair produced PCR products by all five isolates (Table 1). The VanB gene is in a group of operon genes that enable the synthesis of peptidoglycan and inhibit the action of vancomycin by modifying the C-terminal D-Alanine-D-Alanine bonds to D-alanine-D-lactate bonds (Young et al., 2007).

All five bacterial isolates produced PCR products to eight of the ten antibiotic resistance gene primer pairs tested in this study (Table 1). It can be speculated that the impact of the surrounding agriculture likely plays an important role on the antibiotic resistances of the five strains of *Halomonas eurialkalitoleranis*. However, it should be noted that the alkaline conditions of the lake are known to impact the effectiveness of a number of antibiotics including tetracycline and streptomycin. More work is required to determine why these putative antibiotic resistance genes are present in these haloalkiliphilic microorganisms, and what the selection pressures are that maintain these genes in the microbial community.

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Supplemental Table 1. Primer Sequences

Antibiotic	Primers	**Sequences**	**Reference**
Ampicillin	bp5 fw	AACAAAATGACAAACGGG	Mohn et al., 2004
	pbp5 rev	TATCCTTGGTTATCAGGG	
	ddlfm fw	TTCTTTGCTTTATCCGATGT	
	ddlfm rev	CGGTTTTCTGCTTTTGTAAT	
	val629 fw	TGTAAATGGTACAGCACATTC	
	val629 rev	GTTGAAAGCAAACAAGAAACT	
	TEM(321) fw	TGGGTGCACGAGTGGGTTAC	Tenover et al., 1994
	TEM(846) rev	TTATCCGCCTCCATCCAGTC	
	ROB(419) fw	ATCAGCCACACAAGCCACCT	
	ROB(1110) rev	GTTTGCGATTTGGTATGCGA	
	RWO1 fw	AACTGGAGGAAGGTGGGGAT	
	DG74 rev	AGGAGGTGATCCAACCGCA	

Supplemental Table 1. Primer Sequences (cont.)

Chloramphenicol	Cmr1 fw	GGTACCCGGGCGCCGCGGC TGCAGATCTCCTCCACGGG GAGAGCCTGA	Whitby et al., 1998
	Cmr2 rev	ATATCTGCAGGCGCCCGGG TACCGCGGCCACCCCGTCA GTAGCTGAACAGGAGGG	
	catA1 fw	CATTCACCCGACGACGCAC TT	Lu et al., 2014
	catA1 rev	TTATCACTTATTCAGGCGTA GCAC	
	catA2 fw	GAACACTTTGCCCTTTATCG TC	
	catA2 rev	TCCTGCTGAAACTTTGCCAT CGT	
	catA3 fw	TGZTGAGTTGAGAATGGCG ATA	
	catA3 rev	GAGAGCGGCAATAACAGTC TA	
	cmlA fw	GCGGGCTATCTTTGCGTTTC	
	cmlA rev	AAGTAGACTGCCGTGACCG TTCC	

Supplemental Table 1. Primer Sequences (cont.)

	floR fw	TCCTGAACACGACGCCCGCTAT	
	floR rev	TCACCGCCAATGTCCCGACGAT	
Erythromycin	erm(A) fw	AAGCGGTAAACCCCTCTGA	Duran et al., 2012
	erm(A) rev	TTCGCAAATCCCTTCTCAAC	
	erm(B) fw	CTATCTGATTGTTGAAGAAGGATT	
	erm(B) rev	GTTTACTCTTGGTTTAGGATGAAA	
	erm(C) fw	AATCGTCAATTCCTGCATGT	
	erm(C) rev	TAATCGTGGAATACGGGTTTG	
	III1 fw	GAAATTGG(A/C)ACAGGTAAAGGGCA	Seppälä et al., 1998
	III2 b rev	AAA(C/T)TGATTTTTAGTAAA	
	III6 fw	GAAGTTTAGCTTTCCTAA	

Supplemental Table 1. Primer Sequences (cont.)

	III2 b rev	AAA(C/T)TGATTTTTAGTAAA	
	III7 fw	TGCTGTTAA TGGTGGAAA	
	III2 b rev	AAA(C/T)TGATTTTTAGTAAA	
	III8 b fw	GCATGACATAAACCT TCA	
	III9 rev	ACATAAGGAGGTTTCAAT	
	III10 fw	AGGTTATAATGAAACAGA	
	III8 b rev	GCATGACATAAACCT TCA	
	III 13 b fw	TTA GTG AAA CAA TTT GTA	
	III7 rev	TGCTGTTAA TGGTGGAAA	
	III14 fw	TCT CCT TGC CGG TTA TAA	
	III15 rev	ATC AAT TAA GAC AGG TGC TGA AGC	
Kanamycin	KM1 fw	ACCGCGGTTTCAAAATCGG CTCC	Cheng et al., 2011
	KM1 rev	CCCGTTCCACATCATAGGT TGTC	

Supplemental Table 1. Primer Sequences (cont.)

	KM2 fw	GACAACCTATGATGTGGAA CGGG	
	KM2 rev	GTCATGCATTCTAGGTACT AAAAC	
	Fpkan fw	CATATGAGAAAAACTCATC GAGCATC	Samra et al., 2009
	Rpkan rev	GAATTCAGCCATATTCAAC GGAA	
	Kan_NheI fw	GCTAGGCTAGCGGATGAAT GTCAGCTACTGGGC	Lackner et al., 2007
	Kan_PacI rev	GCTAGTTAATTAATCAGAA GAACTCGTCAAGAAGGC	
Neomycin	neo fw	ATCTCGAGGGAGGTCAACA CATCAATGCT	Core TAM., 2015
	neo rev	ATGGTACCTCAGAAGAACT CGTCAAGAAG	
	neo1 fw	AGG ATC TCC TGT CAT CTC ACC TTG CTC CTG	
	neo1 rev	AAG AAC TCG TCA AGA AGG CGA TAG AAG GCG	
	neo2 fw	ACGATATCCCCGGGCAGTG TGGTTTTGCAAGAG	Jackson et al., 1995

Supplemental Table 1. Primer Sequences (cont.)

	neo2 rev	GAGATATCCCCGGGCTGCAGGTCGACGGATCC	
	neo3 fw	TATAACCAGGTTCAGAAGAACTCGTCAAG	
	neo3 rev	TTAAAGGACCCGATGAGGATCGTTTCGCATG	
	neo4 fw	TGCTCCTGCCGAGAAAGTATCCATCATGGC	Core MG. PCR., 2015
	neo4 rev	CGCCAAGCTCTTCAGCAATATCACGGGTAG	
Streptomycin	SA fw	AGCAGAGCGCGCCTTCGCTG	Pezzella et al., 2004
	SB rev	CCAAAGCCCACTTCACCGAC	
	rpsl fw	GGCATGGCCGACAAACAGAACG	Jagielski et al., 2014
	rpsl rev	ACTGGGTGACCAACTGCGATCC	
	rrs fw	TGGCCATGCTCTTGATGC	

Supplemental Table 1. Primer Sequences (cont.)

	rrs rev	CGCCCACTACAGACAAGAAC	
	rrs-seg fw	TTTACGGCGTGGACTACC	
	rrs-seg rev	CAGTAACTGACGCTGAGGAG	
	gidB fw	GGAGTGCGTAATGTCTCC	
	GIdB rev	GTCGGTGGTGTCATTTCC	
Sulfamethoxazole	sul1 fw	CTTCGATGAGAGCCGGCGGC	Aarestrup et al., 2003
	sul1 rev	GCAAGGCGGAAACCCGCGCC	
	sul2 fw	GCGCTCAAGGCAGATGGCATT	
	sul2 rev	GCGTTTGATACCGGCACCCGT	
	SUL2 fw	AGGGGGCAGATGTGATCGAC	Hochhut et al., 2001
	SUL2 rev	TGTGCGGATGAAGTCAGCTCC	

Supplemental Table 1. Primer Sequences (cont.)

	sulA fw	CAC TGC CAC AAG CCG TAA	Gebreyes et al., 2002
	sulA rev	GTC CGC CTC AGC AAT ATC	
Sulfamethizole	sul1* fw	CGGCGTGGGCTACCTGAACG	Kerrn et al., 2002
	sul1b* rev	GCCGATCGCGTGAAGTTCCG	
	Sul 2* fw	GCGCTCAAGGCAGATGGCATT	Hammerum et al., 2006
	Sul 2-B* rev	GCGTTTGATACCGGCACCCGT	
	Sul3* fw	GAGCAAGATTTTTGGAATCG	
	Sul3-B* rev	CTAACCTAGGGCTTTGGATAT	
Tetracycline	Tet A fw	GCGCGATCTGGTTCACTCG	Macauley et al., 2007
	Tet A rev	AGTCGACAGYRGCGCCGGC	

Supplemental Table 1. Primer Sequences (cont.)

	Tet B fw	TACGTGAATTTATTGCTTCGG	
	Tet B rev	ATACAGCATCCAAAGCGCAC	
	Tet C fw	GCGGGATATCGTCCATTCCG	
	Tet C rev	GCGTAGAGGATCCACAGGACG	
	Tet D fw	GGAATATCTCCCGGAAGCGG	
	Tet D rev	CACATTGGACAGTGCCAGCAG	
	Tet E fw	GTTATTACGGGAGTTTGTTGG	
	Tet E rev	AATACAACACCCACACTACGC	
	Tet G fw	GCAGAGCAGGTCGCTGG	

Supplemental Table 1. Primer Sequences (cont.)

	Tet G rev	CCYGCAAGAGAAGCCAGAAG	
	Tet H fw	CAGTGAAAATTCACTGGCAAC	
	Tet H rev	ATCCAAAGTGTGGTTGAGAAT	
	Tet J fw	CGAAAACAGACTCGCCAATC	
	Tet J rev	TCCATAATGAGGTGGGGC	
	Tet Y fw	ATTTGTACCGGCAGAGCAAAC	
	Tet Y rev	GGCGCTGCCGCCATTATGC	
	Tet Z fw	CCTTCTCGACCAGGTCGG	
	Tet Z fw	ACCCACAGCGTGTCCGTC	
	Tet 30 fw	CATCTTGGTCGAGGTGACTGG	
	Tet 30 rev	ACGAGCACCCAGCCGAGC	

Supplemental Table 1. Primer Sequences (cont.)

Vancomycin	Van A1 fw	GGGAAAACGACAATTGC	Ghidán et al., 2000
	Van A2 rev	GTACAATGCCGTTA	
	vanA fw	CATGAATAGAATAAAAGTTGCAATA	Jae Young et al., 2007
	vanA rev	CCCCTTTAACGCTAATACGATCAA	
	vanB fw	GTGACAAACCGGAGGCGAGGA	
	vanB rev	CCGCCATCCTCCTGCAAAAAA	
	vanC1 fw	GGTATCAAGGAAACCTC	
	vanC1 rev	CTTCCGCCATCATAGCT	
	vanC23 fw	CGGGGAAGATGGCAGTAT	
	vanC23 rev	CGCAGGGACGGTGATTTT	
	VanS fw	AACGACTATTCCAAACTAGAAC	
	VanS1 rev	GCTGGAAGCTCTACCCTAAA	
	p3967 fw	ATGAGCGATAAAATACTT	

Supplemental Table 1. Primer Sequences (cont.)

	p5786 rev	TTAGGACCTCCTTTTATC	
	p4649 fw	TTGGTTATAAAATTGAAAAAT	
	p6969 rev	CTATTCATGCTCCTGTCT	
	p6081 fw	ATGAATAACATCGGCATTAC	
	p8607 rev	TTATTTAACGGGGAAATC	
	p8061 fw	ATGGAAATAGGATTTACTTT	
	p9944 rev	TTACCTCCTTGAATTAGTAT	
	p9052 fw	ATGAAGAAGTTGTTTTTTTTA	
	p10585 rev	CTTACACGTAATTTATTC	
	vanZ-1 fw	ATCTGGTTAGTGTTATTCAAA	
	vanZ-2 rev	GATTCATATGCTTATTGCTTA	

V. SOAP LAKE AND THE SALTON SEA: A COMPARISON OF MICROBIAL DIVERSITY WITH RESPECT TO CHEMICAL AND PHYSICAL PROPERTIES OF THE WATER AND SOIL SEDIMENTS

This introduction has been prepared as part of a joint paper with Dr. Matthias Hess. This manuscript will be prepared for submission to *PLosS One* or *Frontiers*.

Introduction

Inland bodies of water are comprised of both freshwater and saline water, with 50% of these water bodies being saline (Williams 1996). Despite the prevalence of inland saline bodies of water, information about the microbial diversity of hypersaline lakes is somewhat limited and even less information known about the effects of different physical and chemical characteristics of these hypersaline environments on the microbial communities.

The total salinity of a body of water is often controlled by the concentrations of four major cations; Ca^{+2}, Mg^{+2}, Na^{+}, K^{+}, and four major anions; HCO^{-3}, CO^{-3}, SO_4^{-2}, Cl^{-}. In systems that are considered open, having outlets, the salinity of the water body is controlled by the composition of the influents and the atmosphere. However, in closed systems, having no outlets, as is the case in most saline lakes, the salinity of the system increases via evaporation and precipitation of salts into the soil sediments (Wetzel 1983). Saline lakes are able to form and persist only when the water outflow is restricted, the rate of evaporation exceeds that of the inflow, and the inflow into the basin is great enough to sustain a standing body of water (Wetzel 1983).

One category of saline lakes is soda lakes. Soda lakes are characterized as highly alkaline environments maintained primarily through evaporation (Jones *et al.* 1998). The typical soda lake has a pH that lies between a pH of 9.0 to 12.0, high salinities, and abnormally high concentrations of several dissolved salts including sodium chloride, carbonate and bicarbonates (Anderson 1958). Soda lakes maintain their alkaline conditions due to the highly buffered nature of the waters and are considered to be among the most stable alkaline environments on Earth (Jones *et al.* 1998). Two examples of

hypersaline, soda lakes are the Salton Sea, located in southern California, and Soap Lake, located in central Washington. Both of these lakes are located in semi arid regions of their respective states and the water levels of both lakes are maintained mainly via evaporation due to a distinct lack of surface outlets (Anderson 1958; Holdren & Montano 2002). These two lakes chosen for this study are both distinct and unique examples of hypersaline, soda lakes.

Soap Lake is a highly eutrophic, meromictic, soda lake. Meromixis can be caused by a number of different factors including changes in the waters density due to salinity, formation of the water body in an endorheic basin, and a lack of episodic mixing mechanisms (Dimitriu *et al.* 2008). It speculated that the aerobic and anaerobic waters of Soap Lake have not mixed in over 2000 years (Peyton & Yonge 2002). Soap Lake is the terminal lake in the chain of lakes that formed in the Lower Grand Coulee, a basalt canyon. Soap Lake has no surface inlets or outlets and it is the lack of outlets that is attributed to being the chief reason for the lakes increasing salinity (Anderson 1958). Soap Lake's water levels are supplied by water runoff from cliffs and plateaus surrounding the lake and from groundwater seepage, with evaporation as the main method for water loss (Anderson 1958).

In comparison, the Salton Sea is a moderately alkaline, hypersaline, eutrophic lake that was formed in the Salton Basin (Holdren & Montano 2002). The formation of this lake occurred between 1905 and 1907 due to a flooding of the Colorado River into the Salton Basin (Swan *et al.* 2010). Since the lakes formation, it has been gradually increasing in salinity due to a lack of surface outlets and evaporation, the main cause of water loss. The Salton Sea is the largest lake in California but it is only about 8m in depth. The Salton Sea is a moderately alkaline environment, with saline concentrations that are higher than most seawater (35 gL^{-1}).

These two lakes are both located in arid environments and their water levels are maintained via evaporation. Soap Lake has no surface inlets or outlets (Anderson 1958), while in contrast, the Salton Sea has three major inlets, the Alamo River, the New River, and the Whitewater River. Due to their high rates of evaporation, these lakes have been increasing in salinity since their creation. The mixolimnion of Soap Lake has a salinity of 15 gL^{-1} and the anaerobic monimolimnion contains 140 gL^{-1} (Sorokin *et al.* 2007).

The Salton Sea's current salinity measurements for the lake are between 48 to 50 gL^{-1} (Swan *et al.* 2010).

The alkalinity of both of these lakes far exceeds that of most freshwater systems, which is normally at or slightly above neutral pH. The pH of the Salton Sea occurs around 8.6 to 8.7 (Holdren & Montano 2002), while the alkalinity of Soap Lake is maintained at a nearly constant pH of 9.8 in both the mixolimnion and the momimolimnion (Dimitriu *et al.* 2008). In both Soap Lake and the Salton Sea, the alkalinity of the lakes is controlled by the concentrations of carbonates and bicarbonates within the system. However, in the Salton Sea, the levels of carbonates are much lower than those of the bicarbonates. The carbonate concentration of the Salton Sea is 2 mgL^{-1}, while the bicarbonates where found to be 245 mgL^{-1} (Holdren & Montano 2002). Similarly, the concentrations of carbonates in the mixolimnion of Soap Lake average around 8,500 mgL^{-1} and 24,000 mgL^{-1} in the monimolimnion. Comparatively, the concentrations of bicarbonates in Soap Lake were always found to be lower than the carbonates with 2000 mgL^{-1} in the mixolimnion and 4,800 mgL^{-1} in the monimolimnion (Anderson 1958).

Both of these two lakes are subject to agricultural drainage into the lake. The Salton Sea was initially designated as an agricultural drainage reservoir in 1924 by the federal government. Agricultural irrigation or municipal wastewaters make up 88% of the water inflow into the lake (Wood *et al.* 2002). Soap Lake is also subject to a large amount of agricultural runoff. Grant County, the county in which Soap Lake is located, has the second largest land area devoted to orchids in Washington State (USDA 2002). Since Soap Lake is maintained in large part due to ground water runoff, it is subjected to agricultural contaminants. Due to the agricultural runoff and lack of surface outlets, both lakes have accumulated toxic inorganic compounds. The levels of these toxic inorganic compounds are likely to impact the structure and function of the microbial diversity of these systems.

The long term meromictic state of Soap Lake has led to the accumulation of a number of toxic inorganic compounds in the anaerobic layers of the lake. Of particular note are the extremely high levels of ammonia, 59.8 mM, and sulfide, 140 mM (Sorokin *et al.* 2007; Dimitriu *et al.* 2008). Soap Lake is currently considered to have the highest

naturally occurring sulfide content in world (Sorokin *et al.* 2007). In the anoxygenic monimolimnion of Soap Lake, the concentrations of sulfide may likely be high enough to directly affect microbial process such as denitrification (Dimitriu *et al.* 2008). In addition, the ammonia in the lake is likely in the non-dissociated form due to the highly alkaline conditions of the lake. This form of ammonia is toxic and it has been suggested that this might directly inhibit microbial processes such as methane oxidation (Dimitriu *et al.* 2008).

While the Salton Sea does not possess toxic levels of ammonia, it does have high levels of sulfides, 10,500 mgL^{-1}, and magnesium, 1,400 mgL^{-1}, (Holdren & Montano 2002). In addition, the Salton Sea possesses high concentrations of selenium. Selenium is considered to be an essential toxin because it is both an essential nutrient and an environmental toxin (VillaRomero *et al.* 2013). The concentrations of selenium have been reported to be as high as 300 μgL^{-1} in the river drainages that enter into Salton Sea (Schroeder *et al.* 1988). This value far exceeds the national water quality criteria of 5 μgL^{-1} (US EPA 2002). Due to the high levels of selenium and other compounds in this lake, the microbial diversity is expected to be very different from that of a typical freshwater lake or even other soda lakes, such as Soap Lake.

The microbial diversity of freshwater environments have been intensively studied, however the microbial diversity of inland saline bodies of water is highly understudied and little information is currently known about these communities or the driving influences of these communities. The goal of this study is to assess the microbial diversity of two distinctly different hypersaline lakes and compare the populations with respects to the chemical and physical properties of each environment.

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SECTION

2. CONCLUSIONS

This research has provided valuable insight into the effects of alkalinity on the antibiotic susceptibilities of a novel species of bacteria, *Halomonas eurialkalitoleranis*. The results of this work have indicated that high alkalinity greatly decreases the effectiveness of several antibiotics including tetracycline, ampicillin, neomycin, demeclocycline, sulfamethizole, kanamycin, chloramphenicol, streptomycin, and roxithromycin. Interestingly, erythromycin and sulfamethizole were found to be more effective in alkaline media. Only vancomycin was found to have no statistical differences between any pH value that was tested. Five strains of *Halomonas eurialkalitoleranis* were screened for known antibiotic resistance genes for ten of the twelve antibiotics assated under alkaline conditions. Antibiotic resistance genes were found for all antibiotics screened except streptomycin and kanamycin. The antibiotic resistances seen by Soap Lake isolates is largely due to the impact of alkaline conditions on the antibiotics themselves and not entirely due to the presence of antibiotic resistance conferring genes.

The implications of this research are great when considering the antibiotic resistances of extremophilic microorganisms. Extreme environments are likely subject to contamination by antibiotics due to both point and non-point sources. The bacteria isolated from these environments harbor antibiotic resistance genes that could contribute to the resistome. In addition, the antibiotics themselves can be altered by the extremes of pH, temperature, ect., and rendered ineffective. This needs to be taken into account when characterizing new species of microorganisms.

A large amount of work is still needed to finalize the characterization assays of the five strains of *Halomonas eurialkalitoleranis*. These strains have been sent to Dr. Antonio Ventosa at the Department of Microbiology and Parasitology, in the University of Sevilla, Spain for analysis. Dr. Ventosa will complete the DNA-DNA hybridization of the strains with most closely related species, the G+C content of the five bacterial isolates, and will re-sequence the 16S rRNA gene of isolate 10. This work will enable the completion of the characterization of *Halomonas eurialkalitoleranis.* After this work has been completed, a manuscript describing the new species will be submitted to the *International Journal of Systematic and Evolutionary Microbiology*.

A large amount of work is still needed to further understand the antibiotic resistances of *Halomonas eurialkalitoleranis.* The PCR results of these experiments has indicated that all five strains have multiple antibiotic resistance genes. However, the exact function and sequence of these genes has not yet been determined. Information available in the primary literature has indicated that antibiotic resistance genes have been known to aid bacteria in evading the actions of toxic heavy metals. Studies to determine the heavy metal tolerances of the five strains should be determined. Subsequently, to determine the function of the genes detected by PCR, these genes will need to be isolated and sequenced. After sequencing results have been gathered, they can be aligned to known antibiotic resistance genes. This will give insight into the possible functions of these genes. Any genes that display significant differences from known genes should be further studied to determine their functions and activity.

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VITA

Tiffany Charlynn Edwards was born on August, 14th, 1991 in Everett, Washington. She was raised by her mother, Teresa Edwards. She attended Malta Bend R-V high school where she graduated at the top of her class and received the honors of Valedictorian. She then spent a year abroad as exchange student at Rekarnegymnasiet, in Eskilstuna, Sweden. She then went on to receive her Bachelor's of Science degree in Biological Sciences from the Missouri University of Science and Technology, in May of 2013. Tiffany began working in research in her sophomore year in Dr. Nuran Ercal's biochemistry laboratory studying the effects of a novel thiol antioxidant, N-acetylcysteine Amide (NACA), on treating rats with cataracts. After two years in the biochemistry laboratory, she began working with Dr. Melanie Mormile in an environmental microbiology laboratory. She began a project where she investigated the pH dependent antibiotic resistance of novel bacteria isolated from Soap Lake, Washington. Tiffany earned her Master's of Science degree in Applied and Environmental Biology from the Missouri University of Science and Technology in August of 2015. She continued and expanded the original project with Dr. Mormile. As a graduate student Tiffany received the honor of Bio Star Graduate Teaching Assistant of the Year for her work as a teaching assistant in the microbiology laboratory. She traveled to both the regional and national meeting for the American Society of Microbiology to present her research. For the 2015 American Society of Microbiology meeting, she was granted a travel grant and her work was featured in the ASM 2015 press room. After graduating in August 2015 she plans to attend Saint Louis University to obtain her PhD where she will focus on medical microbiology and virology.