2017

Using Extremophile Bacteriophage Discovery in a STEM Education Professional Development Partnership to Explore Model Classroom Research Experiences Integrating the Three Dimentions of the Next Generation Science Standards

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USING EXTREMOPHILE BACTERIOPHAGE DISCOVERY IN A STEM EDUCATION PROFESSIONAL DEVELOPMENT PARTNERSHIP TO EXPLORE MODEL CLASSROOM RESEARCH EXPERIENCES INTEGRATING THE THREE DIMENSIONS OF THE NEXT GENERATION SCIENCE STANDARDS

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN

APPLIED IMMUNONOLOGY AND INFECTIOUS DISEASE

UNIVERSITY OF SOUTHERN MAINE

DEPARTMENT OF APPLIED MEDICAL SCIENCES

BY

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MAY 2017

FINAL APPROVAL FORM

THE UNIVERSITY OF SOUTHERN MAINE

We hereby recommend that the thesis of Carrie Boudreau entitled "Using Extremophile Bacteriophage Discovery in a STEM Education Professional Development Partnership to Explore Model Classroom Research Experiences Integrating the Three Dimensions of the Next Generation Science Standards", be accepted in partial fulfillment of the requirements for the degree of Master of Science in Applied Medical Sciences.

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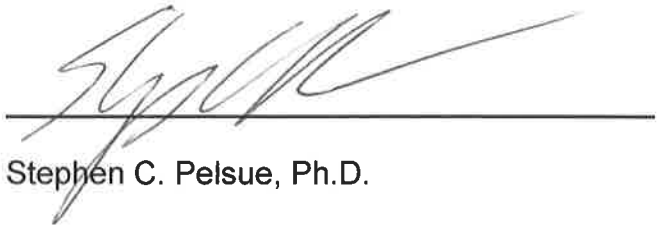

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ACKNOWLEDGEMENTS

Thank you to my mother and grandmother for providing me with moral support and offering me encouragement to see this endeavor to completion. Thank you to the many members of the Duboise lab and my Applied Medical Science department advisors who not only helped me through this project, but also shared their knowledge with my middle school students. My advisor Dr. S. Monroe Duboise instilled in me his enthusiasm of bacteriophage discovery, provided continuous support of my pursuit of a degree even when logistics were a challenge, and remains dedicated to educating K-12 teachers. Dr.Gail Fletcher provided support with integration of microbiology related practices in the lab and in my classroom. Karen Moulton was paramount in helping everyone, including my middle school students, understand the application of a Scanning Electron Microscope and a Transmission Electron Microscope. Jeff Gaynor, Miad Movassaghi, and Jennifer Jamison all patiently walked me through the steps of understanding molecular interactions between bacteria, bacteriophage and biotechnology techniques. Most influential to my understanding of molecular interactions, genetics, and the application of biotechnology were Dr. Naun Lobo, Dr. Stephen C. Pelsue and Dr. Ah-Kung Ng; each has a special way of making the complicated interactions understandable and meaningful. Of equal priority in my gratitude is Dr. Chris Maher for her ability to help me organize a three part project while supporting me through the thesis writing process.

ABSTRACT

The National Research Council's (NRC) *A Framework for K-12 Science Education: Practices, Crosscutting Concepts, and Core Ideas* describes a vision of what it means to be proficient in science. The project discussed in this thesis was developed with a NIH SEPA Grant 8R25OD010937 to the Virology and Electron Microscopy Laboratory at the University of Southern Maine (USM) under the direction of Dr. S. Monroe Duboise. The goal of the project was to explore using discovery of extreme environment bacteria and their bacteriophages as a model for using the three dimensions of learning to teach Next Generation Science Standards (NGSS). Specifically, bacteria were isolated from the haloalkaline environment of a soda lake and the acidic environment of fermented liquids. The isolated bacteria were then used as potential hosts to detect associated bacteriophage. Through the practice of isolating bacteria and detecting their bacteriophage, the development of real world research techniques is established. As culture conditions are manipulated and resulting effects are observed, fundamental knowledge is gained and crosscutting concepts are recognized. The key bacteria isolated in this project, identified through 16s rDNA comparison, most closely resembled *Alkalimonas collagenimarina and Gluconobacter oxydans.* They were isolated at room temperature, making them easy to maintain for study, and they were partially classified through gram-staining, compound light microscopy, and manipulation of culture contents through pH, carbon source, and salinity. Once genetically identical colonies were established, they were used as hosts for bacteriophage discovery. Bacteriophage

were isolated readily from the haloalkaline environment; however, they were more difficult to isolate from acidic environments. Using microscopy, microbiology, and basic biotechnology to isolate, compare, and identify bacteria and their bacteriophage from samples collected in an extreme environment is an approach that can help students to develop a multilevel understanding of the effects that environmental conditions have on cell survival. With support from a university or biotechnology company, “Discovery of an Extremophile Bacteria and Its Bacteriophage” can be developed as an engaging model system for a middle school or high school classroom.

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INTRODUCTION

The project discussed in this thesis was developed with a National Institutes of Health Science Education Partnership Award (NIH SEPA Grant 8R25OD010937) to the Virology and Electron Microscopy Laboratory at the University of Southern Maine (USM) under the direction of Dr. S. Monroe Duboise. This laboratory, along with colleagues in the Department of Applied Medical Sciences and others, has been active for many years in science education collaborations with K-12 educators. Success of past efforts have been reported previously (Duboise, et al., 2009; Serio, et al. 2009; Frankel, et al., 2010). The SEPA project has emphasized professional development workshops for K-12 educators and project-based laboratory experiences, especially in the areas of microbiology, virology, molecular biology, and infectious disease epidemiology. In recent years, the Duboise laboratory group has been most focused upon environmental microbiology and molecular genetics of extremophilic host-bacteriophage systems. This thesis explores potential classroom research activities using bacteria and bacteriophages from haloalkaline and acidic environments with the goal that secondary school educators can apply such activities to meet the challenges inherent in integrating the framework of three dimensions (Science and Engineering Practices, Crosscutting Concepts, and Disciplinary Core Ideas) that are pervasive in the Next Generation Science Standards (NGSS).

The Next Generation Science Standards (NGSS) suggest middle school science students are capable of answering questions such as, “How do atomic and molecular interactions explain the properties of matter that we see and feel?”, “How does a system of living and nonliving things operate to meet the needs of the organisms in an ecosystem?”, “How do living organisms pass traits from one generation to the next?”, and “How do organisms change over time in response to changes in the environment?”. These broad questions require answers stipulating depth of content knowledge. The student must understand the answer to the first question to answer the remaining three questions. The student also must know and understand variables in an ecosystem along with the needs of an organism.

The pieces of information can be taught to all students, allowing all students to master content at a similar pace and chronology, or the entire question can be explored, allowing students to learn pieces as needed. In the first scenario, the student learns a piece of key information as though collecting pieces of a puzzle. The success of this method relies on the ability of the students to retain each piece, at least through the end of middle school, until they have all the pieces to construct the puzzle. This approach also assumes that every student realizes the impact of a missing or altered puzzle piece on the final product, which can be challenging for middle school students. Alternatively, the second scenario, which can be overwhelming initially, allows the learner to see the big picture up front and consider the pieces that created it. With a series of

challenges and the motivation of curiosity from inquisitive middle school students, they learn to manipulate an environment to change an outcome. With the learning more under their control and progressing at their own pace, the students are more likely to retain information and answer similar complex questions in the future (Abels, 2014).

To effectively meet objectives, the ideal method to teach science to middle school students is ~~in~~ constantly being revised; however, many educators agree they want students to have a meaningful experience that results in a lasting gain of content knowledge while obtaining basic science laboratory skills (Skinner & Cannon, 2000). Teaching science through inquiry, i.e., inquiry based instruction, is an approach that gives classroom-bound students the most authentic science experience (Gormally, et al., 2009). Through this approach, students learn content while gaining basic science skills such as microscopy, measurement with metric units, use of scientific models, scale, and collaboration and interpretation of authentic data while conducting meaningful experiments. Common topics of study include space, human body, cells, genetics, science inquiry, matter and energy, energy transformations, disease, weather, and ecosystems (NGSS, 2013). Teachers with strong science backgrounds and those with a modern middle school level philosophy believe students need authentic hands-on experiences to achieve the best outcome. The challenge is to generate a series of lessons that fulfills curriculum goals and stays within the school budget while

engaging all students (Skinner & Cannon, 2000; Armstrong, 2006; Hanauer, et al., 2006; Tachibana, 2015).

The NGSS, along with many generations of standards preceding them, present objectives that include learning about concrete topics while expecting discussions about abstract concepts (Armstrong, 2006; Witt & Ulmer, 2010). Scientific systems are complicated, and models developed by scientists that are aimed toward development of concrete understanding of minute or enormous systems can just as easily generate misconceptions (Armstrong, 2006; Witt & Ulmer, 2010). Students in middle school are in different places developmentally. They are truly in the middle, with some students who cannot imagine beyond the concrete cause and effect they see before them and other students who not only can predict what would happen, but who also already have thought of related experiments that test associated variables (Armstrong, 2006; Witt & Ulmer, 2010). Ideally, middle school faculty teach new concepts through a series of meaningful lessons that balances obtaining new information, simple to complex application of the information, opportunity for social interaction, and that ends with assessments enabling students to reflect their understanding of the information (Armstrong, 2006; Witt & Ulmer, 2010).

The most difficult part of teaching middle school students is keeping them engaged in learning (Armstrong, 2006; Witt & Ulmer, 2010). These students are

ready for a meaningful challenge. They are intrigued by puzzles, ready to test opinions, and eager to debate topics. They like nothing more than to either work toward a goal that will make a difference or just go play games (C. Boudreau, pers. obs.). Educators hope their middle school students have developed a positive learning experience in science topics, but students most likely enter middle school having been led by their teachers through simple science experiments and assessed on their ability to accurately complete worksheets by filling in keywords (Armstrong, 2006; Witt & Ulmer, 2010). Throughout their early education, they probably have not been expected to identify a scientific question or generate their own questions from a proposed problem. Only a lucky few have been involved in a community based science project (Armstrong, 2006; Witt & Ulmer, 2010). In contrast to those prior experiences, the middle school learner is most engaged when involved in projects that allow them to direct their learning, engage socially, and in some way relate those projects to themselves (Armstrong, 2006; Witt & Ulmer, 2010). Personal experience gained from 16 years of teaching middle school indicates students are most interested in topics related to self, such as topics related to the human body, particularly its pathophysiology, and learn best when they can interact with each other and the topic while learning. All students can relate to illness at some level, and many students have friends or relatives struggling with diseases such as asthma, diabetes, lyme disease, or cancer.

Experience and research indicate the greatest motivators for active learning involve novelty topics related to real world events (Armstrong, 2006; Witt & Ulmer, 2010). Students enjoy using microscopes to access the world of the unseen, so they are interested in learning about cells and intrigued by the microbes that infect them. They are curious about disease-causing agents, embrace stories of past epidemics, and immerse themselves in the mysteries associated with them. The documentary *The Great Fever* (Bosch, 2006) explains challenges faced by early scientists in their attempts to control the spread of yellow fever. Engaging students in “virus discovery” after viewing the documentary allows students to become real life investigators. Even though their discovery leads to finding a virus that attacks a bacterial cell versus a human cell, it helps them to gain specific knowledge needed to understand infection of an eukaryotic cell.

Viruses have been topics of interest not only because of their association with disease, but also for their modes of transmission and mechanisms of reproduction. They can contain and preserve the integrity of their DNA or RNA in a variety of environments including extreme environments. They contain enough genetic material to hijack a cell. They can exist in an environment undetected until they cause symptoms in a host. They can withstand time while holding onto cell specificity, which makes them ideal platforms for education, from using their specificity to target cancer cells, to knowing particular viruses that target eukaryotic or prokaryotic cells.

Returning to teaching middle school after having taught elementary school for four years and knowing about advances in development of biotechnology and increased awareness of global infectious diseases, I acted upon an opportunity to learn more about microscopy and infectious diseases by enrolling in a 2009 summer program funded by a SEPA grant and offered by faculty in the Duboise Lab at the University of Southern Maine. This program was the beginning of my journey in boosting my curriculum while learning how to discover bacteriophage. During the two-week program, I learned to gram stain bacteria (I thought this technique would help kids see the cell wall and learn how the cell walls of bacteria are different from eukaryotes). I learned that a virus is so small that it will not break while you grind tobacco with a mortar and pestle (I thought this process might help kids to understand scale). I learned that some viruses only infect bacteria (I thought this idea would be linked to a safe way to study viruses). I learned that you can find bacteriophage by the clearing of bacteria on a bacterial lawn (I thought this technique would be a fun way to use bacteria in my classroom if it were not a threat to human health). I learned that bacteriophage are host specific (I thought that if I have safe bacteria, then their bacteriophage would be safe for students, too). I learned that bacteria and their bacteriophage can be collected from the same environment (I thought my students would love a project where they were assigned to bring in a sample from their environment and told to become virus hunters). Since this workshop, I have tried to develop a safe, economical way to incorporate this activity into my middle school

curriculum. During a 2012 SEPA summer workshop, “Interdisciplinary Collaboration in Research and Education”, we discovered bacteriophage from a haloalkaline environment using laboratory equipment common in the secondary school lab and accessible through a partnership with a local university or biotechnology company. The Mono Lake bacteria were grown on a medium that is saline and alkaline; therefore, it selects against common microbes from other environments such as those in the classroom that potentially would be pathogenic to humans.

No soda lakes occur in New England. Instead, equally extreme environments occur on the other end of the pH spectrum, i.e., areas of high acidity. A colleague, Jeff Gaynor, attempted to isolate bacteriophage from the Katahdin Iron Works from 2009 through 2012, but he was not successful at isolating lytic bacteria through simple methods (Gaynor, 2013). However, he observed bacteriophage particles and putative bacteriophage particles with transmission electron microscopy (TEM), particularly following ultraviolet induction of a lytic cycle by ultraviolet light treatment. He did not isolate the host bacteria easily, nor was he able to easily locate lysogenic bacteriophage, as was possible with samples from Mono Lake (Gaynor, 2013).

An easily accessible source of acid-tolerant bacteria that also contained associated bacteriophage is needed for practical application in a public school

classroom. Dr. Duboise encouraged exploration of fermented liquids such as kombucha tea, vinegar, and apple cider. These liquids are fermented by the metabolism of sugar by bacteria or a symbiotic culture of bacteria and yeast (SCOBY). They are produced for consumption by humans, indicating that associated bacteria are nonpathogenic and therefore safe for classroom investigation.

Bacterial Extremophiles

Extremophiles are organisms that can survive in environments where the majority of life cannot survive (Islas, et al., 2007). These extreme environments are harsh because resources such as oxygen, light, or water may not be readily available, temperatures climb to levels that normally degrade DNA, and nutrients such as salts, minerals, and metals reach toxic levels (Islas, et al., 2007). To survive in these harsh conditions, the organisms must evolve adaptations that protect them from extremes on the pH spectrum and the temperature spectrum while maintaining the ability to obtain energy and excrete waste (Islas, et al., 2007). Their unique adaptations are used to understand where life could exist, to understand limitations of survival, and as model systems to learn how organisms obtain energy while maintaining homeostasis (Padan, et al., 2005). Environmental conditions can be compared to those suspected on other planets and early Earth to predict favorable conditions for survival of prokaryotes (Rothschild & Mancinelli, 2001). The observed survival mechanisms can then be used as a model to anticipate potential for survival in similar conditions by similar

organisms (Rothschild & Mancinelli, 2001). New knowledge gained from the study of extremophiles is applied to advances in the food, medical, and biotechnology industries, making them exciting topics of investigation (Drulis-Kawa, et al., 2012).

The 1977 Alvin exploration of hypothermal vents off the coast of the Galapagos Islands led to the discovery of hyperthermophiles, prokaryotes that can survive temperatures as high as 113°C and thrive in temperatures starting at 80°C. They can obtain energy anaerobically or aerobically through chemosynthesis where they use energy from the oxidation of hydrogen sulfide instead of sunlight to convert carbon dioxide and available nutrients into organic matter (Islas, et al., 2007). Understanding how hyperthermophiles obtain energy without photosynthesis led to the discovery of extremophiles in other extreme environments, because knowing that organisms could obtain energy in the absence of light and in low nutrient conditions inspired scientists to look for organisms in other environments where life was not thought to exist (Islas, et al., 2007).

The two extreme environments explored in this study were the haloalkaline environment of a soda lake (Grant, 2006) and the acidic environment of fermented food products, including kombucha tea, vinegar, wine,

and apple cider. These two environments were chosen for their accessibility, safety, and ability to duplicate conditions in the classroom laboratory setting.

Haloalkaliphiles demonstrate optimal growth at salinity levels above 100 g/L (Oren, 2002) and pH > 8 (Gonzalez, et al., 2010). Some alkaliphiles can withstand pH > 11, but of most interest in this study were obligate alkaliphiles incapable of growth at pH < 8. Biological activity and chemical leaching generate a range of pH in many environments, therefore allowing alkaliphiles to survive in a variety of ecosystems, but haloalkaliphilic organisms also can withstand high salinity levels (Oren, 2002). Evidence collected by Mars rovers *Spirit* and *Opportunity* revealed the presence of water and rocks with minerals similar to those on Earth that are necessary for life. As water on Mars evaporated, it would have become increasingly saline (McDonald et al., 1999). Organisms most able to survive in a haloalkaline environment could have been the last or only life on Mars. On Earth, a similar environment, but without the same type of temperature fluctuation and with an atmosphere, is found in the meromictic soda lakes of western North America, China, and East Africa (Humayoun, et al., 2003; Ma, et al., 2004; Grant, 2006).

Soda lakes such as Mono Lake in California contain many micro-environments, allowing them to serve as ideal sites for exploration of the boundaries of life (Humayoun, et al., 2003; Ma, et al., 2004; Grant, 2006). Mono

Lake is a hypersaline alkaline lake with salinity measuring 84 g/L to 94 g/L and pH 9.8 (Humayoun, et al., 2003). Each layer of the lake, including surface waters, the mixolimnion (2 m), the base of the oxycline (17.5 m), the upper chemocline (23 m), and the monimolimnion (35 m), contains its own life-challenging conditions with varying oxygen, temperature, salinity, alkalinity, and density (Humayoun, et al., 2003).

Acetic Acid Bacteria drive Fermentation in the Food Industry

Acetobacteraceae is a family of obligatory aerobic, rod shaped bacteria once comprised of 6 genera: *Acetobacter, Acidomonas, Asaia, Gluconacetobacter, Gluconobacter, and Gluconobacter*, but it now includes as many as 11 genera (Muramatsu, et al., 2009). Members of the Acetobacteraceae, with the exception of *Asaia*, are characterized by the ability to oxidize ethanol to acetic acid even when accumulation of acetic acid lowers the pH (Markov, et al., 2006; Wee, et al., 2011).

Among the 11 genera of Acetobacteraceae, three distinct groups, *Acetobacter, Gluconobacter*, and *Gluconacetobacter,* are associated with vinegar, wine, and kombucha tea production and spoilage (Mamlouk & Gullo, 2013). Knowing and understanding the microorganisms involved is important to both successful fermentation and spoilage prevention. Identification of Acetic Acid Bacteria (AAB) and their separation from other bacteria commonly begins

with content-specific culture media (Sharafi, et al., 2010), followed by sequencing the ribosomal RNA gene, 16s rDNA (Weisburg, et al., 1991; Woo, et al., 2008; Marsh, et al., 2014). Initial grouping of AAB bacteria can be achieved with changes in the culture media including addition of chemical indicators (Sharafi, et al., 2010), but the high degree of homology among the AAB genera and between species makes it difficult to identify AAB to the species level without specifically considering the 16s–23s rDNA internally transcribed spacer regions (Trcek, 2002).

Kombucha Tea Fermentation by Acetic Acid Bacteria (AAB) and Yeast

Kombucha tea is a beverage produced by fermentation driven by the metabolism of yeast and AAB existing in a symbiotic relationship. Kombucha tea starts when a symbiotic culture of bacteria and yeast (SCOBY) is added to a solution of brewed black or green tea, organic sugar, vinegar (for acidity), and water (Jayabalan, et al., 2014). Through enzymatic hydrolysis, yeasts digest sucrose into ethanol, free glucose, and fructose as the AAB metabolize ethanol with oxygen and link glucose molecules into pure forms of cellulose through a series of phosphorylation steps and oxidation processes (Ross, 1991; We, et al., 2011; Zhang, et al., 2014). As cellulose microfibrils form, they are secreted from pores in the cell wall and the final cellulose ribbon forms (Ross, 1991). Eventually, enough cellulose is secreted to form a film, which floats to the surface of the liquid, bringing the bacteria with it. Now at the surface, the aerobic bacteria have a better supply of oxygen (Ross, 1991; Skinner & Cannon, 2000).

Experimenting In the Classroom

Cellulose and acetic acid production by AAB depends on several factors including nitrogen source (black tea), carbon source (sucrose), surface area of the brewing vessel, broth depth, and temperature (Lončar, et al., 2006; Soheir, 2012). All of these variables can easily be controlled and evaluated in the middle school classroom, making AAB a good model for investigating effects of environment on growth and reproduction, which is described in the NGSS standard MS-LS1-5, *Construct a scientific explanation based on evidence for how environmental and genetic factors influence the growth of organisms.*

Optimal length of kombucha fermentation depends on many factors, with temperature and carbon source being the most influential (Vina, et al., 2013). The desired concentration of metabolites, such as glucuronic acid, as well as the biodiversity of the SCOBY, varies throughout the fermentation process. Fermentation could reach its desired peak between 7 and 21 days at a temperature of 25-32°C (Vina, et al., 2013). Culture pH decreases during the fermentation process, but eventually it stabilizes around pH 3 (Jayabalan, et al., 2008). In the first few days of fermentation, the growing population of symbiotic organisms increases the carbon dioxide concentration. Increased carbon dioxide increases acidity. Next, hydrocarbonate anions (HCO_3^-) are produced, which react with hydrogen ions (H^+) produced by organic acids. The HCO_3-H

relationship is thought to create a natural buffer system, which prevents the medium from becoming too acidic for the yeast (Jayabalan, et al., 2008).

History of Bacteriophage Discovery

Bacteriophage are viruses that use bacterial cells as hosts for replicating genetic material. Environmentally ubiquitous, the bacteriophage exists undetected by humans until it is of specific scientific interest. Bacteriophage bind to specific receptors on specific host cell types. In nature, the only cells that display those receptors are those likely to generate more bacteriophage particles; otherwise, natural selection would eliminate the bacteriophage (Hatfull, 2008; Norkin, 2010).

The bacteriophage, discovered nearly simultaneously in 1915 by Frederick W. Twort and in 1917 by Felix d'Herelle, was named by d'Herelle; "phage" suggested the microbes were bacteria eaters (Norkin, 2010). Although the bacteria-specific virus does not actually eat bacteria, they eventually kill the bacterial host cell to be released back into the environment. Bacteriophage likely evolved simultaneously with their bacterial hosts (Williams, 2013).

d'Herelle initiated the idea of using bacteriophage as therapeutic agents aimed at treating bacterial diseases. He was not successful in his efforts, but he improved our understanding of bacteriophage. Advancements in molecular biology

continue to encourage researchers to revisit bacteriophage as “antibiotics” (Norkin, 2010).

In 1933, Max Schlesinger used high speed centrifugation to purify a bacteriophage and demonstrated its composition of 50% protein and 50% DNA (Norkin, 2010). Wendell Stanley used crystallized tobacco mosaic virus (TMV) and crystallography to demonstrate the composition and regular structure of TMV, demonstrating that viruses produce copies with proteins and nucleic acids or in combination. Previously, Frederick Bawden and Norman Pirie demonstrated that TMV contained not just protein (94%) but also RNA (6%) (Norkin, 2010).

In 1938 Max Delbrück initiated use of viruses as ideal systems for gene studies and thus sparked modern bacteriophage research. From his quantum physics approach, he viewed gene mutations and developed the idea of genes as molecules. Simultaneously, Salvador Luria developed the idea of genes as molecules and bacteriophage as models. Together, they worked on the life cycle of bacteriophages and the physical basis of heredity by analyzing bacteriophage replication (Norkin, 2010).

Today, bacteriophage can be analyzed in many ways, including PCR, gel electrophoresis, restriction digests, and sequencing (Woo, et al., 2008; Goto, et al., 2010). For accurate initial characterization and analysis of progeny

bacteriophage harvested from host bacteria, bacteriophage must be isolated from cell debris such as stray bacterial proteins, nucleic acids, ribosomes, and cell membrane parts. The debris may also include lipopolysaccharides (LPS), a component of the outer membrane of gram negative bacteria, or endotoxins. Because the bacteriophage will be reproduced based on its genetics and possibly used for “phage therapy” treatment of bacterial infections, stray non-phage related debris must be eliminated (Abedon, et al., 2011). Once purified, the structure and functional characteristics of the bacteriophage can be analyzed.

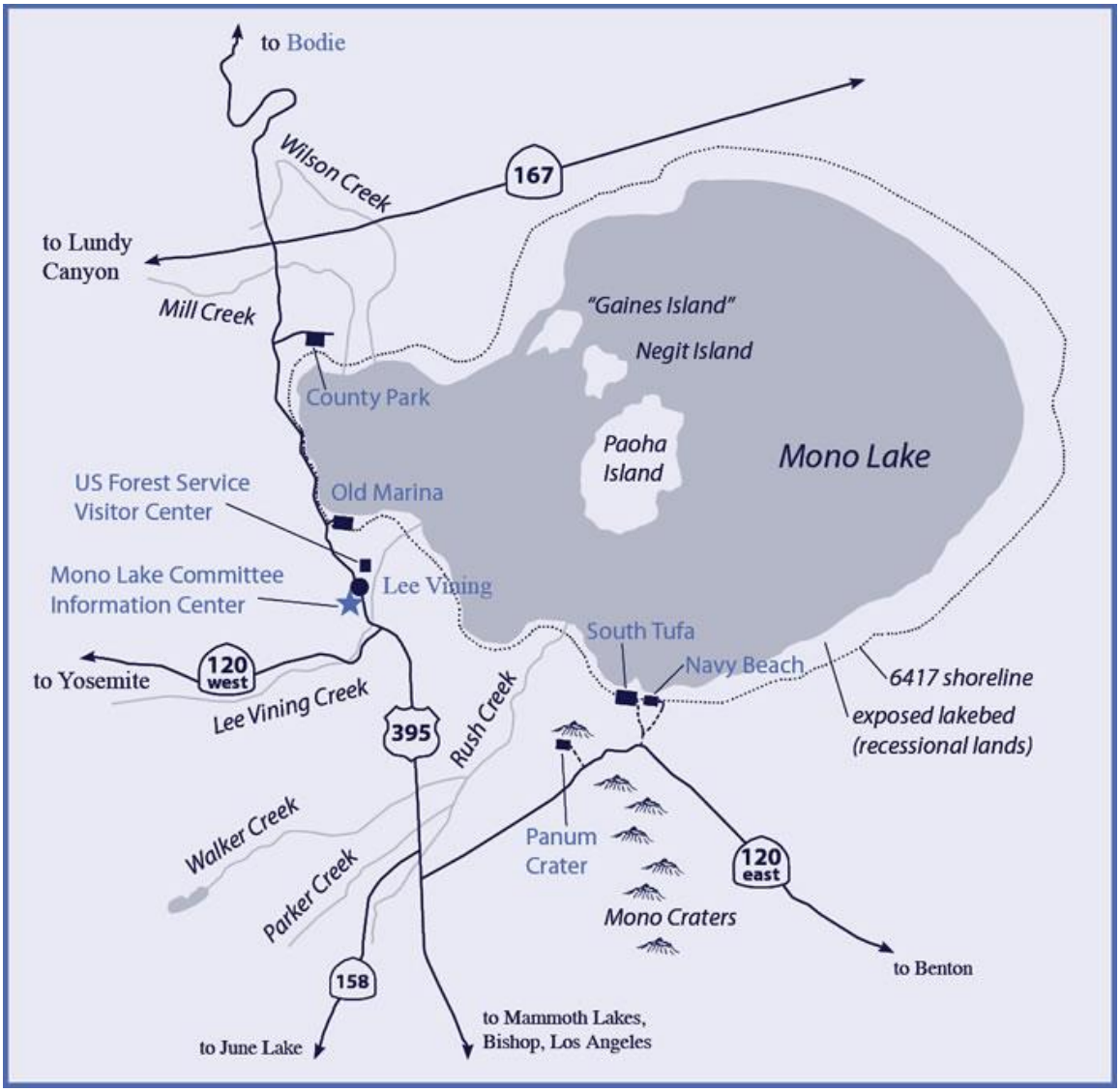
My objectives for this project were to isolate bacteria and bacteriophage from two extreme environments, a haloalkaline environment of a soda lake and an acidic environment of a fermented liquid, with the ultimate goal of translating my isolation methods and isolated organisms into an educational model that could be applied to the middle school classroom. In addition, I attempted to isolate a cellulose-producing AAB from a kombucha tea SCOBY. The pure form of cellulose produced from bacteria is sought for its use in medical, biotechnological, environmental, and biofuel applications (Lin, et al., 2013; Keshk, 2014). A bacteriophage that infects a cellulose-producing organism could contain important lysogenic proteins that in turn might be important to the bacterial cellulose industry (Lin, et al., 2013; Keshk, 2014).

MATERIALS AND METHODS

Isolating Bacterial Hosts and Bacteriophage from a Haloalkaline Environment: Haloalkaline Environmental Samples

Five samples were collected from surface waters of the haloalkaline environment of Mono Lake, California by Dr. S. Monroe Duboise during summer 2012 for purposes of bacteriophage discovery. Samples were labeled MOM 1, MOD 1, MOD 2, MOD 3, and MOD 4 to designate the location of the lake where they were collected. MOM 1 was collected in the Navy Beach area; samples MOD 1, MOD 2, and MOD 4 were collected from the Old Marina area; and MOD 3 was collected from the South Tufa area (Fig. 1).

FIGURE 1: Map of Mono Lake, California showing collection sites of surface water samples for discovery of bacteriophage. Reprinted from Mono Lake Committee 2016.



http://www.monolake.org/visit/map

Media and Culture Conditions for Haloalkaline Bacteria

M^3 Media was used for culture and plates. This specially blended medium was formulated by Dr. S. M. Duboise for microbes isolated by the Duboise lab from haloalkaline environments of the soda lakes in Kenya and Mono Lake in California. The medium mimics the nutrients and pH found in some high salinity and high pH haloalkaline environments. To produce 1 L of M^3 Media, 3 g tryptic soybean broth (TSB) and 7g agar are dissolved in 500 mL of water and autoclaved, followed by mixing and filter sterilization (0.22 µm filter) of 26.5 g sodium carbonate, 10.5 g sodium bicarbonate, and 30.0 g sea salt into 500 mL water. The two components are mixed after the autoclaved portion has cooled to ~65°C and the filter-sterilized portion is warm (~40°C) and dispensed into sterile petri dishes or tubes. The media cannot solidify and then be warmed again because of salt precipitation.

Isolating Haloalkaline Host Bacteria

Environment-specific bacterial strains MOM 1.1, MOM 1.2, and MOM 1.3 were previously isolated by members of the Duboise lab from water samples collected from Mono Lake, California. Additional environment-specific strains were isolated by me from the sediment rich sample MOD 4 collected by S. M. Duboise in summer 2012 in the Old Marina area of Mono Lake, California. Isolation of potential host bacteria was achieved through streak plating

environmental samples on M^3 agar plates and then picking colonies to achieve genetic purity.

16S rDNA Identification of Haloalkaline Bacterial Hosts

DNA was isolated and purified from haloalkaline bacterial hosts using the DNEASY kit from Qiagen. DNA concentration was measured through spectrophotometry. Peak spectrophotometric absorbance for DNA is 257 nm, and peak absorbance for proteins is 277 nm; thus, the ratio of A260/A280 absorption indicates the quality of DNA or protein. Pure DNA has an absorbance ratio of 2.0. Absorbance was based on a 260:280 ratio to determine the concentration of DNA because a 260:280 ratio of ~1.8 is generally accepted as quality DNA (Glasel, 1995; Warburg and Christian,1942). After the spectrophotometer was calibrated with 50 µL blank buffer/elution buffer, 5 µL of buffer was replaced with 5 µL of sample DNA and mixed to create a 1:10 dilution.

16s rDNA from each bacterial strain was prepared for amplification through PCR by combining 1 µL of prepared DNA to 99 µL of master mix in a 0.2 mL PCR tube. Master mix was made by combining enough volume per DNA sample plus an additional 2 samples using the following reagents: 10 µL Thermopol buffer (New England BioLabs), 2 µL of dNTPs (New England BioLabs), 1 µL forward primers 5' AGAGTTTGATCHTGGCTYAG 3' (Integrated DNA Technologies) and 1 µL reverse primers 5' ACGGNTACCTTGTTACGACTT

3' (Integrated DNA Technologies; Weisberg et al., 1991), 1 µL Taq polymerase (New England BioLabs), and 84 µL molecular grade water. The preparation was placed into a thermocycler programmed as follows: initial denaturation at 95°C for 3 min, followed by 34 cycles consisting of denaturation (94°C for 1 min), annealing (58°C for 1 min), and extension (72°C for 1 min), and a final extension step at 72°C for 5 min before being held at 4°C.

16s rDNA was used to identify environment-specific bacteria (Goto, et al., 2000; Woo, et al., 2008) considered to be potential hosts for bacteriophage. Base pair (bp) length and purity of amplified 16s rDNA extracted from the environment-specific host bacteria MOM 1.1, MOM 1.2, and MOM 1.3 were determined through agarose gel electrophoresis. An 0.8% agarose mini-gel was prepared with 1X TAE buffer and ethidium bromide. A 6X loading dye containing glycerol was used to facilitate sample loading. The gel was run at 100 V for 30 min and then viewed on a UV transilluminator. Bands were compared to a simultaneously run 2-Log DNA ladder (New England BioLabs) for bp length comparison.

Locating Bacteriophage from Haloalkaline Samples

Bacteriophage detection began with enrichment of bacteriophage in the environmental samples (hereafter referred to as large lysate) containing each strain of environment-specific potential host bacteria. For previously isolated colonies MOM 1.1, MOM 1.2 and MOM 1.3, 25 mL of each water sample was

added to 5 mL of M^3 media plus 0.5 mL of overnight culture of the potential host bacteria in a 250 mL sterile flask. Each sterile flask was labeled with the water sample location code (MOD 1, 2, 3, or 4) and host bacteria code (MOM 1.1, 1.2, or 1.3). For newly isolated bacterial strains, 10 mL of each water sample MOD 1, MOD 2, and MOD 3 was added to 50 mL conical vials with 200 µL of overnight culture of newly isolated bacteria and 2 mL of M^3 media. Enrichments were incubated at room temperature with shaking for 8-20 hr.

Cell debris from the lysed bacterial cells was removed from the lysate by centrifugation. The remaining lysate was tested for bacteriophage by using 15 µL of lysate and spot testing serial dilutions (1:10). Host-specific bacterial lawns of MOM 1.1, MOM 1.2, and MOM 1.3 were grown on M^3 agar plates and incubated 24-48 hr at room temperature, allowing time for slower growing bacteria to populate the lawn and time for bacteriophage plaques to form. Observed plaques were picked, resuspended, used to generate a new lysate, and then retested on their host-specific lawns. Putative bacteriophages were retested on host-specific bacterial lawns in three rounds to obtain genetically pure isolates.

Isolation and Genetic Purification of Haloalkaline Bacteriophage

An individual plaque produced by bacteriophage on each bacterial lawn was picked, resuspended in 200 µL distilled water, used to create large lysate, and retested with serial dilutions on bacterial lawns to establish genetic homology

(Su, et al., 1998). Resulting plaques were picked and the plugs resuspended in 400 mL of 0.9% sterile saline before being added to log-phase cultures of host bacteria. The inoculated log-phase culture was incubated at room temperature on a shaker until turbidity indicated bacterial cell lysis, approximately 24 hr. Following lysis, the mixture was centrifuged, spun for 10 min at 10,000 x g, decanted into sterile 15 mL conical tubes, and then stored at 4°C.

To determine if large lysate resulting from enrichment of host bacterial log-phase culture contained host-specific bacteriophages, serial dilutions were spot tested on host-specific bacterial lawns (Su, et al., 1998). The lysates were taken out of refrigeration and centrifuged at 3,000 x g for 15 min to pellet any remaining cell debris. The supernatant containing the bacteriophage population was decanted into new sterile 50 mL centrifuge tubes. Host bacterial lawns were created on M^3 agar plates for each bacteriophage isolate. Plates were divided into 8 sections, allowing space for each serial dilution. Serial dilutions of the lysate were created using 0.9% sterile saline and transferred to the plate in a volume of 20 µL. Plates were left at room temperature face up until spots were dry, then inverted and incubated for at least 24 hr at room temperature.

Preparing Haloalkaline Bacteriophage for Observation with Transmission Electron Microscopy, Long Term Storage, and Sequencing

Crude lysates were washed in 100 µL of 0.1 M ammonium acetate and centrifuged at 14,000 rpm for 60 min (Ackermann & Heldal, 2010). Cleaned bacteriophage lysates were isolated from potential host DNA contamination through DNase treatment using a Norgen RNase-Free DNase I kit (product #25710), clarified through centrifugation at 3,000 x g for 15 min, transferred into a 15 mL culture tube, followed by addition of 10 µL DNase I. The mixture was incubated at room temperature for 15 min and then transferred to 75°C water bath for 10 min to achieve heat inactivation of DNase I. After adding 500 µL of Lysis Solution (Norgen lot #A06FC), the mixture was vortexed vigorously for 10 sec. Proteinase K (20 mg/ml; Qiagen lot #136248600) was added to the mixture, followed by 15 min of incubation at 55°C. Subsequent incubation for 15 min at 65°C was completed and included occasional mixing by inversion of the tube. A volume of 320 µL of isopropanol was added to the lysate and followed by brief vortexing. To bind the sample to a column, 650 µL of bacteriophage-containing lysate was applied to a Norgen P/N 46702 spin column with collection tube and centrifuged for 1 min at 6,000 x g (~8,000 rpm). The flow through was discarded.

A volume of 400 µL of wash solution containing ethanol was applied to the column. The column was centrifuged for 1 min at 6,000 x g, and the flow through was discarded. This wash process was completed three times. The column was dried through centrifugation for 2 min at 14,000 x g (~14,000 rpm), and the collection tube was discarded. The column with the washed bacteriophage DNA attached was placed in a clean 1.7 mL elution tube provided in the Norgen kit,

and 75 µL of Norgen Elution Buffer (lot #A06FA) was added to the column. The column was centrifuged for 1 min at 6,000 x g. For optimal DNA collection, the elution process was repeated with resulting flow through collected in an additional elution tube.

To assess bacteriophage DNA quality and quantity, the same procedure was followed for analysis of bacteriophage samples as was followed for 16s rDNA analysis of bacterial samples (see above). There was not enough volume following elution for MOM 1.1-MOD 2 Φ1 to be analyzed through spectrophotometry.

For long-term storage, 500 µL of cleaned large lysate containing bacteriophage was combined with 500 µL 15% sterile glycerol, which prevents damage to DNA by preventing the aqueous lysate from freezing (Golec, et al., 2011), in each of five cryotubes with screw caps. The mixtures were stored at -80°C.

To prepare the samples for viewing with a transmission electron microscope, negative staining of Mono Lake bacteriophage was conducted under the guidance of Karen Moulton and Andrew Flower of the Duboise Laboratory, University of Southern Maine. Carbon-coated, Formvar-coated 200 mesh copper grids (Ted Pella, Redding, California) were held shiny side-up with forceps. One

15 µL drop of each bacteriophage suspension, diluted 1:5 in 0.1 M ammonium acetate, was applied to a grid. After approximately 60 sec, the grid edges were blotted with #50 Whatman filter paper. One drop of 1% uranyl acetate stain/fixative was applied to the virus coated grid. The grid edges were again blotted dry with #50 Whatman filter paper to remove any excess stain that may obscure viewing sample grids. Images then were acquired using a TECNAI G2 Spirit Bio Twin (FEI Corporation, Hillsboro, Oregon), operating at 100 kV.

Preparation of Bacteriophage DNA for Bacteriophage Genomic Library

To generate a gene library and predict viral genome size, cleaned bacteriophage DNA was digested with one of two restriction endonucleases from New England BioLabs, HindIII or EcoR1, to generate fragments for gene sequencing. HindIII is a restriction endonuclease from an *E. coli* strain that carries the cloned HindIII gene from *Haemophilus influenzae* Rd (ATCC #51907) and has a cutting pattern of 5'... A$^{\downarrow}$AGCTT … 3' and 3' …. TTCGA$^{\downarrow}$A … 5'. EcoR1 is a restriction endonuclease from an *E. coli* strain that carries the cloned EcoR1 gene from *E.coli* and has a cutting pattern of 5'... G$^{\downarrow}$AATTC … 3' and 3' …. CTTAA$^{\downarrow}$G … 5'.

A master digestion mix was prepared for each digest, HindIII and EcoR1, that included a total volume of 50 µL x the number of samples digested plus one additional sample. To produce the mix, the following reagents were combined: 36

µL molecular grade water, 20 µL EcoR1 buffer or NEB buffer, 1 µL EcoR1 enzyme or HindIII enzyme. Then, 40 µL of the master mix was allocated to separate 1.5 mL microcentrifuge tubes. A volume of 10 µL of bacteriophage DNA to be digested was added to each microcentrifuge tube, and the mixture with the DNA was incubated for 1-4 hr at 37°C. Digestion results were analyzed through gel electrophoresis.

Isolating Bacterial Host and Bacteriophage from an Acidic Environment: Acidic Environment Samples

Most liquids were obtained from commercial sources, because one objective of this project was to have middle school students perform the tests. Liquids included unfiltered red and white wine, unfiltered vinegars, unfiltered apple cider, and kombucha tea. Slurry produced from orchard apples that had fallen to the ground was also tested.

Media and Culture Conditions for Acetic Acid Bacteria

The culture medium used for incubating and inoculating acetic acid tolerant bacteria was based on a formula suggested by Schramm and Hestrin (1954) in their experiments with *Acetobacter xylinum*, and it was designated AX medium. This medium was made with or without agar, depending on use. Ingredients were added to distilled water in an erlenmeyer flask on a hot plate at 110°C, stirring ~200 rpm until dissolved. Ingredients per 500 mL included

glucose 10 g; peptone 2.5 g; yeast extract 2.5 g; disodium phosphate 1.35 g; citric acid 0.75 g; distilled water 500 mL; agar 7.5 g (when making plates and slants). The AX medium was autoclaved and then poured into plates, tubes, or conical vials as needed or refrigerated until needed. Bacteria were grown on plates at room temperature for 26-48 hr before being used to inoculate liquid AX medium (Schramm & Hestrin, 1954) and grown at room temperature with shaking.

Isolating Acetic Acid Host Bacteria

Bacteriophage are host specific (Rakhuba, et al., 2010), so it is necessary to use environment-specific bacteria to locate them. Bacteria in the kombucha tea symbiotic culture of bacteria and yeast (SCOBY; Chakravorty, et al., 2016; Dutta & Gachhui, 2007) were populated and prepared for isolation by placing the SCOBY in a sugar-rich, green tea environment, i.e., the same conditions used to make kombucha tea. The SCOBY was placed in a medium of organic green tea (five organic green tea bags brewed in 1.89 L of bottled water), organic sugar (126 g), and distilled apple cider vinegar at a volume sufficient to establish pH < 4 in proportions described by the packaging. The SCOBY was then incubated in the culture in a 1 gallon, wide-mouth, non-lead glass jar covered with a loose weave cotton cloth for 10 days with a pH of 2.5 (Somnath, et al., 2016). Subsequently, a 3-cm diameter section of the newly generated SCOBY and 200 mL of culture medium were transferred to a smaller 500 mL jar to use as the source for isolation.

Microbial contents from the SCOBY were streaked onto two plates containing AX medium and incubated at room temperature for 5 days. No colonies appeared on the plates until 36 hr after inoculation. Each of three colonies, having different morphologies, were transferred to separate plates and restreaked three times to achieve colony purity. A sterile loop was used to transfer the three bacterial colony populations into loosely capped (to allow oxygenation; Verschuren et al., 2000), individual 50 mL conical tubes containing 15 mL AX medium and incubated without shaking at room temperature for 8 days, which is the time needed to increase the population of fermenting bacteria in kombucha tea and to observe cellulose production in the culture (Schramm & Hestrin, 1954; Somnath, et al., 2016; Toda, et al., 1997).

To determine if increased exposure to air resulted in observable cellulose production, two colonies of bacteria were placed into individual 50 mL conical vials with 15 mL AX medium, capped with cotton balls, and incubated without shaking at room temperature for 8 days. This condition allows increased oxygenation (Schramm & Hestrin, 1954) and more closely resembles the conditions for kombucha tea fermentation (Somnath, et al., 2016).

The desired host bacteria survive in acidic environments, utilize glucose for energy, and increase the acidity of the medium as the bacteria metabolize the

glucose. Ideally the colony produces cellulose in the process (Sharafi, et al., 2010). To test potential hosts for acetic acid production, they were grown on plates containing yeast extract glucose carbonate medium (ATCC medium 459:YGC medium) composed of d-glucose, 5%; yeast extract, 0.5%; calcium carbonate, 1.5%; and agar, 1.5% for 72 hr at 22°C. The calcium carbonate in the medium (seen as white precipitate at the bottom of the plate) neutralizes the acetic acid, which prevents cell instability and leaves a clearing in the calcium carbonate layer directly below the growing colony (Mamlouk & Gullo, 2013).

The host bacteria must be capable of creating a continuous lawn on the AX agar plate. Therefore, both colonies were tested to determine their abilities to form a continuous lawn. Liquid cultures were plated on AX medium after 24, 48, and 72 hr of incubation at room temperature with and without shaking. The lawns then were incubated at room temperature (22°C) and at 34°C for 24, 48, and 72 hr (Schramm & Hestrin, 1954; Somnath, et al., 2016).

Isolated host bacteria were prepared for long term storage. Three cryotubes of each strain were filled with 850 µL of liquid culture and 200 µL of 100% glycerol, and then the tubes were gently vortexed. They were stored at -80°C.

16s rDNA Identification of Acidic Bacterial Hosts

To prepare bacteria for 16s rDNA identification (Goto, et al., 2000; Woo et al., 2008; Klindworth, et al., 2011; Marsh, et al., 2014), the DNEASY (Qiagen) blood and tissue preparation for gram negative bacteria was first used to purify DNA. The QIAGEN protocol was followed (QIAGEN, 2016). Once purified, forward and reverse primers (Integrated DNA Technologies) for 16s rDNA amplification were used to target the 16s rDNA (Weisburg, et al., 1991). The DNA segments were amplified through Polymerase Chain Reaction (PCR), which was carried out in 50 µL volumes containing 5 µL of 10x ThermoPol reaction buffer, 1 µL of 10 mM dNTPs (New England BioLabs), 0.5 µL of 10 µM of each primer (forward and reverse), 0.5 µL template DNA, 0.5 µL of *Taq* DNA polymerase (New England BioLabs), and 42 µL of nuclease-free water. The following PCR conditions were used: initial denaturation at 95°C for 3 min, followed by 35 cycles consisting of denaturation (94°C for 1 min), annealing (58°C for 1 min) and extension (72°C for 1 min), and a final extension step at 72°C for 5 min, and then held at 4°C.

Analysis of the PCR product for the expected 1500 bp 16s rDNA was conducted through agarose gel electrophoresis. PCR product was purified with the Qiagen Qiaquick PCR Purification Kit and then sent to the University of Maine for sequencing. The Basic Local Alignment Search Tool (BLAST) was used through the National Center for Biotechnology Information (NCBI) to compare 16s rDNA sequences in the genetic sequence databases.

Locating Bacteriophage from Naturally Fermented Liquids

Previously isolated bacteria obtained from the kombucha tea SCOBY (CBAX 1.3 and CBAX 2.3) and *Gluconacetobacter xylinus* (ATCC #700178) were used as potential hosts for bacteriophage existing in organic, unfiltered (i.e., sediment was observable in containers), fermented beverages (Aydin & Deveci, 2009). Following the same procedure used to detect bacteriophage in the haloalkaline environment (see above), spot testing was used to test samples.

Potential bacteriophage sources included organic liquids that were naturally fermented by a combination of Acetic Acid Bacteria (AAB) species including *Gluconobacter oxydans* and *Gluconacetobacter xylinus*. The array of liquids included organic unfiltered wines: Larzac, Esperance, and Zinfandel; a variety of kombucha tea products from three different brewers: Blueberry and Chaga Chai Kombucha by Urban Farm Fermentory, organic and raw Trilogy kombucha tea by Synergy, and unfiltered unpasteurized Organic Apple Cider Vinegar by BRAGG; organic vinegars by Fleischmann's Vinegar; home brewed beer; and slurry produced from rotting fallen apples.

Slurry from rotting fallen apples was prepared for testing by blending three rotting apples with 500 mL spring water in a sterilized blender (Hamilton Beach) on medium and then high speeds until a thick slurry formed (Robakis, et al.,

1985). The slurry was prepared two ways for testing. The first sample was composed of 2 mL of complete slurry added to a 50 mL conical tube containing 10 mL AX Medium and 1 mL CBAX 1.3 log-phase culture. A second sample was prepared with 2 mL of surface liquid from settled slurry added to 10 mL AX Medium and 1 mL CBAX 1.3 log-phase culture. A third sample was prepared from the liquid that had seeped from the rotting apples in the bag. Again, 2 mL of liquid to be tested was added to 10 mL AX Medium and 1 mL CBAX 1.3 log-phase culture. All tubes containing samples were placed in a beaker in a shaker set at 120 rpm for 48 hr at room temperature.

To first separate heavier particles and cells from bacteriophage, 45 mL of each source were transferred into 50 mL conical tubes, and then centrifuged at 2800 rpm for 20 min. The top 15 mL of each source were filtered with a 0.45 µL filter (Millex Ha 0.45 µm with REF #SLHA033ss) and 60 cc syringe into new 15 mL conical tubes. A bacteriophage enrichment (a process used to increase the number of bacteriophage in a sample to a titer large enough to detect) was attempted by combining 1 mL of liquid from each filtered organic potential source of bacteriophage with 20 mL of log-phase liquid culture of potential host bacteria. At 24 hr, the Colony 2 culture was turbid, but *G. xylinus* was translucent until 96 hr, a characteristic of *G. xylinus* previously observed in the lab. Samples placed in the Colony 2 culture for bacteriophage enrichment were incubated at room temperature overnight (16 hr) on a shaker at 120 rpm. Samples grown in *G.*

xylinus for enrichment were incubated at room temperature overnight (16 hr) or for 48 hr on a shaker at 120 rpm.

Host bacteria in log-phase growth, as determined through culture turbidity and grown for 36-96 hr (Schramm & Hestrin, 1954), were used to produce a bacterial lawn on AX medium. A variety of spreading techniques was attempted to obtain the best quality lawn. Once spread, plates were allowed to dry for 30 min. Serial tenfold dilutions 10^0 to 10^{-6} of each sample were placed on different areas of the plate in volumes of 15 µL each. After 48–72 hr, when bacterial growth was evenly distributed throughout the plate, the plate was examined for plaques, indicating the presence of lytic bacteriophage. Cleared areas on the bacterial lawn that were consistent with appearance of a plaque were picked as a small agar plug with a 1000 µL micropipette tip. These plugs were resuspended in 200 µL distilled water and vortexed. These resuspensions, which potentially contained bacteriophage, were used to enrich a log-phase population of corresponding host bacteria. The enrichment was then serially diluted and replated on a new bacterial lawn.

Soft Agar Bacteriophage Isolation from Commercial Kombucha Tea, Commercial Cider, and Private Kombucha Tea Stock

To determine if lack of initial bacteriophage plaque formation or re-appearance of plaques after resuspension and enrichment was due to age of the

host cells or to an inability of the bacteriophage to contact non-motile host bacteria, the soft AX agar overlay technique (Lammert, 2007) was used to retest the fermentation sources. Standard incubation temperatures inhibit growth of *G. xylinus* and other *Gluconobacter* species (Schramm & Hestrin, 1954), so the AX agar was heated just enough to be fluid, the host bacteria were added, and then the plates were incubated at 30°C for 48-72 hr.

Approximately 25 mL of each potential source were clarified by centrifugation. The supernatant was transferred to new 50 mL conical tubes before being enriched with 1 mL overnight culture of *G. xylinus* or Colony 2 bacteria. Enough new AX medium was added to the mixture to reach a total volume of 30 mL. The samples were incubated for 60 hr at 24°C. Following incubation, the samples were centrifuged at 2500 rpm for 20 min. The supernatants were then filtered with a 0.45 µL membrane filter to further reduce bacterial populations.

Subsequently, 300 µL of overnight (72 hr) bacteria culture was added to 2 sets of 1.5 mL microcentrifuge tubes for each type of potential bacterial host. Then, 100 µL of each filtered sample (prepared above) was transferred to the 1.5 mL microcentrifuge tubes containing the bacterial strain to be tested. Two concentrations of each host bacteria were prepared to establish an adequate host population. The first host population density was prepared with 100 µL host

mixture diluted in 900 uL of AX medium. The second host population density was prepared by diluting the first host population tenfold. Samples were incubated at least 15 min, allowing time for bacteriophage and host contact before being added to 3 mL of 50°C soft agar. Soft AX medium agar was prepared using 6 g/L of AX medium instead of 15 g/L of agar.

The soft agar with samples were gently mixed for 2-3 sec. The tube contents were then poured onto the hard agar and swirled for even distribution across the surface. Even though the agar looked completely liquid in the tube, many samples were lumpy when poured. Samples incubated for 48 hr at approximately 24°C.

RESULTS

Characteristics of Host Bacteria Isolated from Haloalkaline Environments

Three colonies of environment-specific bacteria, MOD 4.00, MOD 4.02, and MOD 4.05, were isolated from sediment rich water in Mono Lake, California (Table 1). The colonies were identified by the water sample of origin followed by one of a range of numbers assigned to each lab student. Three colonies with slightly different physical characteristics were selected for further investigation from all samples plated (Table 1). All three colonies were characterized as a shade of white, and they were translucent and undulate. Two colonies, MOD 4.00 and MOD 4.05, were irregular in shape. MOD 4.05 appeared flat, whereas MOD 4.00 was slightly raised. Colony MOD 4.02 was circular and raised. All three colonies were isolated from the Old Marina section of Mono Lake.

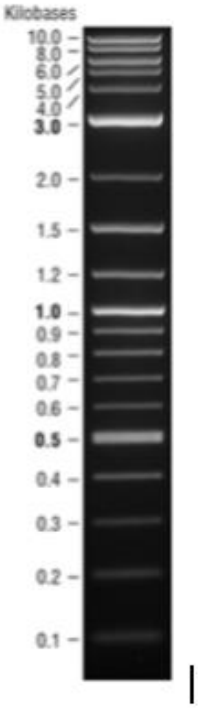
TABLE 1: Physical characteristics of potential environment-specific bacterial hosts isolated from environmental sample MOD 4, Mono Lake, California.

Host Bacteria	Colony Form	Colony Pigmentation	Colony Surface Appearance	Colony Margin	Colony Elevation
MOD4.00	irregular	clear-white	translucent-glistening	undulate	flat-raised
MOD4.02	circular	white-cream	translucent	undulate	raised
MOD4.05	irregular	white	translucent	undulate	flat

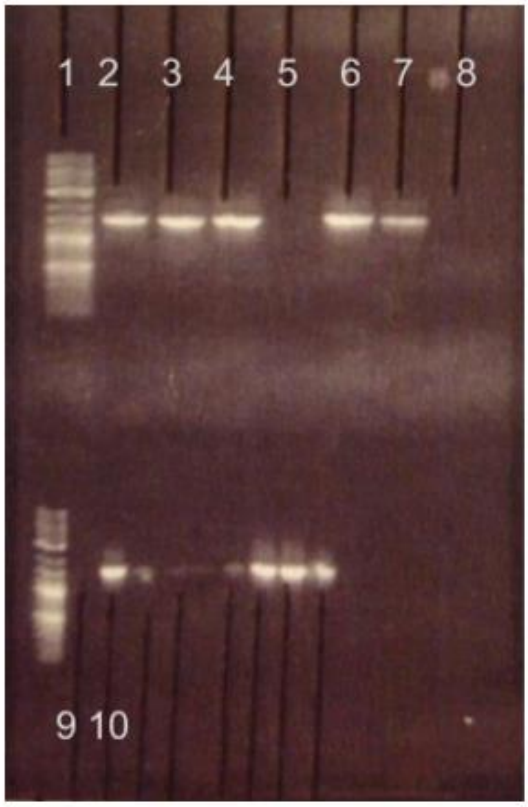
Analysis of 16s rDNA Amplification of Bacterial Hosts MOM 1.1, MOM 1.2, and MOM 1.3

The presence of banding that corresponded with the 1500 bp mark on the DNA 2-Log DNA ladder of an 0.8% agarose gel following electrophoresis suggested the 16s rDNA segment of MOM 1.1, MOM 1.2, and MOM 1.3 amplified successfully (Fig. 2). The concentration of amplified 16s rDNA product of host bacterial colony MOM 1.1 was 260 µg/mL with a purity A260/A280 ratio of 2.15, which suggested a sufficient quantity of high-quality product (Table 2). The concentration of amplified 16s rDNA product of host bacterial colony MOM 1.2 was a moderate quantity of 90 µg/mL with a low 260/280 purity ratio of 1.17 (Table 2). The concentration of amplified 16s rDNA product of host bacterial colony MOM 1.3 was the lowest amount, 30 µg/mL, but with a high-quality A260/A280 ratio of 2.03 (Table 2). DNA from colonies MOM 1.1 and MOM 1.3 was of sufficiently high quality to sequence.

FIGURE 2: Agarose gel electrophoresis analysis of 16s rDNA PCR amplification products from potential bacterial hosts MOM 1.1 (lane 2), MOM 1.2 (lane 3), and MOM 1.3 (lane 4). NEB = New England BioLabs



***2-log DNA Ladder (NEB)**



***0.8% agarose gel in TAE. Lane 1 = 2-log DNA Ladder (NEB).**

TABLE 2: Spectrophotometry results showing concentrations of DNA and purity of samples of potential host bacteria isolated from Mono Lake, California. Absorbance ratios > 1.8 represent high-quality samples.

Host Bacteria Strain	Concentration of DNA (µg/mL)	A260/A280 Ratio
MOM 1.1	260	2.15
MOM 1.2	90	1.17
MOM 1.3	30	2.03

Detection of Bacteriophage from Haloalkaline Environments

A total of 12 potential bacteriophage were isolated from the Mono Lake samples (Table 3). Water sample MOD 1 enriched with bacterial host MOM 1.1 and water sample MOD 2 enriched with MOM 1.1 both produced plaques (Table 3). The bacteriophage (Φ) retrieved from MOD 1 enriched with MOM 1.1 was named MOM 1.1-MOD 1 Φ1, and the bacteriophage retrieved from MOD 2 enriched with MOM 1.1 was named MOM1.1-MOD 2 Φ1 (Moulton et al., 2013). Plaques formed from spot tests with serial dilutions of all water samples tested (MOD 1, MOD 2, and MOD 3) on lawns of all bacterial strains isolated from MOD 4 (MOD4.00, MOD4.02, and MOD4.05; Table 3). The plaques were clearly visible at dilutions of 10^{-6} and 10^{-7}.

TABLE 3: Plaque formation on host bacterial lawns of isolates from water samples from Mono Lake, California. Each positive result represents a potential bacteriophage.

	Water sample		
Bacterial Strain	MOD 1	MOD 2	MOD 3
MOM 1.1	**yes**	no	no
MOM 1.2	no	no	**yes**
MOM 1.3	no	no	no
MOM 3.1	no	**yes**	no
MOD 4.00	**yes**	**yes**	**yes**
MOD 4.02	**yes**	**yes**	**yes**
MOD 4.05	**yes**	**yes**	**yes**

Analysis of Haloalkaline Bacteriophage DNA and Imaging of the Bacteriophage

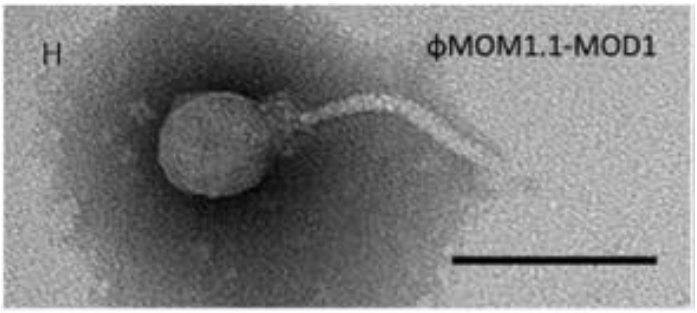
Spectrophotometry results for MOM 1.1-MOD 1 Φ1 (isolated using a Norgen phage DNA purification kit) revealed a concentration of 110 µg/mL of DNA with a high-quality ratio of 1.87. Results for MOM 3.1-MOD 2 Φ1 indicated a much lower concentration of 60 µg/mL of DNA and a lower quality absorbance ratio of 1.68 (Table 4). There was not enough volume of MOM 1.1-MOD 2 Φ1 for spectrophotometric analysis. Only DNA from MOM 1.1-MOD 1 Φ1 was of sufficiently high quality for library production and DNA sequencing.

The quality of bacteriophage MOM 1.1-MOD 1 Φ1 DNA and its high titer allowed it to be viewed with the transmission electron microscope (TEM) by TEM manager Karen Moulton (Fig. 3). The haloalkaline bacteriophage was comprised of an icosahedral head and a 100 nm noncontractile tail.

TABLE 4: Spectrophotometry results showing concentrations and purity of bacteriophage DNA isolated from lysates of host bacteria. Absorbance ratios > 1.8 represent high-quality samples.

Bacteriophage DNA Elution	Concentration of DNA (μg/mL)	A260/A280 ratio
MOM 1.1-MOD 1 Φ1	110	1.87
MOM 1.1-MOD 2 Φ1	n/a	n/a
MOM 3.1-MOD 2 Φ1	60	1.68

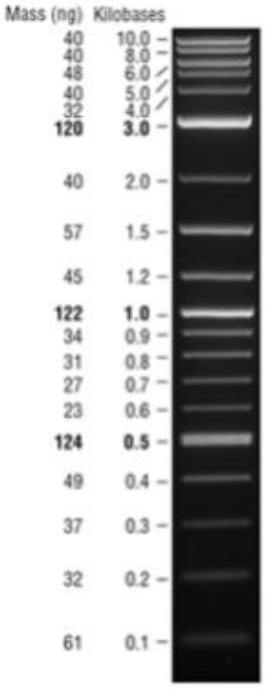
FIGURE 3: Transmission electron microscope image of haloalkaline bacteriophage MOM 1.1-MOD 1 Φ1 isolated from Mono Lake, California. Scale bar represents 100 nm.

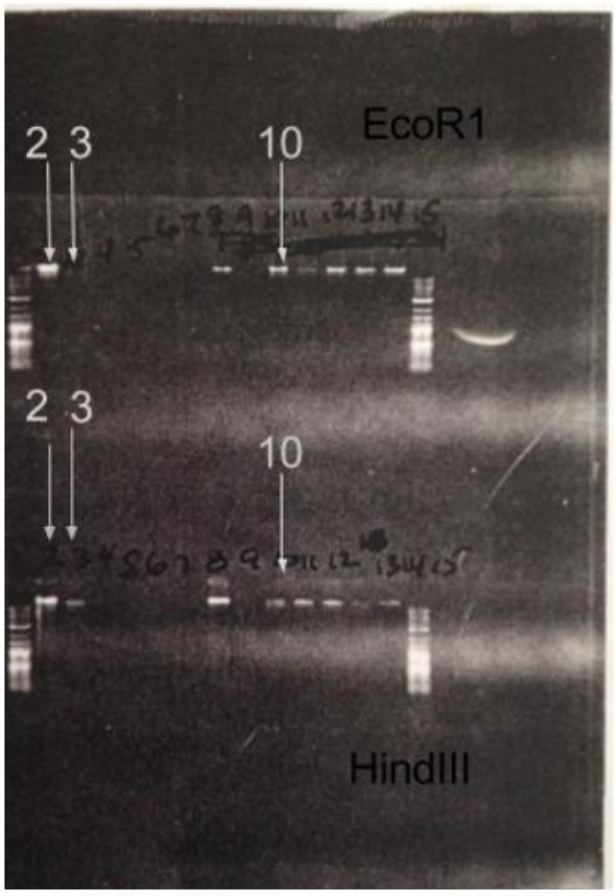


Bacteriophage (Φ) DNA Digestion by EcoR1 or HindIII

Enzyme digestion of DNA from bacteriophage MOM 1.1-MOD 1 Φ1 (Fig. 4, lane 2), MOM 1.1-MOD 2 Φ1 (Fig. 4, lane 3), and MOM 3.1-MOD 2 Φ1 (Fig. 4, lane 10) by restriction nucleases EcoR1 or HindIII resulted in no DNA fragments smaller than intact bacteriophage DNA for each sample, indicating that no digestion was detectable.

FIGURE 4: Gel electrophoresis results for digestion of bacteriophage (Φ) DNA by EcoR1 (top row) and HindIII (bottom row). Lane 2 contained MOM 1.1-MOD 1 Φ1, lane 3 contained MOM 1.1-MOD 2 Φ1, and lane 10 contained MOM 3.1-MOD 2 Φ1. NEB = New England BioLabs





***2-log DNA Ladder (NEB)**

***0.8% agarose gel in TAE. Lane 1 = 2-log DNA Ladder (NEB).**

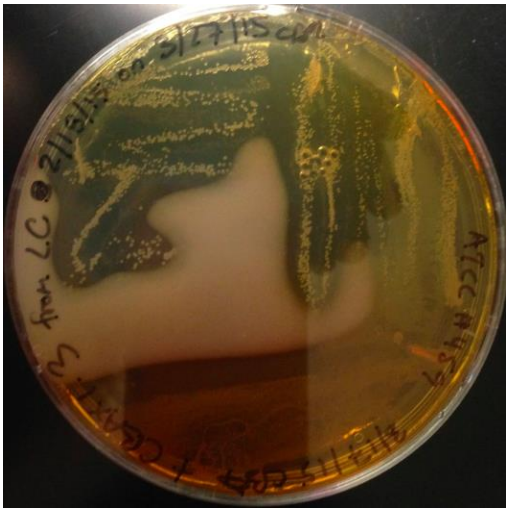
Characteristics of Host Bacteria Isolated from the Acidic Environment of Kombucha Tea

Three colonies with different morphologies were isolated from the kombucha tea SCOBY. Colony 1 (CBAX 1.3) was translucent, off white to pink, glossy with round margins, and convex. Colony 2 (CBAX 2.3) was opaque, beige, glossy, round with smooth margins, and convex. Colony 3 (CBAX 3.3) was white, high, glossy, and gave off the aroma of baking bread. Gram staining of the colonies revealed that CBAX 1.3 and CBAX 2.3 were gram-negative, whereas CBAX 3.3 was gram-positive or indeterminate.

Liquid cultures CBAX 1.3 and CBAX 2.3 incubated at temperatures greater than room temperature did not grow as well as those incubated at room temperature, indicating intolerance to the higher temperature. Best growth was seen at 26°C for 72 hr.

CBAX 1.3 and CBAX 2.3 created clear spots in the calcium carbonate of the YGC medium, as did *G. xylinus* (Fig. 5), but CBAX 3.3 did not, which suggests CBAX 1.3 and CBAX 2.3 generated acetic acid as they metabolized the sugar. Thus, they were suitable bacterial hosts for the desired bacteriophage.

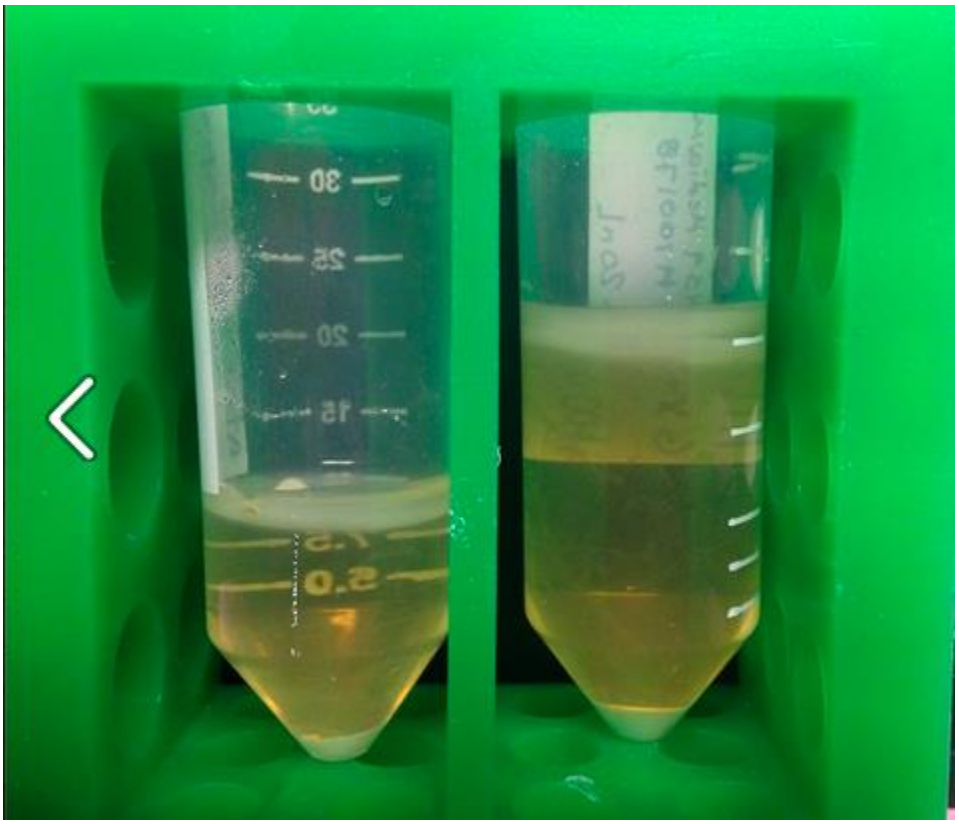
FIGURE 5: Clearings in the calcium carbonate (white) on the agar plates indicate acetic acid production by the growing bacteria.



G. xylinus (left image) and CBAX 1.3 (right image) grown on YGC medium at room temperature for 5 days.

G. xylinus, a known cellulose-producing bacterium, produced an obvious pellicle of cellulose when grown in YGC medium (Fig. 6). However, CBAX 1.3 and CBAX 2.3 did not produce observable cellulose as bacteria processed the glucose and fermented the medium. Instead these bacteria created a thin, brownish film on the surface. When the tubes were shaken, the film redistributed throughout the medium. From previous observations involving *G. xylinus,* cellulose film does not dissociate easily. CBAX 3.3 did not produce any type of film. Instead it was seen growing only at the bottom of the medium.

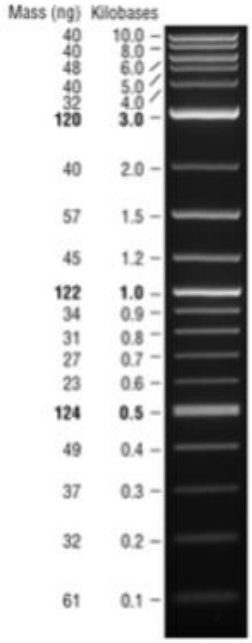
FIGURE 6: Cellulose pellicle forming at the surface of the variable volumes of YGC medium. Cellulose was produced by *G. xylinus* after incubation for 72 hr at room temperature without shaking.



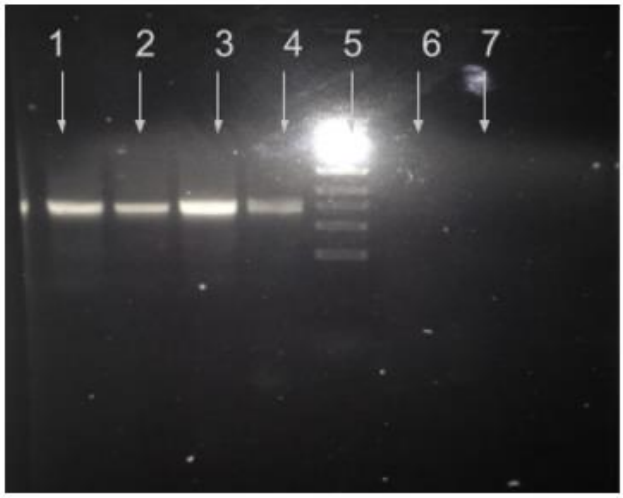
Analysis of 16s rDNA Amplification of Potential Bacterial Hosts

When compared against a 2-log DNA ladder (NEB), the 16s rDNA products from CBAX 1.3 and CBAX 2.3 displayed banding aligned with the 1500 bp marker, indicating that amplification was successful (Fig. 7). CBAX 3.3 did not show any evident banding, suggesting no 16s rDNA was amplified. This observation, combined with lack of film produced in the cellulose test and inconclusive gram staining results, suggested that CBAX 3.3 was yeast instead of bacteria. Therefore, CBAX 3.3 was no longer considered as a possible bacteriophage host.

FIGURE 7: Agarose gel electrophoresis analysis of 16s rDNA PCR amplification products from potential bacterial hosts CBAX 1.3 (Lanes 1 and 2), CBAX 2.3 (lanes 3 and 4), and CBAX 3.3 (lanes 6 and 7). NEB = New England BioLabs



***2-log DNA Ladder (NEB)**



***0.8% agarose gel in TAE. Lane 5 = 2-log DNA Ladder (NEB).**

Sequence Results of 16s rDNA of Isolated Potential Host Bacteria

DNA sequences from CBAX 1.3 (Fig. 8) and CBAX 2.3 (Fig. 9) exhibited 99% sequence similarity with over 90 *Gluconobacter* strains, including 52 hits with *Gluconobacter oxydans* based on 16s rDNA sequence analysis through the Basic Local Alignment Search Tool (BLAST). The top two hits were with *B10* GenBank: EU131163.1 and *Gluconobacter oxydans* strain 39PCAac1 GenBank: AY206688.1. They had a maximum score and total score of 2540 and 2543, respectively, with 100% quarry coverage and E value of 0.0 with *Gluconobacter sp.* and 16s rRNA gene partial sequences.

Figure 8: DNA sequence of the 16s rRNA gene for bacterium CBAX 1.3 isolated from a kombucha tea SCOBY.

GCGGCATGCTTAACACATGCAGTCGCACGAAGGTTTCGGCCTTAGTGGCGGACGGGTGAGT
AACGCGTAGGGATCTATCCACGGGTGGGGGACAACTTCGGGAAACTGGAGCTAATACCGCA
TGATACCTGAGGGTCAAAGGCGCAAGTCGCCTGTGGAGGAACCTGCGTTCGATTAGCTAGT
TGGTGGGGTAAAGGCCTACCAAGGCGATGATCGATAGCTGGTTTGAGAGGATGATCAGCCA
CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA
ATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGC
ACTTTCGACGGGGACGATGATGACGGTACCCGTAGAAGAAGCCCCGGCTAACTTCGTGCCA
GCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATGACTGGGCGTAAAGGGCGC
GTAGGCGGTTGTTACAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGATA
CGTGACGACTAGAGTTCGAGAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAG
ATATTGGGAAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCGATACTGACGCTGAGGCG
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTGT
GCTGGATGTTGGGAAACTTAGTTTTTCAGTGTCGAAGCTAACGCGCTAAGCACACCGCCTG
GGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGCATGGGGAGGACC
GGTTCAGAGATGGACCTTTCTTCGGACCTCCCGCACAGGTGCTGCATGGCTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCTTTAGTTGCCAG
CACTTTCAGGTGGGCACTCTAGAGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATG
ACGTCAAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGGCGGTGACAG
TGGGAAGCTACATGGTGACATGGTGCTGATCTCTAAAAGCCGTCTCAGTTCGGATTGTACTC
TGCAACTCGAGTACATGAAGGTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAA
TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTCGACCTTAAGC
CGGTGAGCGAACCGCAAGGACGCAGCCGACCACGGACGGTCAG

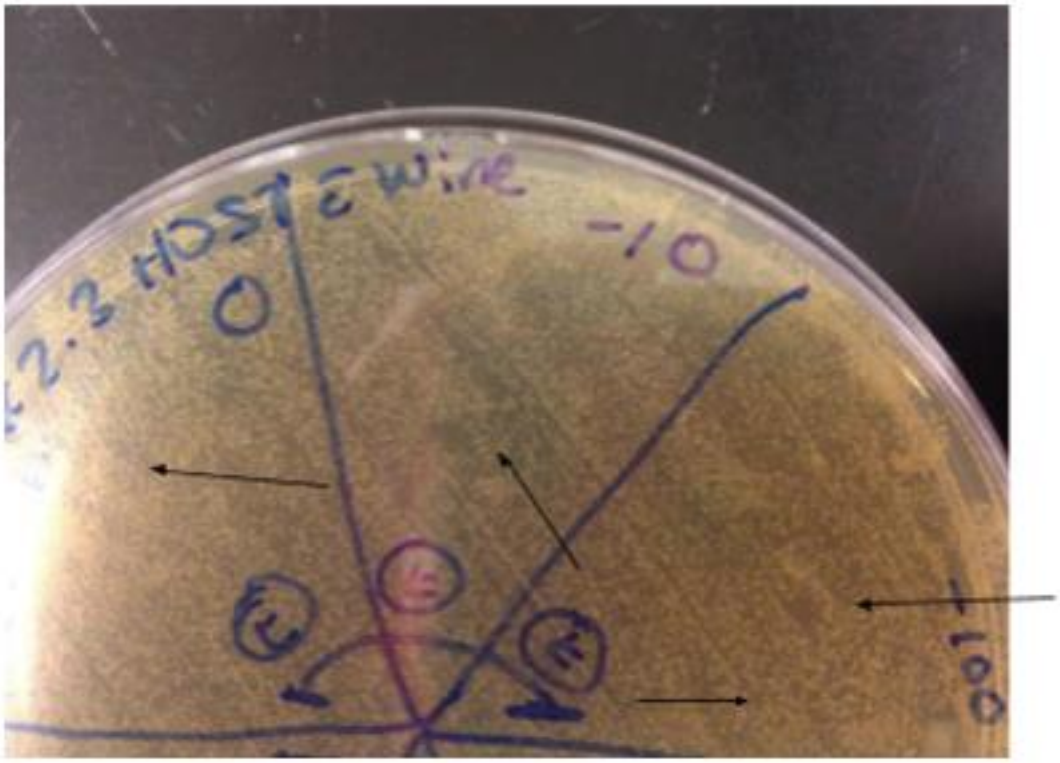
Figure 9: DNA sequence of the 16s rRNA gene for bacterium CBAX 2.3 isolated from a kombucha tea SCOBY.

GCGGCATGCTTAACACATGCAAGTCGCACGAAGGTTTCGGCCTTAGTGGCGGACGGGTGA
GTAACGCGTAGGGATCTATCCACGGGTGGGGGACAACTTCGGGAAACTGGAGCTAATACCG
CATGATACCTGAGGGTCAAAGGCGCAAGTCGCCTGTGGAGGAACCTGCGTTCGATTAGCTA
GTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCGATAGCTGGTTTGAGAGGATGATCAGC
CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA
CAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAA
GCACTTTCGACGGGGACGATGATGACGGTACCCGTAGAAGAAGCCCCGGCTAACTTCGTGC
CAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATGACTGGGCGTAAAGGGC
GCGTAGGCGGTTGTTACAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTG
ATACGTGACGACTAGAGTTCGAGAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAAATTCG
TAGATATTGGGAAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCGATACTGACGCTGAG
GCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATG
TGTGCTGGATGTTGGGAAACTTAGTTTTTCAGTGTCGAAGCTAACGCGCTAAGCACACCGCC
TGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGT
GGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGCATGGGGAGGA
CCGGTTCAGAGATGGACCTTTCTTCGGACCTCCCGCACAGGTGCTGCATGGCTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCTTTAGTTGCCA
GCACTTTCAGGTGGGCACTCTAGAGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGGGAT
GACGTCAAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGGCGGTGACA
GTGGGAAGCTACATGGTGACATGGTGCTGATCTCTAAAAGCCGTCTCAGTTCGGATTGTACT
CTGCAACTCGAGTACATGAAGGTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGA
ATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTCGACCTTAAG
CCGGTGAGCGAACCGCAAGGACGCAGCCGACCACGGACCGGTC

Isolation of Bacteriophage from Acidic Environments Created by Naturally Fermented Liquids

One organic wine, Esperance, initially appeared to contain bacteriophage, because plaque-like formations were observed on the bacterial lawn (Fig. 10). However, in subsequent platings, plaques did not form (Table 5).

FIGURE 10: Plaque-like clearings resulting from initial plating of Esperance Enrichment on CBAX 2.3 host lawn.



arrows indicate plaque-like clearings

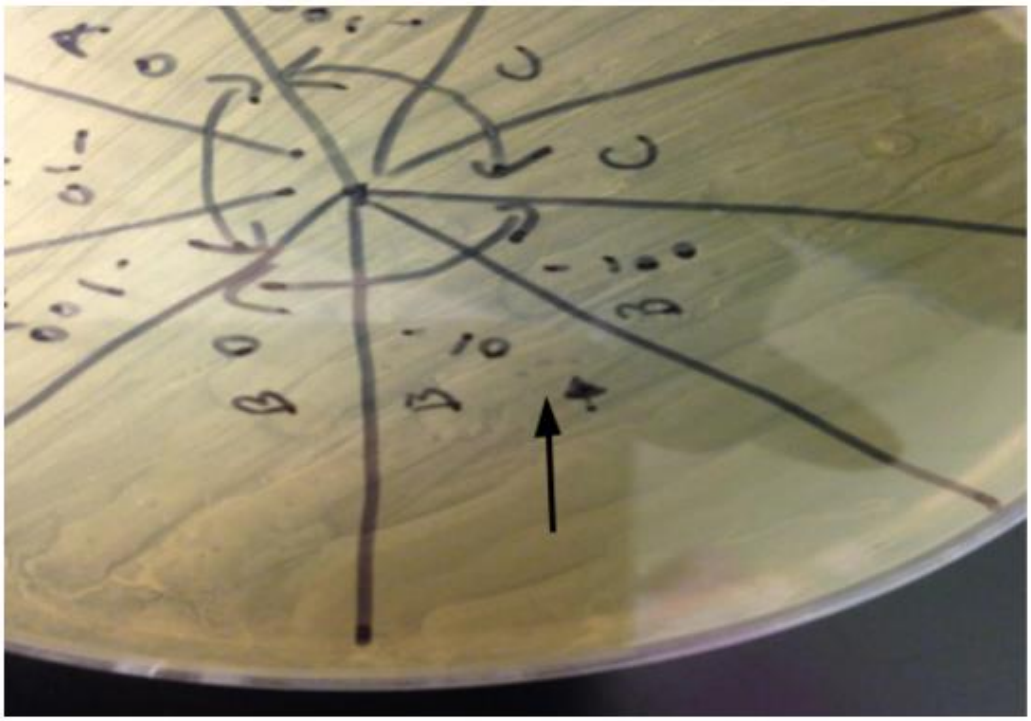
TABLE 5: Results of attempts to isolate bacteriophage from unfiltered fermented liquids inoculated with CBAX 2.3 bacterial host. + indicates positive results; - indicates negative results

Unfiltered Fermented Liquid	Initial Plaque Formation	Resuspension and Retest	Subsequent Plaque Formation
Esperance	+	+	-
Larzac	-	-	n/a
Zinfandel	-	-	n/a
Kombucha Tea	-	-	n/a

** Enrichments were spot tested in tenfold serial dilutions on host-specific lawns grown on AX medium*

Plaque-like clearings (Fig. 11) initially appeared on the CBAX 1.3 host lawn for all three preparations of apple slurry tested at various dilutions (Table 6). Subsequent platings performed after plaques were picked, resuspended, enriched, and re-plated did not result in clearings, suggesting the absence of bacteriophage.

FIGURE 11: Plaque-like clearings resulting from the initial tenfold serial dilutions of apple slurry enrichment on bacterial host CBAX 1.3 lawn.



Serial dilutions of apple slurry on CBAX 1.3 bacterial lawn grown on AX medium plate with 15 g/L agar incubated at room temperature. Arrows indicate area of plaque-like clearings.

TABLE 6: Results of attempts to isolate bacteriophage from slurry of rotten apples grown on bacterial host CBAX 1.3 lawn at different serial dilution concentrations. + = plaque-like clearings observed; - = no plaque-like clearing observed

		Serial Dilution Concentration			Plaque Formation After Replating
Component Tested	pH	0	-10	-100	
Full slurry (A)	3.0	+	-	-	-
Surface liquid from slurry (B)	2.5	+	+	+	-
Liquid resulting from rotting apples (C)	3.0	-	+	+	-

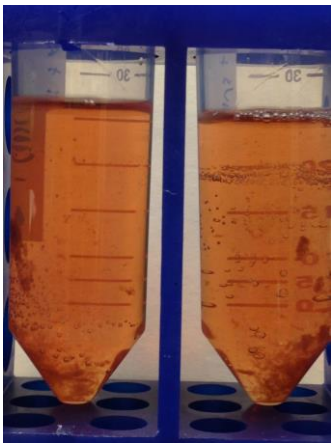
Letters A, B, C were used to label the plate with corresponding spot tests as shown in Fig. 11.

Possible plaque formation occurred on the lawn of a CBAX 2.3 plate created with 20-hr shaken broth containing CBAX 2.3 and serial dilution of 24-hr enrichment of 0.5 µL CBAX 2.3 with 10 mL organic apple cider vinegar and 10 mL AX medium without sea salt. Some plaque-like clearings appeared on the corresponding plate with AX medium and sea salt enrichment, but plaque-like clearings looked smaller on the better quality lawn. However, the culture tubes that contained the apple vinegar enrichment were fairly translucent, although they developed brown sediment not seen in any previous trials (Fig. 12). They did not appear to show progressive growth of CBAX 2.3.

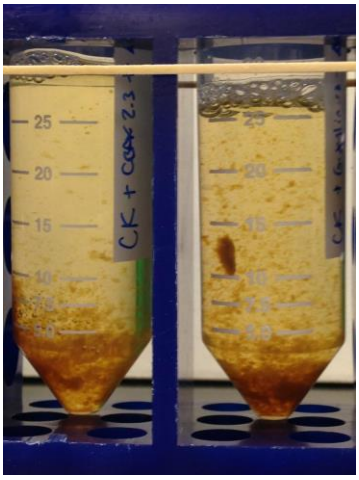
FIGURE 12: Images depicting sediment formed in four enriched fermentation cultures after 16 hr on a shaker at room temperature. For each pair of images, the image on the left represents enrichment in host CBAX 2.3, and the image on the right represents enrichment in host *G. xylinus*.

Blueberry Kombucha

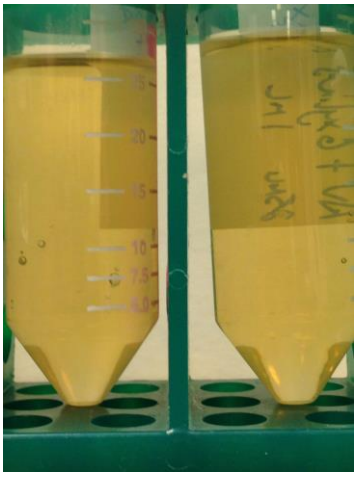
Trilogy Kombucha



Chaga Chai Kombucha



Organic Apple Cider Vinegar



**All cultures were grown in AX medium. Images were taken within 10 min of removal from shaking.*

After assessing both the soft agar and spot test techniques of finding bacteriophage for all kombucha tea samples tested, only Ginger Root kombucha enrichments resulted in plaque-like formations on CBAX 1.3 or CBAX 2.3 lawns (Tables 5, 7, and 8). The lawn, however, was not continuous. All enrichments presented in Table 8 seemed to produce plaque-like formations on the *G. xylinus* lawn, but the lawn was not continuous; therefore, the plaque formations were not conclusive. All enrichments that initially appeared to produce plaques did not produce plaques in subsequent platings.

TABLE 7: Results of attempts to isolate bacteriophage from blueberry, chaga chai, and Trilogy kombucha teas, and from organic apple cider vinegar. + = plaque-like clearings observed; - = no plaque-like clearing observed

Source	pH		Host CBAX 2.3			Host *G. xylinus*
	Initial	Final	Evidence of plaques	Resuspension	New plaques formed	Evidence of plaques
Blueberry Kombucha	2.5	2.5	-	-	n/a	-
Chaga Chai Kombucha	2.5	2.5	-	-	n/a	-
Trilogy Kombucha	3.0	3.0	-	-	n/a	-
Organic Apple Cider Vinegar	3.5	3.5	small clearings observed	+	-	+

** Enrichments were spot tested in tenfold serial dilutions on host-specific lawns grown on AX medium.*

TABLE 8: Results of attempts to isolate bacteriophage using soft agar assays of kombucha enrichments with bacterial hosts CBAX 1.3, CBAX 2.3, and *G. xylinus*. - = no plaque-like clearings observed

		Host Bacteria		
Source	pH	CBAX 1.3	CBAX 2.3	*Gluconobacter xylinus*
Blueberry Kombucha	3.0	-	-	1 mm plaque-like clearings appeared on an uneven lawn with initial plating only
Chaga Chai Kombucha	3.5	-	-	2 mm plaque-like clearings appeared on an uneven lawn with initial plating only
Ginger Root Kombucha	3.0	-	plaque-like clearings appeared on an uneven lawn with initial plating only	plaque-like clearings appeared on an uneven lawn with initial plating only
Gray Kombucha	3.5	-	-	plaque-like clearings appeared on an uneven lawn with initial plating only
Honey Ginger Cider	3.5	-	-	plaque-like clearings appeared on an uneven lawn with initial plating

The lysates from three varieties of unfiltered vinegar enriched in two host cultures produced various plaque morphologies when spot tested initially at full concentration and when resuspensions were tested at different dilutions (Tables 9 and 10). Patterns of plaque-like clearing varied with bacterial host, vinegar sample, and lysate dilution. Bacterial host CBAX 2.3 produced the best bacterial lawn and also generated the widest range of plaque morphology (Table 9). Resuspension and enrichment of agar plugs from the cleared area resulting from red wine vinegar lysate resulted in cloudy streaks before dilution, plaque-like areas with apparent inhibition of bacterial growth at the first dilution (10^{-1}), and no bacterial growth on the perimeter of the enrichment drop (bull's eye plaque morphology) at the second dilution (10^{-2}). Resuspension and enrichment of agar plugs from the cleared area resulting from initial enrichment with white distilled vinegar resulted in plaque-like clearings before dilution, but no subsequent clearings. Resuspension and enrichment of agar plugs from the cleared area resulting from initial enrichment with apple cider vinegar resulted in no plaques before dilution, partial clearings at the first dilution (10^{-1}), and no bacterial growth on the perimeter of the lysate drop at second dilution (10^{-2}), similar to results observed at the same dilution of lysate from red wine vinegar.

Bacterial host *G. xylinus* produced poor lawn growth on all plates, but lysates generated from all three vinegar types resulted in obvious clearings on spot test areas at full concentration (Table 10). Lysate from apple cider vinegar resuspension produced plaque-like clearings at no dilution, and lysate from red

wine vinegar resuspension diluted two fold produced plaque-like clearings. No plaques appeared during attempts to further isolate and purify plaques.

TABLE 9: Results of bacteriophage enrichment and resuspension from three types of vinegar fermentations on CBAX 2.3 host lawn at different serial dilutions.

Source	pH	Initial Plaque Formation	Resuspension and retest	Dilution 0	Dilution 10^{-1}	Dilution 10^{-2}
Red Wine Vinegar	2.5	Full clearing	Yes	cloudy streaks	plaque- like areas of less growth	cleared margin of drop's perimeter
White Distilled Vinegar	2.5	Full clearing	Yes	plaque-like spots	less dense growth but no clear plaques	no plaques
Apple Cider Vinegar	3.0	Cloudy clearing	Yes	no plaques	partial clearing on spotted area	area of clearing near spot margin perimeter

** Enrichments were spot tested in tenfold serial dilutions on host-specific lawns grown on AX medium at room temperature.*

TABLE 10: Results of bacteriophage enrichment and resuspension from three types of vinegar fermentations on host lawn *G. xylinus* at different serial dilutions.

Source	pH	Initial Plaque Formation	Resuspension and retest	Dilution 0	Dilution 10^{-1}	Dilution 10^{-2}
Red Wine Vinegar	2.5	+	Yes	poor lawn growth	poor lawn growth	plaque-like clearings within spot area, but poor lawn growth
White Distilled Vinegar	2.5	+	Yes	poor lawn growth	poor lawn growth	poor lawn growth
Apple Cider Vinegar	3.0	+	Yes	Plaque-like clearings within spot area	poor lawn growth	poor lawn growth

** Enrichments were spot tested in tenfold serial dilutions on host-specific lawns grown on AX medium at room temperature.*

DISCUSSION

My goals were to isolate bacterial hosts and their bacteriophage that inhabited two extreme environments, haloalkaline habitats of Mono Lake and acidic environments of fermented liquids, using isolation and cultivation techniques that were simple and safe for secondary school students to use. My efforts were moderately successful. I isolated multiple bacterial hosts from each environment using non-toxic media, but I definitively isolated and cultivated associated bacteriophage from the haloalkaline environment only. Isolating and cultivating bacteriophage from the acidic environments proved more challenging. Bacteriophage appeared to be present in the acidic environments but were not as common as bacteriophage from Mono Lake, and lack of subsequent cultivation left questions about the virulence of viruses from the acidic habitats.

Despite extreme conditions with respect to high pH and salinity, the haloalkaline environment of Mono Lake initially showed good biodiversity potential because seven morphologically dissimilar host colonies isolated from the environmental samples were used to detect the presence of lytic bacteriophage. All but one (MOM 1.3) of four bacteria previously isolated by K. Moulton appeared to be suitable hosts to at least one bacteriophage, and all three bacteria newly isolated from the MOD 4 samples were host to bacteriophage from all samples tested. Sequencing analysis of the bacterial hosts MOM 1.1, MOM 3.1, MOD 4.00, and MOD 4.05 was carried out subsequently by other members of the Duboise lab.

The primary goal of the project in the Duboise lab was to discover bacteriophage from the haloalkaline environment. Assessment of bacterial diversity and maintenance of multiple hosts were not high priority, which limited subsequent analyses of bacterial hosts. Maintaining multiple hosts for bacteriophage adds cost to the project, takes up valuable space, and is time consuming. Despite these concerns, an alternate bacterial host would be beneficial if the host develops resistance to the bacteriophage.

Samples were collected from the mixolimnion of Mono Lake; therefore, bacterial hosts isolated by the Duboise lab should resemble bacteria from similar environments. Subsequent work with host bacteria MOM 1.1, MOM 3.1, MOD 4.00, and MOD 4.05 by other members of the Duboise lab revealed low diversity of bacterial hosts (S. M. Duboise, pers. comm.). MOM 1.1 was reported to be *Alkalimonas collagenimarina* (Genbank accession number KJ486288.1), and MOM 3.1, MOD 4.00, and MOD 4.05 reportedly were most similar to *Vibrio metschnikovii* (Genbank accession numbers KJ486275.1, KJ475442.1, and KJ486278.1, respectively; S. M. Duboise, pers. comm.).

Little information has been published about *A. collagenimarina*, but it has been isolated from deep sea sediment (Kurata, et al., 2007). It was not reported among the bacteria found in a soda lake biodiversity study (Humayoun, et al.,

2003) or extremophiles in a study of alkaline environments (Grant, 2006). Instead, two other species of *Alkalimona*s were found in similar soda lake environments, *A. amylolytica* sp. nov. N10 (GenBank accession number AF250323) from Lake Chahannor in China and *A*. *delamerensis* sp. nov, 1E1 (GenBank accession number X92130) from Lake Elmenteita in East Africa (Ma, et al., 2004).

Vibrio metschnikovii, on the other hand, is more commonly reported, including a few cases linked to rare causes of infection (Linde, et al., 2004; Wallet, et al., 2005). It has been described in biodiversity studies from soda lakes in Kenya (Mwirichia, et al., 2010) and previously reported as host to a bacteriophage from a soda lake in Kenya (Moulton, et al., 2011). Whereas the habitats of *Alkalimona*s seem more limited due to its dependence on haloalkaline conditions (Shi, et al., 2012), over 20 strains of *Vibrio metschnikovii* have been isolated from more diverse habitats, including river water, shellfish, sewage, fowl, human feces, salt marshes, and other marine environments (Lee, et al., 1978; Farmer, et al., 1988; Marhual, 2012).

In addition to isolating bacteria in this study, 12 potentially different bacteriophage were isolated from the haloalkaline environment. Detecting the presence of lytic bacteriophage can be straightforward, but identification of their distinct characteristics for comparisons requires a series of isolation, cultivation,

purification, and preparation steps. Furthermore, bacteriophage are host specific, and their structure cannot be viewed without an electron microscope. When trying to locate and cultivate bacteriophage through plaque assays using unknown bacteria that also were recently isolated from the same environment, duplication of morphologically similar individuals is possible. One way to preserve biodiversity while reducing duplication is to choose only genetic isolates that infect different hosts (Brussow & Desiere, 2001; Drulis-Kawa, et al., 2012). Another key to successful cultivation and observation of genetically cloned bacteriophage is to choose those organisms that produce high titer particles in lysate (Anderson, et al., 2011).

Of the 12 potentially different bacteriophage isolated with bacterial hosts MOM 1.1, MOM 1.2, MOM 1.3, and MOM 3.1 and those isolated with MOD 4.00, MOD 4.02, and MOD 4.05, I cultivated, prepared, and analyzed three bacteriophages: MOM 1.1-MOD 1 Φ1, MOM 1.1-MOD 2 Φ1, and MOM 3.1-MOD 2 Φ1. I discovered MOD 4.05-MOD 2 Φ1 during this project, but it was further processed and viewed by other members of the Duboise Lab (Moulton, et al., 2013).

Of the two phages infecting host MOM 1.1, only MOM 1.1-MOD 1 Φ1 was examined under the TEM (Moulton, et al., 2013) because it generated the best sample for further exploration. This phage was detected in a sample collected

from Mono Lake in July, and it infected a host most closely related to bacteria in the *Alkalimona*s group. Based on the TEM image, it, along with MOD 4.05-MOD 2 Φ1, is a tailed bacteriophage, making them members of the order Caudovirales (Moulton, et al., 2013). More specifically, their non-contractile tails put them in the family Siphoviridae *(*Bebeacua, et al., 2013). In addition to non-contractile tails, Siphoviridae are characterized by non-encapsulated contractile heads about 60 nm in diameter and double-stranded nonlinear DNA of about 50 kb (Bebeacua, et al., 2013).

Bacteriophage MOM 1.1-MOD 2 Φ1 was prepared for TEM imaging, long term storage, and EcoRI and HindIII digestion, but it did not undergo spectrophotometric analysis. Because host duplication is one way to eliminate morphologically similar bacteriophage, MOM 1.1-MOD 2 Φ1 may have been dropped from further study because it used the same host as MOM 1.1-MOD 1 Φ1. My records indicated MOM 1.1-MOD 2 Φ1 produced plaques more quickly than the other two bacteriophage and was prepared for long term storage and imaging at least 24 hr ahead of the other two bacteriophage. Thus, it potentially has higher virulence than other bacteriophage, but additional work would be required to learn more about this virus.

Bacteriophage MOM 3.1-MOD 2 Φ1 was prepared for TEM imaging and underwent digestion attempts with EcoRI and HindIII digestion, but to my

knowledge it was not imaged. Spectrophotometry results indicated a low concentration of DNA compared to MOM 1.1-MOD 1 Φ1 and slightly less than optimal quality of DNA, unlike MOM 1.1-MOD 1 Φ1. Therefore, MOM 3.1-MOD 2 Φ1 was not a good candidate for imaging with the TEM.

One step in characterizing bacteriophage is to create a genomic library of bacteriophage fragments, which are useful in identification of subsequent bacteriophage, studies of bacteriophage evolution, gene comparisons, and gene protein conservation analysis (Hatfull, 2008). Enzymatic digestion of the bacteriophage genome breaks the genome into manageable parts. Each enzyme cuts the genome in a particular location based on the sequence. The ends then can be aligned with additional fragments for comparison and extension. Unique fragments can be catalogued and added to a library for future reference. Unfortunately, attempts to create a genomic library of digestion fragments were not successful in this study. When digestion of the bacteriophage MOM 1.1-MOD 1 Φ1, MOM 1.1-MOD 2 Φ1, and MOM 3.1-MOD 2 Φ1 was attempted with EcoR1 or HindIII, agarose gel analysis indicated that the genomes were not broken into smaller fragments. Perhaps the genomes of the bacteriophage did not contain the matching cutting sequences, or another factor may have inhibited digestion.

Mono Lake proved to be a suitable environment from which to isolate bacteria and discover bacteriophage in a situation where only standard methods

of detection and isolation could be used. Based on the ease of detection with respect to both bacterial host and bacteriophage diversity in the haloalkaline environment, the California soda lakes are ideal sampling environments for future bacteriophage discovery projects. Generally, microbes obtained and cultured from extreme environments are less likely to present health risks to humans than microbes obtained from non-extreme environments, because high sodium, high pH, and extreme temperature conditions necessary for their growth do not exist in the human body (Wilson, 2005). Nonetheless, salinity levels in the surface waters and mixolimnion of Mono Lake from which samples were collected for this project change frequently due to rainfall, currents, and wind (Mono Lake Committee, 2017), particularly during the migratory season for waterfowl (Little, 2016). Bacteria that do not require conditions of pH > 8 or high sodium concentration can survive in this environment (Islas, et al., 2007; Mwirichia, et al., 2010). Three hosts from Mono Lake, MOM 3.1, MOD 4.00, and MOD 4.05, were most closely related to *Vibrio metschnikovii* (S. M. Duboise, pers. comm.), which occurs in birds and tolerates a wide range of salinity levels (Lee, et al., 1978). Although many strains of *V. metschnikovii* are not known to cause human disease, and reports of disease caused by *V. metschnikovii* are rare (Lee, et al., 1978), one would not want to endanger the health of students in middle school classrooms. Both bacteria and bacteriophage can mutate rapidly (Brussow & Desiere, 2001) and now that *V. metschnikovii* is known to be zoonotic, it would not be a good choice for classroom use. Therefore, if a true extremophile host was sought, a sample from deeper within the soda lake should be obtained, and

environment-specific medium should be used to ensure that only extremophiles dependent on those conditions are isolated.

Although *Vibrio* would not be a suitable host to use in the middle school classroom, a safer alternative and more environmentally specific host, e.g., *Alkalimona*s, could be used to detect bacteriophage from the haloalkaline environment. Students could try to grow the host bacteria in a variety of conditions to ensure they have a haloalkaline-dependent host. They then could use that host to isolate bacteriophage from environmental samples with the same alkalinity and salinity required by the host. Bacteriophage only infect a specific range of bacteria, so insuring that the host is safe for students to study is essential.

The primary goals in the second part of this project were to isolate acetic acid bacteria (AAB) with the ability to produce cellulose from fermented kombucha tea, and then to use those bacteria as potential hosts to detect bacteriophage in other fermented liquids. Cellulose-producing bacteria were not isolated, but a related species of bacteria commonly used in fermentation of liquids was isolated. A purchased strain of the cellulose-producing species *G. xylinus* and the isolated bacteria (CBAX 1.3 and CBAX 2.3) then were used to attempt detection of bacteriophage in various fermented liquids. However, obtaining bacteriophage from fermented liquids proved even more challenging

than isolating cellulose-producing bacteria. Whereas detection of lytic bacteriophage was not successful, lysogenic bacteriophage were potentially detected, although subsequent attempts to detect the bacteriophage were not successful.

The desired host bacteria are members of the group of AAB first classified as *Acetobacter xylinus* and more recently classified as *Gluconacetobacter xylinus,* which is a gram-negative, aerobic species able to survive low pH levels and produce high-quality cellulose while oxidizing glucose (Jozala, et al., 2015). Their ability to withstand low pH conditions while fermenting fruit enables them to occupy a specific niche in the environment and the food industry, although they hold positive and negative roles in the food industry (Sengun & Karabiyikli, 2011). Some bacteria cause the spoilage of wine, beer, and milk products, whereas specific bacteria are used to produce these products through an oxidative or fermentative metabolic process (Sengun & Karabiyikli, 2011). The bacteria's dependence on aerobic conditions at moderate temperatures from 24°C to 32°C enables control of the fermentation process (Sharafi, et al., 2010). Limiting oxygen, adjusting carbon sources, and adjusting temperature prevent over-metabolism of glucose and control the fermentation rate (Mamlouk & Gullo, 2013).

The prokaryotes initially grouped as *Acetobacter* were later separated according to the pathway used for sugar metabolism to CO_2, products resulting from oxidation of carbohydrates, and ability to produce acid from particular substrates (Mamlouk & Gullo, 2013). When comparing 16s rDNA sequences of species within each genus, there is high genetic conservation, and species within the genus *Acetobacter* show 95.5% to 96.3% similarity with other members of Acetobacteraceae. Furthermore, *Gluconobacter* spp. show 16s rDNA sequence similarities >97.9%, and *Gluconacetobacter* (Ga.) spp. have 16s rDNA sequence similarities >96.3%. The four species, *Ga. europaeus, Ga. intermedius, Ga. oboediens, and Ga. xylinus*, are so closely related that they share >99% 16s rDNA sequence similarity (Kersters, et al., 2006, Wee et al., 2011). BLAST results for 16s rDNA sequences of CBAX 1.3 and CBAX 2.3 indicated 99% sequence similarity with over 90 *Gluconobacter* strains, including 52 hits with *G. oxydans*. Nonetheless, the newly isolated strains could hold unique qualities. CBAX 1.3 and CBAX 2.3 were isolated from kombucha tea SCOBY so they are most likely one of the following species: *A. xylinum, A. xylinoides, Bacillus gluonicum, A. aceti, A. pasteurianus, G. oxydans*, or *Lactobacillus* spp. (Vina, et al., 2013; Marsh, et al., 2014). The highly conserved 16s rDNA gene within this family suggests the need for a polyphasic approach to classification and identification.

In addition to 16s rDNA sequences, several characteristics of the bacteria CBAX 1.3 and CBAX 2.3 indicate they belong in the AAB group. The lack of

cellulose production, along with other phenotypic characteristics, suggest they are closely related to *G. oxydans*. Furthermore, both strains of bacteria were rod shaped and gram-negative. Acetic acid production was observed when the growing colonies created clearings in the calcium carbonate floor of the agar plates (Gupta, et al., 2001; Mamlouk & Gullo, 2013). A cellulose pellicle did not develop on the surface of the liquid medium, as was observed with *G. xylinus* grown in the same medium; instead, a light brown film developed on the surface. Growth was inhibited at 32°C and optimal at 26°C, which is characteristic of *G. oxydans* (Gupta, et al., 2001). *G. oxydans* is a common component of fermented liquid biofilms (Gullo & Giudici, 2008; Sengun & Karabiyikli, 2011), and *Gluconobacter* species display a higher affinity for oxidizing sugars over ethanol compared to other genera. They also are more prolific at the beginning of fermentation than other AAB (Gullo & Giudici, 2008). To learn the capabilities and identify the isolated and cultured CBAX 1.3 and CBAX 2.3 bacteria more specifically, characterization limits of temperature, pH, ability to oxidize ethanol and carbohydrates, and metabolic pathways need to be determined (Gullo & Giudici, 2008; Sharafi, et al., 2010; Sengun & Karabiyikli, 2011; Mamlouk & Gullo, 2013).

Another goal of the acidic environments project was to isolate bacteriophage; however, those attempts were not clearly successful. As seen in the haloalkaline experiments, distinctive plaques created by lytic bacteriophage on bacterial lawns would indicate successful phage detection and confirm

subsequent isolation. Instead, poor, inconsistent, and slow lawn growth by all three host bacterial strains made identification and interpretation of plaque-like clearings difficult. Inconsistent and variable appearance of the clearings also complicated detection of bacteriophage. Despite many trials, it was difficult to discern areas in which bacteria did not grow from clearings that arose due to bacteriophage lysis.

The most encouraging results that suggested bacteriophage interacted with the host bacteria were observed in plaque assay tests of enrichments using three varieties of unfiltered vinegar from the same manufacturer: red wine vinegar (RWV) at pH 2.5, white distilled vinegar (DWV) at pH 2.5, and apple cider vinegar (ACV) at pH 3.0. Two host cultures produced full clearings, cloudy clearings, or plaque-like clearings when spot tested at full concentration, and they produced some plaque-like clearings at various dilutions. Following resuspension and subsequent enrichment, however, plaque-like clearing patterns varied with vinegar sample.

RWV samples yielded cloudy streaks before dilution, plaque-like areas with apparent inhibition of bacterial growth at the first dilution, and no bacterial growth on the perimeter of the spot. Many factors could explain these outcomes: high titer bacteriophage in the sample, toxins in the sample prevented cell growth, intolerance to low pH, lysogenic bacteriophage, low bacteriophage to

host ratio, or presence of bacteriolytic particles (Bradley, 1967; Gupta, et al., 2001; Wang, et al., 2015). Some enrichments created from resuspended plaques appeared to produce complete spot clearings at high concentrations. However, when these samples were further diluted, they did not produce plaques, suggesting the clearings were not originally due to lytic bacteriophage infection.

When the titer of bacteriophage in a sample is high enough to create a complete spot clearing, one would expect to see lower plaque frequency or smaller plaque size in serial dilutions created from the same sample (Bradley, 1967). A complete clearing suggests all bacteria within reach of the bacteriophage were lysed, which could occur due to high titer, lysis time, or virion morphology (Gallet, et al., 2011). Thus, bacteriophage should have produced some evidence of plaques at least in the first undiluted suspension. The lack of new clearings suggested the suspected plaques did not consist of high titer bacteriophage.

Extremely low pH may have inhibited cell growth in these particular strains of AAB-like cells (Wang, et al., 2015). If pH inhibited growth, then subsequent serial dilutions could result in increased bacterial growth because increased dilutions would have decreased acidity and therefore would produce a similar result as serial dilutions containing infectious or bacteriolytic materials. Even though the host bacteria demonstrated the ability to withstand acidity as low as

pH 2 in the lab, they grew best at pH 5-6. The change in pH between dilutions was not tested, but it should be considered as one explanation for the inhibited growth.

Another possibility for the inconclusive results is that other cells released toxins that affected the CBAX strains as a result of intolerance to increased levels of acetic acid production during fermentation of the DWV and RWV samples, but those cells were not present in the ACV and thus did not inhibit cell growth in that medium (Wang, et al., 2015). From prior samples and pH testing, both *G. xylinus* (96 hr) and CBAX 2.3 can survive in pH 3.0-6.0. The pH of DWV and RWV samples was close to 2.5, but the ACV had pH closer to 3.0.

Bacterial growth inconsistencies at plaque assay spot sites suggested interactions with bacteriophage or bacteriolytic particles (Bradley, 1967; Gupta, et al., 2001; Wang, et al., 2015). Characteristic traits of bacteriophage such as adsorption rate, lysis time, and morphology impact features of plaque formation (Gallet, et al., 2011). Assay results of the various fermented liquids included a variety of plaque morphologies, which also was described in a study of a siphovirus-like phage (VHS1) of *Vibrio harveyi* (Khemayan, et al., 2006). In their results, exposure to VHS1 resulted not only in clear lysis areas that included lysogenic clones, but also no evidence of lysis as well as turbid plaques.

Turbid plaques and variation in plaque patterns from generation to generation provide evidence of bacteriolytic particles that are not capable of or do not display full lysis (Weigle, 1953; Bradley, 1967; Khemayan, et al., 2006). These particles that inhibit bacterial growth could be underdeveloped bacteriophage, created by partial bacteriophage DNA, bacteriophage in plasmids, bacteriophage mutants, or even temperate bacteriophage (Weigle, 1953; Paepe, et al., 2014). Two *Gluconobacter* bacteriophages isolated from rotting apples each displayed different overall plaque morphology, but both plaques had some degree of turbidity (Robakis, et al., 1985). Many factors influence the generation and development of these bacteriolytic particles, including culture media, length of host cell cycle, and fitness of host cells (Weigle, 1953; Bradley, 1967; Khemayan, et al., 2006; Paepe, et al., 2014).

Obtaining bacteriophage that infect AAB from the ever-changing environment of fermenting liquids is challenging. The highly permeable membrane and lipopolysaccharide (LPS) components characteristic of gram-negative bacteria should make AAB good bacteriophage hosts (Rakhuba, et al., 2010) However, they have evolved adaptations to withstand the drop in pH such as the peculiar timing of intracytoplasmic membrane development demonstrated by *G. oxydans* (White & Claus, 1981) that may help them to resist bacteriophage infection. Bacteria reproduce and mutate rapidly, and these mutations include bacteriophage resistance (Paepe, et al., 2014). For example, bacteria in vinegar fermentation displayed the ability to resist infection within two weeks of

bacteriophage detection (Sellmer, et al., 1992). Bacteriophage vary in their ability to infect cells, produce detectable copies, and lyse cells (Norkin, 2010). They are too small for researchers to detect visually with an electron microscope; the viruses vary in their host interactions; and they come from a variety of sources that are not fully understood. The plaque assay technique of detecting bacteriophage is the most common technique and generally the most successful at detecting bacteriophage, but when the hosts are not easily cultivated on a bacterial lawn, this method becomes less successful.

Discovery of bacteriophage that infect AAB has been limited until recently. The majority of detected bacteriophage come from vinegar fermentaria where reduced production indicated bacteriophage proliferation (Sellmer, et al., 1992). Most detection has come from fermentaria using either submerged generators or trickling generators with nonsterile air circulators and infect *G. oxidans* (Sellmer, et al., 1992). Other bacteriophage have been isolated from rotting fruit or spoiled wine (Robakis, et al., 1985). In these cases, the suspected enrichment was analyzed with electron microscopy, followed by DNA sequencing (Robakis, et al., 1985; Selmer, et al. 1992; Bebeacua, et al., 2013). Electron microscopy is an efficient and effective way to detect bacteriophage in infected liquids when the numbers of bacteriophage reach at least 10^6/mL (Sellmer, et al., 1992). Once the particles are detected, they can be used to test infectability to a host of interest. Digestion of DNA from environmental samples can help to reveal conserved sequences and capsid proteins that could lead to bacteriophage detection and

identification.

The 21st century brings time-sensitive challenges in the fields of science, technology, engineering, and mathematics (STEM; Bybee, 2010; PCAST, 2012; Jordan, et al., 2013). Obstacles faced with respect to disease control will best be met with education and preparedness of pre-college students having specific research skills in the fields of science and technology to monitor antibiotic resistance through surveillance; to prolong antibiotic effectiveness; to develop alternative solutions to antibiotics; and to uncover links between nonhuman animals and humans through genomics (Bybee, 2010; PCAST, 2014). One such method of collaborative hands-on, research-based instruction involving bacteriophage discovery and genome library development already has been implemented successfully at the undergraduate level (Jordan, et al., 2013). The same strategy was used with development of the Wisconsin FastPlants®, *Brassica rapa,* that included feedback from K-12 students (Wendell & Pickard, 2007) and involved communication with astronauts on the International Space Station (NASA, 2012). These goal-oriented, inquiry-based STEM projects are the best way to prepare students for the future (Bybee, 2010; Abels, 2014). With support from local universities and biotechnology companies through interactive partnerships, teachers can improve their own skills while providing their students with engaging experiences that in turn strengthen the future workforce.

Isolation of bacteria, followed by discovery of bacteriophage from both the haloalkaline soda lake environment and the acidic fermented liquid environment, can be developed into an education model that uses inquiry-based lessons in the context of STEM to aid students in meeting the NGSS standard “Molecules to Organisms”. The entire process could be scaffolded to meet the needs and enhance the abilities of all students (Abels, 2014). Specifically MS-LS1-5, *Construct a scientific explanation based on evidence for how environmental and genetic factors influence the growth of organisms,* can be addressed through manipulation of bacterial growth using media contents, temperature, pH, salinity, and oxygen, all factors easily controlled in classrooms with minor support from a university or biotechnology laboratory. The ease of discovering lytic bacteriophage in the haloalkaline environment suggests it is the best choice for developing a model that includes bacteriophage detection. However, isolation of a *Vibrio* species, although non-toxic, suggests that pre-isolation and identification should occur before using the samples in a classroom. Acidic fermented liquids are part of the food industry, so their bacteria are the safest to isolate, but their slow growing rates could be problematic in the classroom timeline, and the difficulties in discovering and propagating matching bacteriophage suggest pre-isolation would result in the most success.

Understanding the role of microbes in the environment, their impacts on human health, and their genetic similarities and differences can be paramount in meeting 21st century needs with respect to environment (water), health (disease

and medical applications), and energy (biofuels). Developing an educational model for secondary school students (grades 7-12) based on the practices of isolating bacteria and discovering bacteriophage is one way to help students learn core ideas of environmental impacts and molecular interactions while they practice crosscutting concepts through the processes of research and collaboration, ultimately meeting the expectations of the NGSS.

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