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Isolation and Characterization of Bacteriophages Infecting Enterobacter cloacae to Reduce Bloater Defect in Cucumber Fermentations

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Isolation and Characterization of Bacteriophages Infecting *Enterobacter cloacae* to Reduce Bloater Defect in Cucumber Fermentations

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Abstract

Fermented cucumbers are one of the most important fermented vegetables consumed worldwide. During cucumber fermentations, certain undesirable changes may occur. One of such changes is known as bloater defect (hollow cavities in fermented cucumbers), which is primarily caused by gas-producing bacteria including *Enterobacter cloacae*. Bloater defect lowers product quality and leads to significant economic loss to the pickle industry, and effective preventative methods are needed. Bacteriophages (phages) are highly host-specific bacteria killers. Use of phages to control unwanted bacteria in foods is a promising approach because phages do not change food properties. The goals of this research were to isolate, characterize, and evaluate phages infecting *Enterobacter cloacae.* The morphology, growth kinetics, host range, and effectiveness of two isolated phages, named Φ107E-p1 and Φ115E-p2, were examined. Additionally, DNA and protein analysis were performed. Based on acquired data, Φ115E-p2 is a potential candidate for use as a biocontrol method to prevent bloater defect during cucumber fermentations. Φ107E-p1 is not a candidate based on the effectiveness data obtained. More research is needed to further evaluate the efficacy of the phage infections against their hosts in cucumber fermentations.

Introduction

Food fermentation

Fermentation, defined by Pasteur as “life without air,” has a rich history and high importance dating back thousands of years. Biochemically, fermentation is a metabolic process that breaks down carbohydrates, such as glucose, without the use of oxygen. During this process, glucose will undergo glycolysis but not enter into the citric acid cycle or the electron transport chain. During glycolysis, glucose is broken down into pyruvate. The breakdown of glucose into pyruvate also produces two net ATP and two NADH molecules. The NADH molecules will then undergo oxidation to lose electrons since they are not needed for the electron transport chain. This is done to regenerate NAD+ to continue to carry out glycolysis. This process produces carbon dioxide as a byproduct. The fermentation process can produce alcohol or lactic acid depending on the specific type of fermentation. Alcohol fermentation involves NADH donating its electrons to a pyruvate derivative to produce ethanol, which is often used by yeast and drives the creation of alcoholic beverages such as beer. Lactic acid fermentation involves NADH donating its electrons to pyruvate which generates lactate. Lactate will then be deprotonated to form lactic acid, which is used to drive many food fermentations.

Fermented foods have been a vital part of the human diet for thousands of years, and fermentation is often regarded as one of the oldest biotechnologies (Ray and Joshi, 2014). The fermentation process helps to preserve food and prevent spoilage (Chilton et al., 2015), which, in the early years of fermentation, was essential to providing food during colder months when food may be harder to grow or find due to a decrease in

available vegetation and a lowered animal population due to hibernation and migration. Additionally, some fermented foods hold importance in many cultural dishes around the world and are a part of the rich history of many cultures. A prime example of a popular fermented food, fermented cucumbers, has been important on a global scale for thousands of years, dating as far back as 6,000 BC (Franco et al., 2016).

Earliest fermentation is believed to have originated in the Indian sub-continent, but exact origin time and location have been lost throughout the years. At this time, the scientific basis for the fermentation process was not understood and would not be understood until around 1665 when van Leeuwenhoek and Hooks identified microorganisms (Gest, 2004). After this identification, Lister made the discovery that bacteria were the driving force behind milk fermentation in 1877 (Santer, 2010). Since these discoveries, food fermentation has expanded to become an important part of the food industry on a global scale, and there are many types of fermented foods on the market (i.e. Sauerkraut, yogurt, kimchi, miso, beer, kombucha, fermented cucumbers (pickles, etc.)). This large variety and the popularity of these foods create a profitable market, which is seen in the global pickle market.

The global pickle market was valued at $10.3 billion USD in 2018, according to a report by the International Market Analysis Research and Consulting Group (Anonymous, 2019). Additionally, fermented cucumbers are used in many countries in East Asia to create cultural foods such as khalpi in Nepal, paocai in China, and oiji in Korea. While similar, each of these dishes have their own cultural aspects and have some differences, such as spices used for flavor. These dishes center around the

fermented cucumber component and are popularly consumed in their countries of origin.

Lactic acid bacteria (LAB) are naturally found on cucumbers, and they aid in the fermentation process through the release of lactic acid as a product of a metabolic processes called lactic acid fermentation (Franco et al., 2016). LAB metabolize and degrade carbohydrates to produce lactic acid during this process. This lactic acid contributes to the smell, taste, and preservation of fermented foods. Industrial fermentation of cucumbers occurs in fermentation tanks capable of holding 15 to 20 tons of cucumbers. The cucumbers in the tanks are covered in brine containing salts and other materials necessary for the fermentation process to occur (Franco et al., 2016). The fermentation tanks are checked often to ensure proper pH and to check for brine turbidity. During the fermentation process, carbon dioxide can build up in the tanks. If not properly managed, this can lead to defects that impact taste, texture, appearance, smell, and consistency of the fermented cucumbers. While not harmful to consume, defective fermented cucumbers are undesirable for consumption due to consumer preference. This can result in economic loss to the pickling industry due to the product becoming undesirable to consumers.

Cucumber Fermentation defects

Fermentation defects can change the texture and smell of fermented cucumbers, leading to the fermented cucumbers being undesirable for consumption. There are many types of defects ranging from change in color to more serious physical defects, leading to holes inside the fermented cucumbers or changes in texture consistency.

These defects often occur due to the accumulation of gases produced by lactic acid bacteria in the fermentation tanks.

One defect that is the result of an accumulation of CO_2 during the fermentation process is known as bloated cucumber defect, also known as bloater defect, seen in figure 1. Bloater defect is the formation of gas pockets in fermented cucumbers. This can cause undesirable changes in texture and appearance of the fermented cucumbers which leads to a lower product quality. This lower product quality can then result in an economic loss in the pickling industry (Zhai et al., 2018). Gas-producing Gram-negative bacteria, such as *Enterobacter cloacae*, are the primary cause of bloater defect during cucumber fermentations.

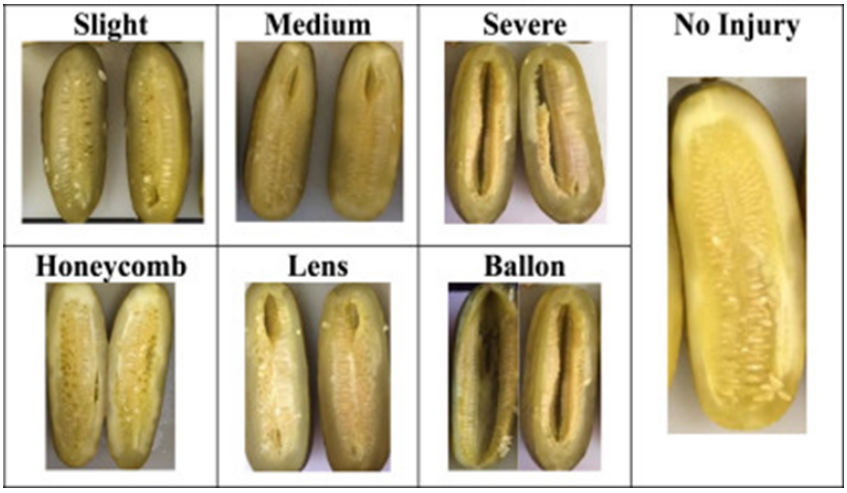

Fig. 1. Examples of bloater defect. Adapted from Zhai et al. (2018)

E. cloacae, seen in figure 2, is a rod-shaped, Gram-negative bacterium that belongs to the family *Enterobacteriaceae* which contains many gas-producing members. This organism is a facultative anaerobe which means that although oxygen is preferred to undergo aerobic respiration, the organism is capable of fermentation in the absence of oxygen. *E. cloacae* is also one of the bacteria that naturally occurs on

cucumbers and is one of the major contributors of bloater defect during cucumber fermentation (Etchells and Jones, 1941). This organism is also a member of the human intestinal microbiome and can be an opportunistic pathogen causing infections in the urinary tract (Pereira et al., 2015), lower respiratory tract, and on skin (Liu et al., 2013). This organism produces CO_2 during cucumber fermentation and contributes to the gas buildup that creates the hollow cavities in fermented cucumbers that cause bloater defect. There are current preventative methods to attempt to reduce the incidence of bloater defect and inhibit the growth of undesired microorganisms in fermentation tanks, but these methods have limitations..

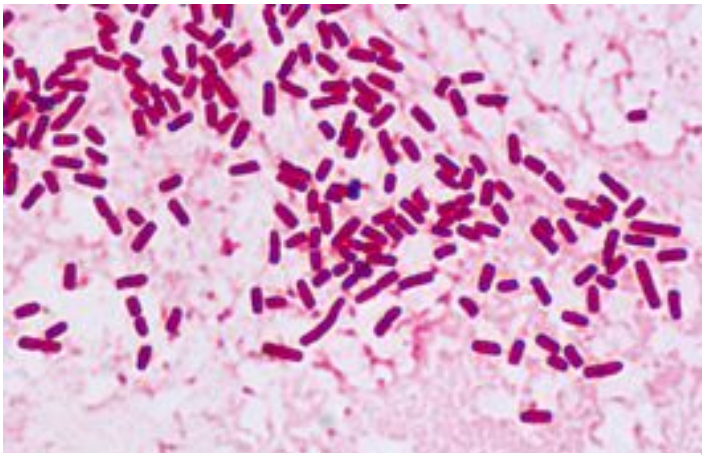

Fig. 2. Image of *Enterobacter cloacae*. Adapted from microbe-canvas.com.

Current control methods

There are several methods that have been used to reduce bloater defect during cucumber fermentations. These methods include nitrogen purging, controlled fermentation using a starter culture, acidification of cover brines, and an increase in salt concentration. **Nitrogen purging**, as seen in Figure 3, utilizes nitrogen gas to displace CO_2 during the fermentation process in an attempt to prevent gas accumulation from

occurring inside the fermentation tanks (Fleming et al., 2006). However, nitrogen purging greatly increases the production costs due to additional equipment investment, energy input, and the cost for the constant use of N_2 for days at a time (Zhai et al., 2018).

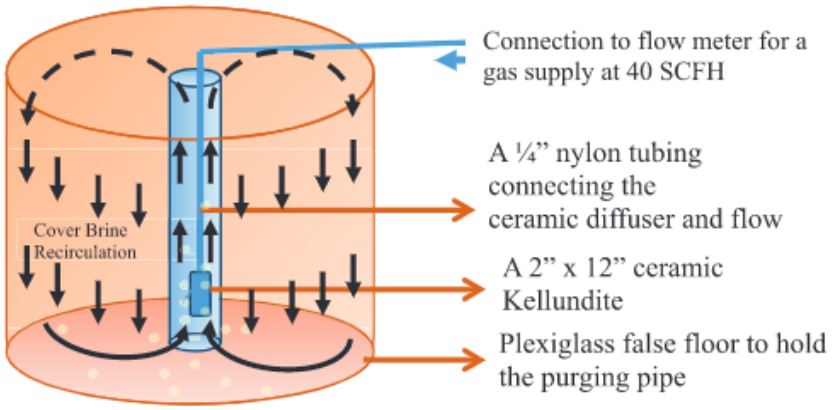

Fig. 3. Nitrogen purging system design proposed by Costilow et al. (1977). This design shows an incorporation of a false floor of Plexiglass at the bottom of the tank to hold a pipe to serve as a case for a nitrogen diffuser. Adding nitrogen bubbles to the bottom of the tank forces the liquid to move up the pipe to create circulation.

Controlled fermentation with added starter cultures consisting of lactic acid bacteria can quickly produce a large amount of acids causing a decrease in pH, thereby inhibiting the growth of *E. cloacae* and other Gram-negative gas-producing bacteria. However, some starter cultures may also produce CO_2, therefore contributing to bloater defect (Zhai et al., 2018).

Acidification of the cover brine and **increasing salt concentration** in brine both can inhibit the growth of certain microorganisms including some gas-producing bacteria such as *E. cloacae* and others during fermentation (Zhai et al., 2018). Issues with acidification arise because lower pH may inhibit the growth of lactic acid bacteria,

which are commonly used starter cultures (Perez-Diaz and McFeeters, 2010). Disposal of wastewater containing a high concentration of salt pollutes freshwater which disturbs natural ecosystems of local bodies of fresh water creating a serious environmental issue (McFeeters and Perez-Diaz, 2010).

While these preventative methods are commonly used, they do have their limitations and are not completely effective at preventing bloater defect. More effective methods need to be developed. One such method is the used of bacteriophages for biocontrol of these undesirable bacteria.

Bacteriophages

Bacteriophages (phages) are viruses that infect specific bacteria and can lead to cell lysis. Like other viruses, phages are obligate intracellular parasites and rely on the host cellular machinery to replicate. Most phages have an icosahedral head and a tail that may have tail fibers attached. The phage head contains the genome, which is usually double-stranded DNA. The DNA is injected into the host via the tail to begin the process of phage replication.

After the DNA is inserted into the host genome, there are two cycles the phage DNA can begin (figure 4), the lytic and the lysogenic. In the lysogenic cycle, the phage DNA remains in the host genome for a period of time without replicating. The lytic cycle will need to be induced for phage replication and release. The lytic cycle begins with an adsorption phase where the phage attaches to the host and injects its DNA into the host cell. The DNA will then undergo transcription into RNA that will then be translated into necessary viral proteins. The DNA then undergoes replication and is then assembled

inside the host cell. The cell then undergoes cell lysis to release the progeny virions during a lytic cycle. The number of progeny virions released from an infected cell is known as burst size which is a characteristic often examined when studying phages.

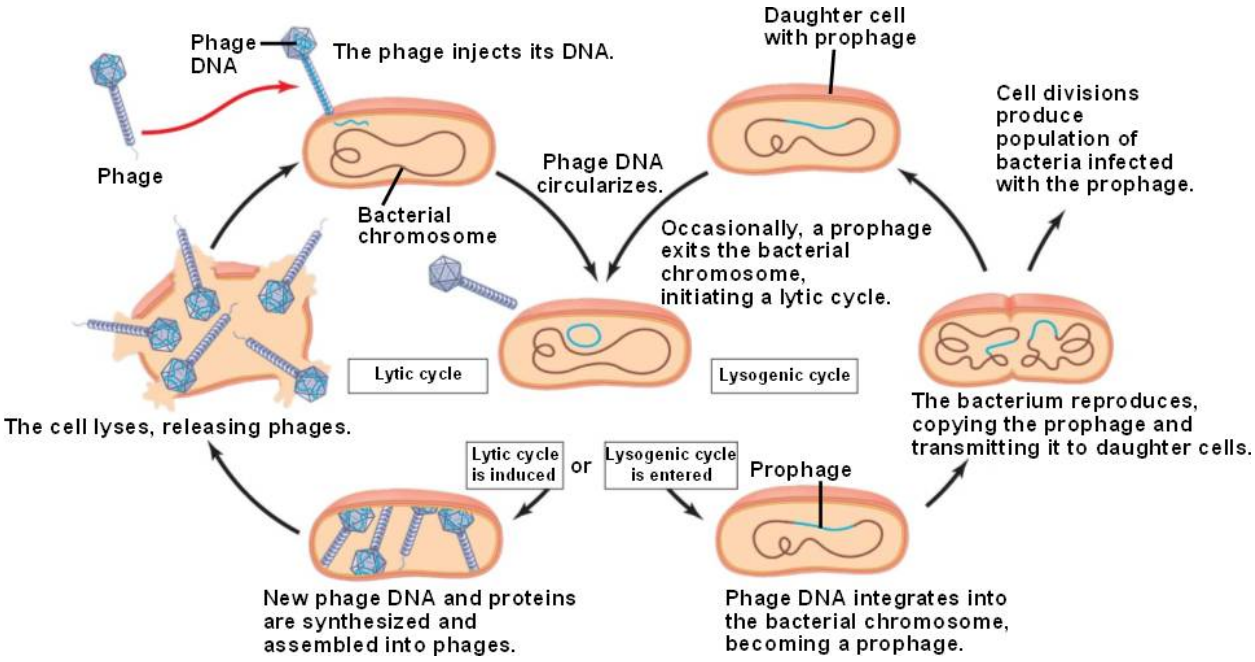

Fig. 4. Diagram of the replicative cycle of a lytic bacteriophage. Adapted from the internet (https://mrmitchellsbiology.weebly.com/uploads/1/0/4/2/10422385/5830831_orig.jpg).

There are many studies on the use of phages as biocontrol agents against foodborne pathogens such as *Escherichia coli* O157:H7 (Ramirez et al., 2018), *Salmonella enterica* (Wall et al., 2009), and *Listeria monocytogenes* (Lee et al., 2017). These foodborne pathogens pose significant problems to consumers as they can lead to gastroenteritis as well as other health issues in humans. In addition to this, *L. monocytogenes* can lead to birth defects and miscarriage. The use of phages as a biocontrol method has shown to reduce the concentration of these pathogenic bacteria

in model food systems such as cucumber juice and beef broth as well as certain foods such as vegetables, dairy, and meat. Therefore, using phages may be a promising method to control the bacteria causing bloater defect in the pickling industry.

A phage that is an ideal candidate would need to be lytic so as to more rapidly kill its host. In addition to this, an ideal phage would have a relatively broad host range so it can infect more than one bacterial strain. However, a narrow host range can still be useful and the use of a phage with a narrow host range would depend on other factors. A relatively short adsorption period and large burst size are also preferred so the phage can rapidly enter the cell and lyse the cell while producing a large number of progeny virions to more rapidly infect and kill the host organism. Additionally, a phage should be effective against its host with the ability to greatly reduce the host concentration. Previous studies (Guenther et al.) have shown that phage concentration at application is critical for phage effectiveness. Testing phages at various multiplicity of infections (MOI), the ratio of the concentration of virus particles to the concentration of host cells, provides crucial information regarding the phage concentration for future application. Ideally, a phage that is a good candidate will have a combination, if not all, of these characteristics.

Advantages of using bacteriophages to control bacteria

There are many advantages of using phages for the use of preventing and controlling undesired bacteria. Phages are highly host-specific, infecting only certain bacteria, and not infecting most microflora found in the gastrointestinal tract (Lu et al., 2015). Phages are self-limiting because they will not replicate in food unless their host is

present. Phages will not change basic food characteristics such as flavor, texture, and nutrition (Coffey et al., 2010). It is also relatively cheap and easy to isolate and propagate phages in a laboratory, which is cost-effective to study and to apply phages in the pickling industry. Phages are the most abundant organisms on the earth. They are ubiquitous in nature, found almost anywhere in the environment that bacteria persist. This allows for phages to be readily isolated and propagated in laboratories. Many phages have been isolated from sewage water (Topka et al., 2019) and fecal material from animals (Lee et al., 2017) and used to control foodborne pathogens.

The many advantages of phages contribute to their potential use in the pickling industry to prevent bloater defect. However, the effectiveness of phage infection against a target bacterium in a model food system needs to be evaluated before application in the industry. Cucumber juice has the same chemical composition as whole cucumbers. It is convenient to use cucumber juice as a model system to evaluate of the effectiveness of phage infection against *E. cloacae*.

Objectives of this research

The ultimate goal of this research was to isolate phages that are potential candidates for use in the pickling industry to prevent bloater defect. The objectives of this research were 1) to isolate phages infecting *E. cloacae*, 2) to characterize the isolated phages based on their morphology, growth kinetics, host range, structural proteins, and genome, and 3) to evaluate the effectiveness of the phages against *E. cloacae* in cucumber juice (as a model food system). The results from this study will provide valuable information for the application of phages in the pickling industry.

Materials and Methods

Bacterial strains and culture conditions

Two strains of *E. cloacae* (107E and 115E, highlighted in red) were used for phage isolation. A total of 30 Gram-negative bacterial strains were used for determination of host ranges (table 1). Strains can be identified by host ID. Those bacterial cultures were from our culture collection stored at -80ºC. Frozen cultures were streaked onto tryptic soy agar (TSA) plates. All plates were incubated at 37ºC for 20-24 hr. A well isolated colony from each plate was picked to inoculate 10 mL of tryptic soy broth (TSB). Those tubes were then incubated at 37ºC for 12 hr. The fresh broth cultures were temporarily stored at 4ºC until use.

Table 1. Bacterial strains used in the study.

Host ID	Host name
102E	*Citrobacter freundii*
227E	*Citrobacter freundii*
232E	*Citrobacter freundii*
103E	*Enerobacter kobei*
107E	***Enterobacter cloacae***
109E	*Enterobacter cloacae*
116E	*Enterobacter cloacae*
220E	*Enterobacter cloacae*
115E	***Enterobacter cloacae subsp. cloacae***
119E	*Enterobacter cloacae subsp. cloacae*
221E	*Enterobacter cloacae subsp. cloacae*
225E	*Enterobacter cloacae subsp. cloacae*
228E	*Enterobacter cloacae subsp. cloacae*
230E	*Enterobacter cloacae subsp. cloacae*
231E	*Enterobacter cloacae subsp. cloacae*
237E	*Enterobacter cloacae subsp. cloacae*
112E	*Enterobacter kobei*
235E	*Enterobacter kobei*
238E	*Enterobacter kobei*
222E	*Enterobacter nimipressuralis*
43893	*Escherichia coli* O157:H7
B241	*Escherichia coli* O157:H7
233E	*Escherichia vulneris*
226E	*Leclercia adecarboxylata*
239E	*Leclercia adecarboxylata*
111E	*Providencia rettgeri*
117E	*Providencia rettgeri*
113E	*Pseudocitrobacter anthropi*
B205	*Salmonella enterica* Belem
B207	*Salmonella enterica* Cerro

Measurement of host cell growth was done using spread plate method followed by colony enumeration.

Sample collection and processing

About 3 pounds of cow manure was collected from 5 individual cows from Carlton Farm in Rockmart, GA. The samples were placed in an ice box and brought back to the lab for same-day processing.

Approximately 150 g of cow manure sample and 100 mL of BHI broth were added into each of two stomach bags. The contents in the two bags were homogenized using a stomach machine (Seward) at 150 RPM for 2 min. After the coarse homogenization, the content of each was poured into a large beaker containing 900 mL of saline and a stir bar. After stirring for 5 min, the well homogenized sample was transferred into 6 centrifuge bottles and then centrifuged (Sorval model RC 5B Plus) for 30 min at 8,000 rpm and additional 50 min at 10,000 rpm. Approximately 500 g of the supernatant was collected and then centrifuged (Sorval model RC 5B Plus) for 100 min at 11,000 rpm. The supernatant from the last centrifugation was pre-filtered using coarse porosity filter paper (P8-creped, Fischer). The filtrate was subjected to a second filtration through a bottle top filter (0.45 µm pore size) (rapid flow, Nalgene). The final filtrate was stored at 4ºC to be used for phage isolation.

Phage isolation and purification

The methods described by Lu et al. (2015) and Akhtar et al. (2017) were used with some modifications for phage isolation. Two hundred µL of a fresh host culture (*E. cloacae* 107E or *E. cloacae* 115E) was added into a 250-mL bottle containing 60 mL BHI and 20 mL cow manure filtrate. The two bottles were incubated at 37°C for 12 hr. Nine hundred µL of the phage lysate from each bottle was transferred to a 1.5 mL

microcentrifuge tube and centrifuged at 13,200 rpm for 3 min. The supernatant was transferred to new microcentrifuge tubes and used for spot tests. One hundred µL of each host culture was added to 3 mL of soft Luria Bertani (LB) agar (0.7% agar) and poured onto a BHI agar plate. After the top layer was solidified, about 10-12 µL of the corresponding phage solution was spotted onto the overlay plate. Each plate was spotted in 4 areas and incubated at 37°C for 12 hr. Positive spot tests were indicated by zones of cell lysis on the host lawn. Titer of the phage solution used for spot testing was determined using plaque assays. The phage solution containing phages against *E. cloacae* 107E had a titer of approximately $1x10^6$ pfu/mL. The phage solution containing phages against *E. cloacae* 115E had a titer of approximately $2x10^7$ pfu/mL.

Plaque assays were performed to obtain well isolated plaques. Specifically, 100 µL of the host culture and 100 µL of the corresponding diluted phage lysate were added to 3 mL of soft TSA (0.5% agar). After briefly mixing using a vortex, the mixture was overlayed onto a TSA plate. The plates were incubated at 37°C for 6 to 12 hr.

A well-isolated plaque was picked and added into a 1.5 mL tube containing 1 mL TSB. After briefly mixing using a vortex to release phage particles from the plaque, the tube was incubated at 37ºC for 12 hr. The phage lysate was centrifuged at 13,200 rpm for 3 min. The supernatant was used for the next round of plaque purification. A total of 3 rounds of plaque purification was performed.

Phage enrichment and phage stock preparation

To increase the phage titer and the volume of the phage solution, the plaque-purified phages were added into a 50 mL tube containing 30 mL of TSB and fresh host

culture. The tube was incubated at 37°C for approximately 10 hr and then centrifuged (Eppendorf 5810R, 15-amp version) for 30 min at 4,000 rpm (3,220 x g) to remove remaining host cells and cell debris. Hosts and cell debris formed a pellet while leaving the supernatant clear. The supernatant was transferred to new tubes and filtered with a syringe disk filter (0.45 µm pore size). The filtered phage solution was stored at 4ºC for short-term use. In addition, frozen phage stock for long-term storage was prepared by adding glycerol (16% final concentration) and stored at -80ºC.

Determination of host range

Host range of the isolated phages was determined using both spot tests and plaque assays similarly described above. TSA and soft TSA (0.5%) were used as media for the tests. The study was carried out using the bacteria listed in Table 1. A 4-class scale was used for results with (-) indicating no phage activity, (+) indicating a positive spot test but non plaque-forming, (++) indicating the formation of turbid (hazy) plaques, and (+++) indicating the formation of clear plaques.

One-step growth curve

The growth kinetics of the isolated phages were measured in cucumber juice using the methods described by Lu et al. (2003 and 2015) with some modifications. Briefly, 12 µL of phage was added to a tube containing 12 µL host and 1,176 µL cucumber juice at the multiplicity of infection (MOI) of 0.005 for both phages. After a 20-min incubation at 37ºC, the un-adsorbed free phage particles were removed by centrifugation at 13,200 rpm followed by and washing with 1 mL cucumber juice three

times. The cell pellet with adsorbed phages was then resuspended in 1.2 mL of cucumber juice. The tube was incubated at 37°C. Samples were taken at time 0 and then at 10 min intervals for up to 120 min. After serial dilution, samples were analyzed using plaque assays as described above. Experiments were repeated in triplicate. A one-step growth curve was constructed based on the collected data. Latent period and burst size were calculated based on the curve.

Large scale phage preparation, concentration, and purification

One liter of high titer phage solution was prepared as described above. Phage lysate was centrifuged (Sorval) at 10,000 rpm for 100 min. The supernatant was then filtered (0.45 µm pore size). The filtrate was then treated with the enzymes DNase I and RNase A prepared in 50% glycerol to a total concentration of 0.3 g/5 mL. DNase I and RNase A at a volume of 215 µL were then added to 850 mL of Φ107E-p1 filtrate. DNase I and RNase A were added at a volume of 142 µL to two bottles containing 565 mL Φ115E-p2 filtrate each. Enzyme treated phages were incubated for approximately 4.5 hr. Enzyme treated Φ107E-p1was then split into two bottles containing approximately 430 mL phage each. For Φ107E-p1, 13 g NaCl and 45.4 g PEG 8,000 were added for precipitation. For Φ115E-p2, 17 g NaCl and 58.4 g PEG 8,000 were added for precipitation. Phage solution was filtered (0.45 µm pore size) and centrifuged (Eppendorf) for 20 min at 8,000 rpm. Supernatant was discarded and more phage solution was added and recentrifuged for 30 minutes at 9,000 rpm. Supernatant was discarded. Additional phage solution was centrifuged at 9,000 rpm for a 50 min. Supernatant was discarded and the pellet was resuspended in 900 µL Tris buffer for

density gradient ultracentrifugation or a total of 3.5 mL TE buffer for DNA extraction. Phage suspended in Tris buffer was used for density gradient centrifugation in an ultracentrifuge. The densities of CsCl used were 1.7, 1.5, and 1.4. 1 mL of each density were layered into an ultracentrifuge tube. Three mL of phage in Tris buffer was then added to the tube. Approximately 2.9 mL mineral oil was then used to fill the ultracentrifuge tube. Tubes were then centrifuged using an ultracentrifuge (Beckman Coulter) at 74,000 rpm for 15 hr at 4°C. Phage bands were collected using a syringe and 21 1/2 G needle. Phage bands underwent dialysis in sterile deionized water for a total of 4 h and 15 min to remove any CsCl in the phage bands. Highly purified and concentrated phages were then stored at –20°C for future use.

Electron Microscopy

Highly purified and concentrated phage samples were negatively stained with 2% (w/v) aqueous uranyl acetate on carbon coated grids and examined by transmission electron microscopy at an accelerating voltage of 80 kV. Electron micrographs were taken at 50,000x magnification (Center for Electron Microscopy, North Carolina State University, Raleigh, NC).

Phage DNA extraction and analysis

The methods described by Lu et al. (2003 and 2015) were followed with minor modifications for phage DNA extraction and analysis. Approximately 3.7 mL of phage solution underwent phenol-chloroform extraction. Phage solution was added to 15 mL tubes. Equal amounts of phenol were added to each phage and then rigorously shaken.

200 µL phenol:chloroform:isoamyl alcohol (25:24:1) was then added. Tubes were shaken and then centrifuged (Eppendorf) for 20 min at 4,000 rpm at 4°C. The aqueous layer for each phage was then split into two phase lock gel tubes. For each tube, 200 µL phenol and 2 mL phenol:chloroform:isoamyl alcohol were added. Tubes were centrifuged for 10 min at 4,000 rpm at 4°C. Two mL phenol:chloroform:isoamyl alcohol was added to each tube and centrifugation was repeated. This step was repeated for a total of 5 times. The aqueous layer was transferred to clean 15 mL tubes with like phages being combined. Sodium acetate at a concentration of 3 M was added to the aqueous layers at a ratio of 10:1, and 3 times 95% ice-cold ethanol was added to the tubes. Tubes were placed at -20°C for 1 hr. Tubes were then balanced and centrifuged for 20 min at 4,000 rpm at 4°C. Supernatant was decanted. DNA pellets were rinsed with 13 mL ethanol followed by washing with 10 mL ethanol. Pellets were dried at 37°C for 30 min. For each tube, 300 µL TE was added to resuspend the DNA pellets. DNA was then stored at 4°C.

Phage DNA was digested with the restriction endonuclease EcoRV at 37°C for 12 hr. One µL of the DNA fragments was mixed with 10 µL of sample buffer before analyzed using the Agilent TapeStation 4200.. A 60-kbp DNA ladder (Agilent) was used to measure the size of phage DNA fragments. The ladder was mixed with 0.5 µL water and 10 µL of sample buffer before loaded to the automated electrophoresis system.

Fragments of DNA were also separated by agarose (60 mL 1% agarose in TBE) gel electrophoresis in Tris-borate–EDTA buffer at 150 V for 45. The gel was stained with RedSafe Stain (Frogga Bio). The gel was analyzed under UV light.

Protein analysis

The methods described by Lu et al. (2015) were followed for the analysis of viral structural proteins with some modifications. Phage samples were prepared by adding 4X sample buffer and 10X DDT. Samples were then boiled for 10 min. Pre-stained protein standard (FroggaBio) and phage samples were loaded into a precast gradient minigel (4–12% Bis-Tris, Invitrogen Corporation, Carlsbad, CA). Electrophoresis was carried out at constant 100 V for 90 min. Gel was removed and rinsed with deionized water. After rinsing, 50 mL SimplyBlue SafeStain (Invitrogen) was added and placed on a shaker for 60 min. Stain was then changed and placed back onto the shaker to stain overnight. Stain was then changed 4 more times to allow for a total staining time of approximately 16 hr. Stain was removed and gel was destained with deionized water for a total of 1 hr and 50 min. Water was decanted and approximately 60 mL of drying solution was added. Gel was dried for a total of 25 min. Gel was mounted into parafilm and allowed to continue to dry before analysis.

Evaluation of effectiveness of phages against E. cloacae in a model food system

Cucumber juice was used as a model food system to evaluate the effectiveness of the phages against the target bacteria. Fresh overnight host culture was prepared in TSB with 12 hr incubation at 37°C. The initial concentration of the host in the cucumber juice was approximately 10^5 cfu/mL. For Φ115E-p2, two MOIs were used (0.01 and 0.1). For Φ107E-p1, four MOIs were used (0.01, 0.1, 1, and 10). A tube that does not contain

the phage was included as a control. Samples were taken at 1 hr intervals over a 6 hr time period with samples starting at 0 hr. The samples were diluted as needed and plated onto TSA plates. After 16 hr incubation, those plates were examined. The colonies were enumerated and the data was recorded. Experiments were repeated in triplicate and graphs were generated based on acquired data.

Results and Discussion

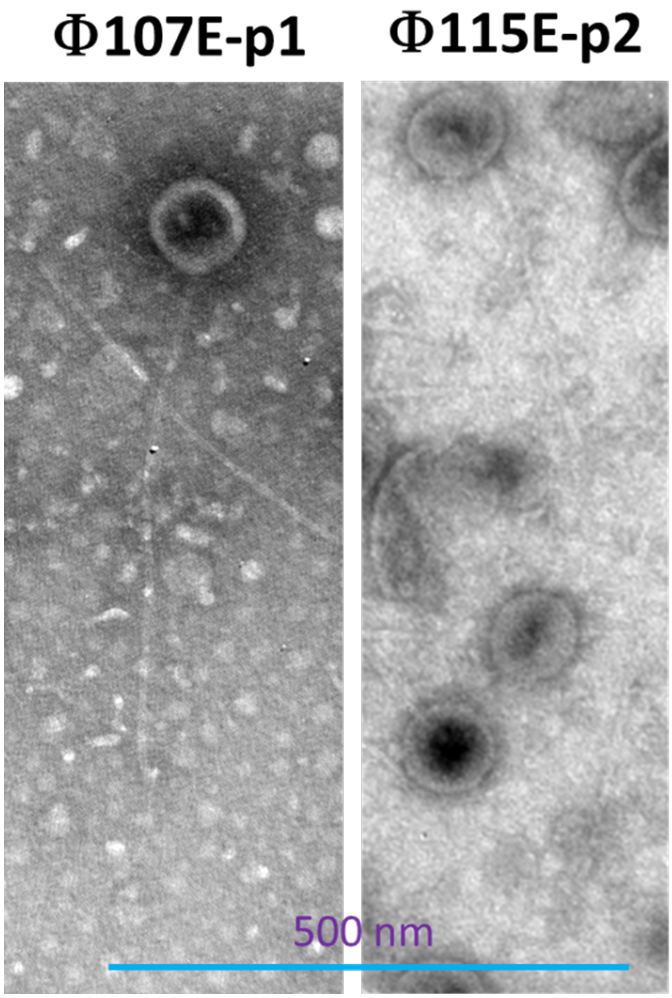

Fig. 5. Transmission electron microscopy images of Φ107E-p1 and Φ115E-p2. The bar represents 500 nm.

Phage isolation and morphology

Two phages against *E. cloacae* were isolated from approximately 3 pounds of cow manure taken from 5 cows. Since *E. cloacae* is a member of the natural microflora in many organisms, cow manure is an excellent source of phages due to the fact that phages can be found where their host resides. Additionally, cow manure is often used as a fertilizer for many vegetables, including cucumbers. Phages are also commonly found in sewage water, but for this study cow manure was deemed to be a better source due to the reasons described above.

The two isolated phages were named Φ107E-p1 and Φ115E-p2. Phages were named based on the specific strain of *E. cloacae* that they were isolated against (Φ107E-p1 was isolated against *E. cloacae* 107E and Φ115E-p2 was isolated against *E. cloacae* 115E). Highly purified phage samples were used for transmission electron

microscopy imaging to determine the morphology for characterization purposes as well as to determine the family in which Φ107E-p1 and Φ115E-p2 belong.

Electron microscopy images were used to determine phage morphology and establish size of the phages as this is a standard method for phage morphology characterization. Φ107E-p1 has an icosahedral head with a diameter of approximately 90 nm and a tail with a length of approximately 500 nm (as seen in figure 5). Φ115E-p2 has an icosahedral head with a diameter of approximately 100 nm and a tail with a length of approximately 260 nm (as seen in figure 5). Φ115E-p2 has a head that is slightly larger in diameter than the head of Φ107E-p1. However, Φ107E-p1 has a tail that is approximately 240 nm longer than the tail of Φ115E-p2. Both heads are icosahedral which is to be expected as this is a morphological characteristic of phages. The tails are considered noncontractile due to the thin diameter of the tails which likely indicates the absence of a tail sheath, a feature seen in contractile tails.

Both phages in this study are tailed phages, which are incredibly abundant on Earth (Casjens, 2008), with long non-contractile tails, as seen in figure 5. Based on morphological analysis and comparison with phages belonging to various families, both phages belong to the *Siphoviridae* family, the second most common family of phages that were found to be infecting members of the *Enterobacteriaceae* family (Grose and Casjens, 2014).

Further studies can be done for verification including sequencing and open reading frame prediction to look for an ORF coding for a tail plate. A base plate would indicate that the phages belong to *Myoviridae,* another family of tailed phages. The absence of a base pate would confirm that the phages are *Siphoviridae*. Additionally,

length of the tails (500nm and 260 nm) is more consistent among comparison with various *Siphoviridae* phages. *Myoviridae* phages tend to have shorter and thicker tails that do not typically exceed approximately 200 nm. Both phages in this study have tails that exceed this length to provide evidence that the phages do not belong to *Myoviridae*.

An additional study that could confirm whether or not the tails are contractile would include taking samples every few minutes while the phage is attaching to the host. If the phages have a visible tail of one length at a particular time point and a shorter length at a later time, the tails would be contractile. Consistent tail length over time would indicate a noncontractile tail and verify that the phages belong to the *Siphoviridae* family.

Host range

Table 2 Host ranges of Φ107E-p1 and Φ115E-p2.

Host ID	Host name	ϕ107-p1 activity	ϕ115-p2 activity
102E	*Citrobacter freundii*	-	-
227E	*Citrobacter freundii*	-	-
232E	*Citrobacter freundii*	-	-
103E	*Enerobacter kobei*	+	-
107E	***Enterobacter cloacae***	**+++**	**-**
109E	*Enterobacter cloacae*	+	-
116E	*Enterobacter cloacae*	++	-
220E	*Enterobacter cloacae*	++	-
115E	***Enterobacter cloacae subsp. cloacae***	**-**	**+++**
119E	*Enterobacter cloacae subsp. cloacae*	+	-
221E	*Enterobacter cloacae subsp. cloacae*	++	-
225E	*Enterobacter cloacae subsp. cloacae*	+	+++
228E	*Enterobacter cloacae subsp. cloacae*	+	-
230E	*Enterobacter cloacae subsp. cloacae*	-	-
231E	*Enterobacter cloacae subsp. cloacae*	+	-
237E	*Enterobacter cloacae subsp. cloacae*	-	-
112E	*Enterobacter kobei*	+	+
235E	*Enterobacter kobei*	+	-
238E	*Enterobacter kobei*	+	-
222E	*Enterobacter nimipressuralis*	+	-
43893	*Escherichia coli* O157:H7	-	-
B241	*Escherichia coli* O157:H7	-	-
233E	*Escherichia vulneris*	++	-
226E	*Leclercia adecarboxylata*	-	-
239E	*Leclercia adecarboxylata*	-	-
111E	*Providencia rettgeri*	-	-
117E	*Providencia rettgeri*	-	-
113E	*Pseudocitrobacter anthropi*	-	-
B205	*Salmonella enterica* Belem	+	-
B207	*Salmonella enterica* Cerro	+	-

Table 2 shows the host range results for Φ107E-p1 and Φ115E-p2. The host range study was carried out using spot tests and plaque assays to determine the ability of the phages to infect the host as well as to access their ability to form plaques on the

hosts. There was a total of 30 bacterial strains tested in the host range study. The host IDs in the above table distinguish bacterial strains. Twenty-six of the strains selected are found naturally occurring on cucumbers. The other 4 strains, the *E. coli* and *S. enterica* strains, are foodborne pathogens. All strains were selected based on the likelihood that they may be present during the fermentation process. The range of hosts used in this study can be considered adequate for determination of host range as the range is broad, spanning across multiple genera while placing an emphasis on multiple strains of the same species.

Φ107E-p1 was able to infect 17 of the tested strains across three different genera. Most of these are *Enterobacter* species, one *Escherichia* species, and two *Salmonella* species. Of the 17 positive strains, Φ107E-p1 had the ability to form turbid plaques on 4 of the positive hosts and clear plaques on only 1 of the positive hosts, which is the host strain the phage was isolated against. Φ115E-p2 was only able to infect 3 of the tested *Enterobacter* species. Of the 3 positive hosts, Φ115E-p2 was able to form plaques on 2 of the hosts, including the strain used for isolation. Plaques for both of these hosts were clear. The host range study also revealed that there is not a cross-infection between phages and the hosts they were initially isolated against. Φ107E-p1 does not infect *E. cloacae* 115E, and Φ115E-p2 does not infect *E. cloacae* 107E.

The results indicate that Φ107E-p1 has a broad host range. This is due to the fact that this phage is able to infect multiple strains of the same species as well as showing infection ability across different genera. In contrast, Φ115E-p2 has a narrow host range. This phage is considered to have a narrow host range because it was only

able to infect three strains belonging to two different species of the same genera. A broad host range is an important characteristic when exploring phages for use as biocontrol methods because it will allow for more undesired strains to be targeted. However, a phage with a narrow host range can still be a viable candidate. This is due to the fact that multiple phages are often used together to lower the chance of host strains becoming resistant to a specific phage.

Phages can have diverse host ranges ranging from narrow to broad, and diversity should be taken into account when considering the use of phages for biocontrol (Ahktar et al., 2013). A combination of phages with diverse host ranges can be beneficial in targeting multiple strains at once, and if there is overlap between host ranges, there is the possibility that the host will be less likely to develop phage resistance. Host range is one of many factors in determining a phage's eligibility for biocontrol and other factors should be taken into account (i.e. growth kinetics, ability to kill the host organism, etc.).

Growth kinetics

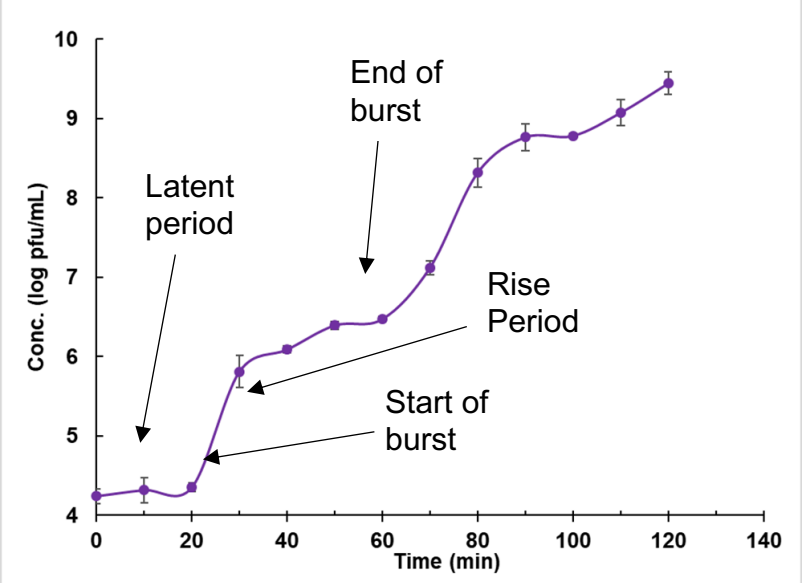

Fig. 6. One-step growth curve of Φ107E-p1 measured in cucumber juice at 37°C. Latent period is approximately 20 minutes. Error bars represent standard deviation.

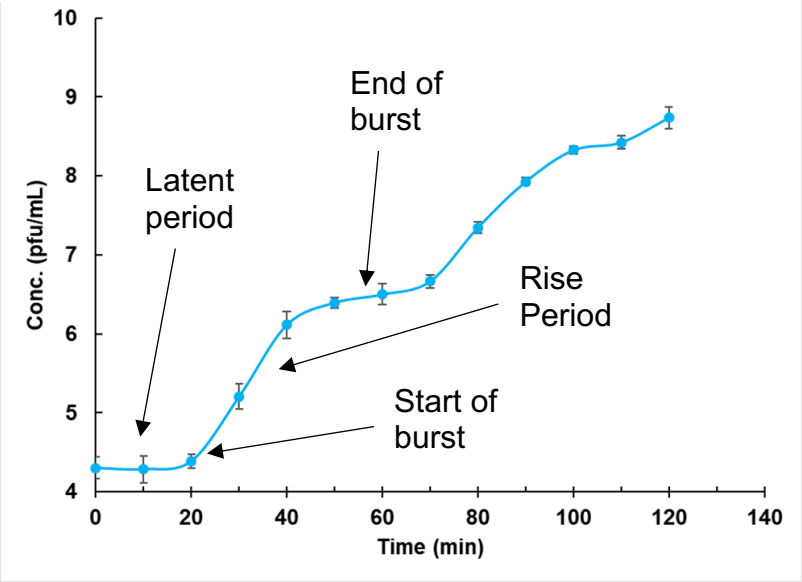

Fig. 7. One-step growth curve of Φ115E-p2 measured in cucumber juice at 37°C. Latent period is approximately 20 minutes. Error bars represent standard deviation.

Growth kinetics in host strains were determined by constructing one-step growth curves. Cucumber juice was used as the media for experiments due to the similar composition cucumber juice has to whole cucumbers and due to the fact that cucumber juice is simpler to work with. The use of a media similar to the fermentation environment in which the phages could potentially be used is imperative to determining the most accurate growth kinetics for both phages in a laboratory setting.

Figures 6 and 7 revealed that Φ107E-p1 and Φ115E-p2 have a similar latent period of approximately 20 minutes (excluding 20 minutes for adsorption). The rise period for both phages ended at approximately 50 to 60 minutes giving a rise period lasting a total of approximately 30 to 40 minutes. Burst size was calculated as the number of free phages immediately after the burst divided by the number of free phages before the burst (number of phages in the system after adsorption and before cell lysis). Both phages had similar burst sizes. Φ107E-p1 and Φ115E-p2 had burst sizes of 143 and 141 virions per infected cell, respectively. The growth kinetics of the two phages are very similar despite the phages being different, as demonstrated by host range studies. Additionally, the short latent period and relatively large burst size of the phages are desirable characteristics for phages being considered for industrial use.

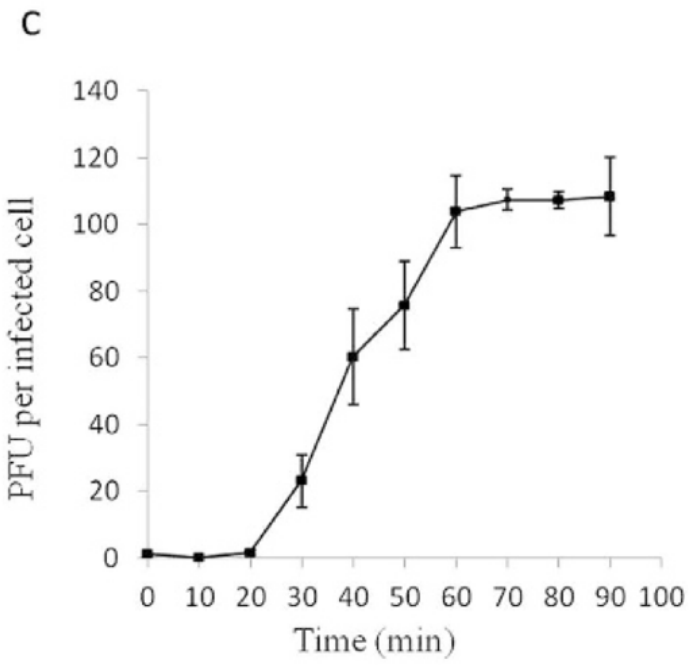

Fig. 8. One-step growth curve of phiEap-2 measured in Luria Bertani (LB) broth at 37°C. Adapted from Li et al., 2016.

The growth kinetics of these two phages was compared with the growth kinetics of another *Enterobacter* phage, phage phiEap-2, infecting *Enterobacter aerogenes* (Li et al., 2016) which can be seen in figure 8. Phage phiEap-2 had a latent period of 20 minutes, rise period of 60 minutes, and an average burst size of 100 virions per infected cell. The

two phages in this study have similar latent periods and larger burst sizes (143 and 141 virions per infected cell) than phiEap-2. Additionally, the two phages in this study had rise periods that are approximately half of the rise period for phiEap-2.

Another *Enterobacter* phage, phi MJ2 (Jamal et al., 2018) was used for comparison. In LB media at 37°C, the latent period for phi MJ2 was approximately 21 minutes which is similar to the latent period of Φ107E-p1 and Φ115E-p2. However, phi MJ2 had a very large burst size of 350 virions per infected cell which is much larger than the burst sizes of the two phages used in this study.

When compared to another *Siphoviridae* phage, the latent period is similar to that of phage Z63-B1 against *Lactobacillus fermentum,* a lactic acid bacterium, (Foschino et al., 1995) which had a latent period of 20 minutes in de Man, Rogosa & Sharpe (MRS) broth at 37°C, similar to the two phages in this study. The rise period for Φ107E-p1 and Φ115E-p2 is shorter than that of Z63-B1 however, which had a rise period lasting for approximately 80 minutes. Φ107E-p1 and Φ115E-p2 also have much larger burst sizes that Z63-B1 which only had a calculated burst size of 10 virions per infected cell. In comparison, Φ107E-p1 and Φ115E-p2 had burst sizes of 143 and 141 virions per infected cell, respectively. However, Z63-B1 has slow adsorption rate and small burst size when compared to the average for phages infecting lactic acid bacteria.

Although the phages used for comparison were tested in different media, valuable information regarding the growth kinetics of the phages can still be used for comparative purposes. Media used for each study was determined based on host requirements. However, it is likely that the phages would respond differently in different types of media due to the changes in the chemical composition of various media types.

Large scale phage preparation, concentration, and purification

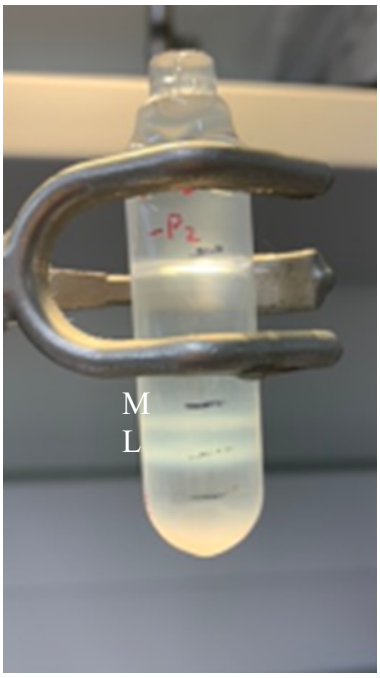

Fig. 9. Phage bands of Φ115E-p2 obtained after CsCl density gradient ultracentrifugation. Phage bands appeared milky white in color.

Both phages were propagated to increase the titer of the phages and to increase the volume of the phage lysate to approximately 1 liter for concentration and purification purposes. Phages then underwent enzyme treatment with DNase I and RNase A to remove cellular DNA and RNA from the lysate to prevent future contamination during molecular work. Two bands were obtained after CsCl separation based on density as seen in figure 9. Both bands were milky white in color. The milky white color of the bands is consistent to what has previously been observed about the appearance of phage bands. Two bands appear due to a mixture of intact phage and phage components (detached heads and tails) in the phage lysate. Since intact phages and phage components have different densities, two bands appear. One band, the upper band, was later confirmed through protein analysis to contain more intact phages. This band was labeled as M based on relative position to the other band. The other band, the lower band, was later confirmed through protein analysis to contain mostly phage components and less intact phages. This band was labeled as L based on relative position to the other band.

Transmission electron microscopy images for band L can be seen in figure 5 while transmission electron microcopy images for band M can be seen in figure 10. Based on electron micrographs, band M contains primarily phage components for both

phages while band L contains primarily intact phages. This result is not conclusive however as the electron micrographs focus on a small area. However, location of the phage bands would support this result due to the fact that the L bands are higher in density than the M bands which could indicate more intact phage. Protein analysis on both the L bands and M bands later provided evidence that did not support this and instead suggests that the M band contains intact phages while the L band contains phage components. This is due to the number of major structural proteins belonging the phages/phage components in each of these bands. These results will be further discussed in the protein analysis section.

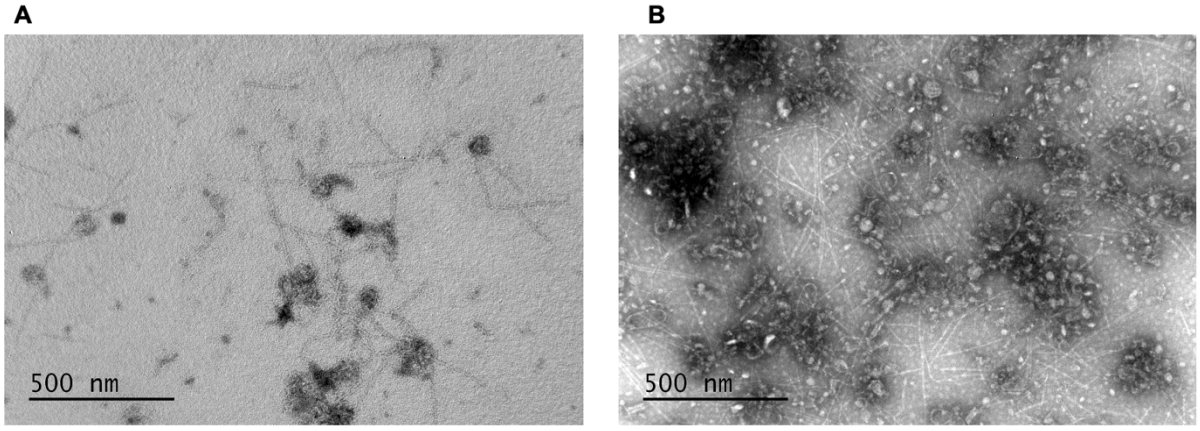

Fig. 10. Transmission electron microscopy images of M bands from Φ115E-p2 (A) and Φ107E-p1 (B).

DNA analysis

Phage DNA was extracted from Φ107E-p1 and Φ115E-p2 via the phenol-chloroform method using multiple rounds of extraction. Extraction was immediately followed by DNA precipitation. Φ107E-p1 DNA produced a large jelly-like pellet. Φ115E-p2 DNA produced a small, firm pellet that was white in color. Pellets were resuspended in 300 µl of TE buffer for use in restriction analysis. Both phages have DNA genomes, which is common among bacteriophages, as demonstrated by their susceptibility to the restriction enzyme EcoRV.

In a study by Loessner et al. (1993), 9 *Enterobacter* phages were compared. Of the nine phages used in the study, 8 of them could have their DNA digested with EcoRV. The range for fragments of these phages range from 2 to >35 fragments when digested using this restriction enzyme. The two phages in this study were both able to be digested by EcoRV as seen with other *Enterobacter* phages indicating that a proper restriction enzyme was chosen for analysis.

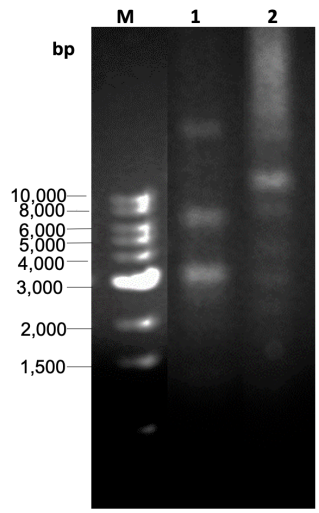

Fig. 11. Restriction analysis of Φ107E-p1 DNA and Φ115E-p2 DNA using EcoRV. M - DNA ladder. Lane 1 – Φ107E-p1 DNA fragments. Lane 2 – Φ115E-p2 DNA fragments.

Restriction digestion with EcoRV followed by gel electrophoresis, seen in figure 11, revealed that the phages are genetically different based on the different banding patterns of the digested phage DNA.

Digested Φ107E-p1 DNA produced 3 clear bands with two major bands between 6,000 and 8,000 bp and 3,000 and 4,000 bp, respectively. Digested Φ115E-p2 DNA produced 5 clear bands with a major band above 10,000 bp. However, the Φ115E-p2 DNA had streaking on the gel which created a quality undesirable for proper analysis. As a result, analysis using the Agilent TapeStation 4200 was done to more thoroughly analyze the DNA of both phages.

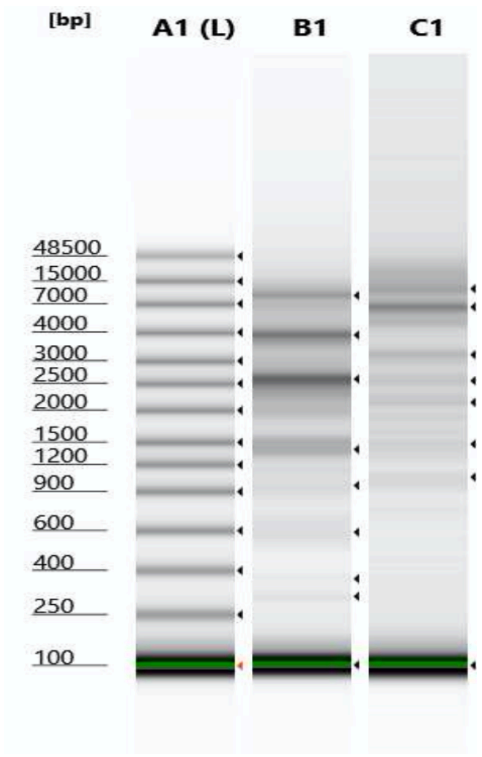

Fig. 12. Restriction analysis of Φ107E-p1 DNA and Φ115E-p2 DNA using EcoRV. A1 - DNA ladder. B1 – Φ107E-p1 DNA fragments. C1 - Φ115E-p2 DNA fragments.

Restriction digestion followed by analysis with the Agilent TapeStation 4200 was done. Results are seen in figure 12. Analysis using the Agilent TapeStation 4200 revealed more DNA fragments than could be seen with the agarose gel. Additionally, results obtained using agarose gel suggest that digested Φ115E-p2 DNA has more bands than the digested Φ107E-p1 DNA when both are digested with EcoRV, but analysis using the TapeStation software suggest otherwise. Earlier runs using the TapeStation software revealed that the concentration of DNA fragments was low for both phages. Low concentration could explain the low number of bands seen in the

agarose gel image. Digested phage DNA was concentrated approximately 10-fold for TapeStation analysis to give the observed bands in figure 12. Additionally, the TapeStation software is highly sensitive and more likely to pick up on DNA fragments than analysis using a traditional agarose gel.

When the phage DNA was cut with the endonuclease EcoRV and analyzed using the TapeStation software, Φ107E-p1 had 9 bands (100 bp, 301 bp, 363 bp, 587 bp, 955 bp, 1389 bp, 2577 bp, 3868 bp, 9087 bp) with major bands at 3,868 bp and 2,577 bp. Φ115E-p2 had 8 bands (100 bp, 1051 bp, 1472 bp, 2137 bp, 2554 bp, 3193 bp, 6579 bp, 11660 bp) with a major band at 6,579 bp.

Table 3. Table indicating the fragment sizes of EcoRV digested Φ107E-p1 DNA. Concentration of fragments in ng/μl as well as % integrated area are also provided.

Size [bp]	Calibrated Conc. [ng/μl]	Assigned Conc. [ng/μl]	% Integrated Area	From [bp]	To [bp]	PeakComment	Observations
100	8.50	8.50	-	68	156		Lower Marker
301	0.269	-	2.27	267	334		
363	0.213	-	1.79	334	421		
587	0.511	-	4.30	453	643		
955	0.510	-	4.29	765	1029		
1389	1.45	-	12.20	1197	1704		
2577	3.18	-	26.74	2071	3018		
3868	2.16	-	18.14	3212	5383		
9087	1.84	-	15.51	5571	24300		
-	-	-	-	-	-		Sample Well

Table 4. Table indicating the fragment sizes of EcoRV digested Φ115E-p2 DNA. Concentration of fragments in ng/μl as well as % integrated area are also provided.

Size [bp]	Calibrated Conc. [ng/μl]	Assigned Conc. [ng/μl]	% Integrated Area	From [bp]	To [bp]	PeakComment	Observations
100	8.50	8.50	-	68	148		Lower Marker
1051	0.583	-	6.72	842	1175		
1472	0.685	-	7.89	1175	1582		
2134	0.606	-	6.99	2014	2378		
2554	0.625	-	7.20	2378	2799		
3193	0.945	-	10.90	2799	3705		
6579	1.33	-	15.32	5273	8689		
11660	1.16	-	13.42	8689	18125		
-	-	-	-	-	-		Sample Well

Size of DNA fragments were derived from the tables provided by the TapeStation analysis tool which can be seen in tables 3 and 4. Electropherograms depicting the peaks associated with each fragment can be seen in figures 13 and 14.

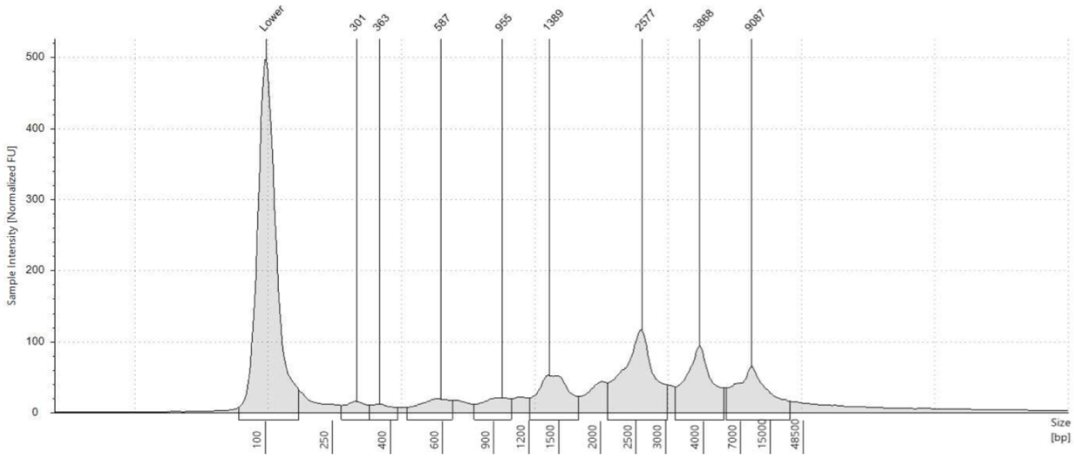

Fig. 13. Electropherogram for EcoRV digested Φ107E-p1 DNA.

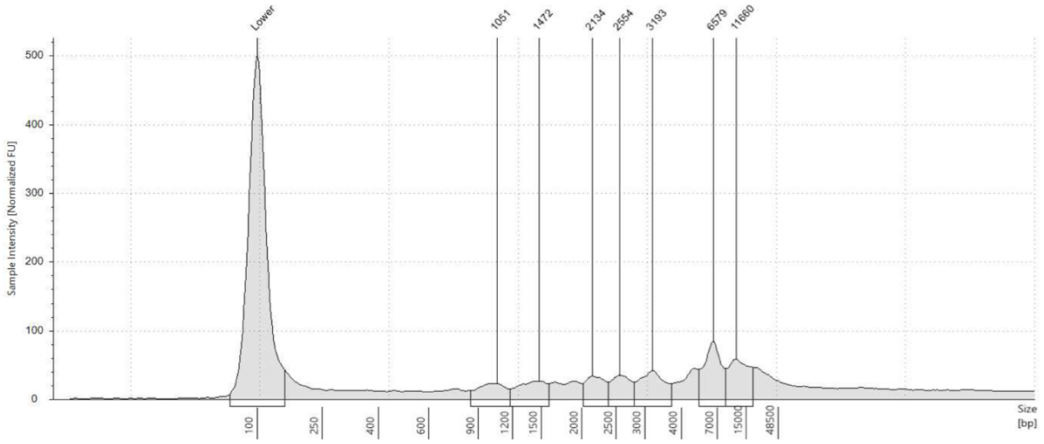

Fig. 14. Electropherogram for EcoRV digested Φ115E-p2 DNA.

Analysis of the DNA fragments using both agarose gel images as well as analyzing the gel images and tables obtained from the TapeStation software reveals that the endonuclease EcoRV cut the DNA of the two phages differently creating different banding patterns, indicating that the phages are genetically different from each other. The two methods also produced different banding patterns. This is seen in both the total number of bands that appear for the phage DNA in each method in addition to size estimates for the fragments using each method.

Analysis with the TapeStation software produced more bands for both phages when compared to analysis with an agarose gel. However, both methods produced similar numbers of major bands with the digested Φ107E-p1 DNA having two major bands and digested Φ115E-p2 DNA having one major band. Major bands are those bands that have a higher (darker) intensity. However, the base pair estimate for the major phage DNA bands were different when analyzed with TapeStation software than with the agarose gel electrophoresis. For the digested Φ107E-p1 DNA, the major bands appear at 2,577 bp and 3,868 bp when using the TapeStation software. Both of these bands are close to the 3,000 bp to 4,000 bp range that was estimated with agarose gel analysis. The TapeStation software also reveals another band at 9,087, though not necessarily major due to intensity compared with the other two major bands. This band is close to the band estimated to be around 8,000 bp in the agarose gel image. For digested Φ115E-p2 DNA, the major band appears at 6,579 bp when analyzed with the TapeStation software. This is much smaller than the major band that appeared in the agarose gel image. However, the TapeStation software reveals a band at 11,660 which

could be the band that is estimated to be over 10,000 bp observed when analyzing the agarose gel image.

Accurate estimation of genome size cannot be done due to the errors associated with estimating genome size based on fragmentation (i.e. missing bands, errors with estimation of fragment size, etc.). Additionally, concentration of the whole phage genome was too low to provide reliable data. However, average genome of a *Siphoviridae* phage which is approximately 50 kbp and can provide a basis for future comparison. However, there can some be variation among genome sizes among this family of phages as there is a phage, phage RRH1, that has a genome of 14,270 bp and is considered to be the smallest known *Siphoviridae* phage (Petrovski et al., 2011).

Sequencing of the phage genomes would give a more accurate estimation of genome size and is something to consider moving forward. Sequencing would provide more accurate data on genome size as well as provide valuable information regarding the genetic similarities and differences between the two phages in this study as well as other phages. Additionally, sequencing would confirm if the two phages in this study are indeed novel phages.

Protein analysis

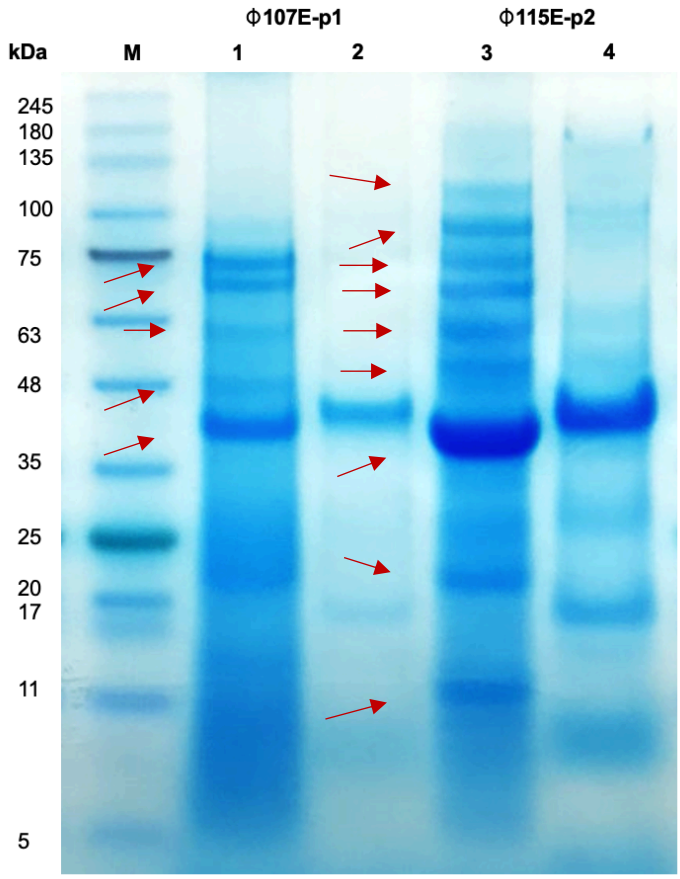

Fig. 15. SDS-PAGE of Φ107E-p1 and Φ115E-p2 structural proteins and molecular mass markers (lane M).

Protein analysis was done using SDS-PAGE. The results of which can be seen in figure 15. Phage bands obtained after CsCl density gradient ultracentrifugation were used for protein analysis using SDS-PAGE. Band 1 and 3 were from the band labeled M in figure 10. Band 2 and 4 comes from the L band in figure 5. Protein analysis of the phage was done to reveal certain structural proteins and their sizes.

Based on protein banding, Φ107E-p1 appears to have 5 major structural proteins (pointed out using the arrows in the figure) as seen in figure 15. These proteins have estimated approximate sizes of 100, 75, 63, 48, and 35 kDa. Based on protein banding, Φ115E-p2 seems to have 9 major structural proteins (as pointed out using the arrows in the figure). These proteins have estimated approximate sizes of <135 but >100, 100, 75, <75 but >63, 63, 48, 35, 25, and 20kDa.

The M bands of both phages appear to have more major structural proteins than the L bands which suggests that the M bands likely contain more intact phages, which is contradictory to the results obtained from electron micrographs alone. Comparison between M and L bands for each phage show that the M band for Φ107E-p1 has 3 more major structural proteins than the L band. The M band for Φ115E-p2 appears to have 5 more major structural proteins than the L band. More thorough TEM analysis could reveal which components the L bands contain to help estimate the number of major structural proteins for each phage component.

Phage proteins can vary in number and size and is a useful means of comparison between phages. However, exact number of structural proteins cannot be determined based of the obtained data. It is likely that not all structural proteins appeared in the gel due to issues such as degradation. More detailed protein analysis would need to be performed to identify all phage proteins. Additionally, more detailed protein analysis coupled with ORF prediction after sequencing can provide information regarding protein function to help increase the understanding of how these phages work.

Effectiveness of phages against their host in a model food system

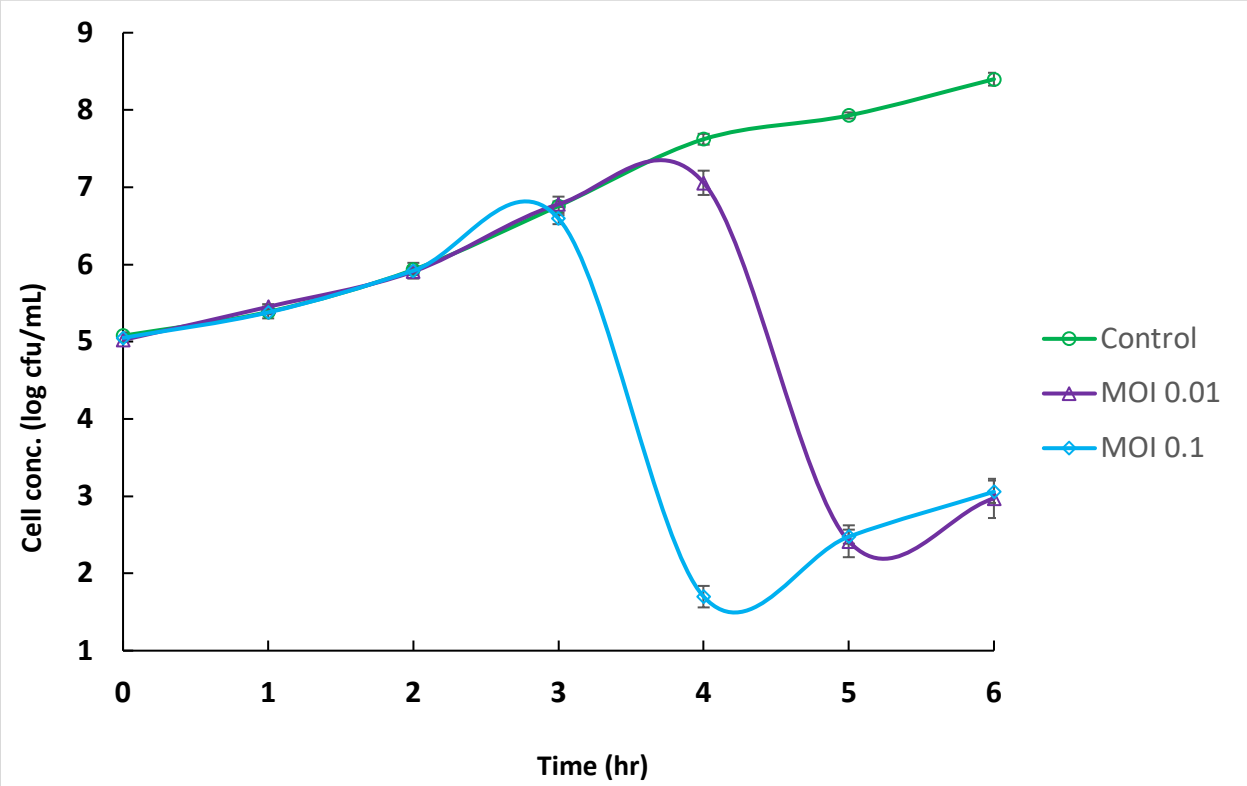

Fig. 16. Effectiveness of Φ115E-p2 against *E. cloacae* 115E in a cucumber juice at 37°C over a 6 hr time period.

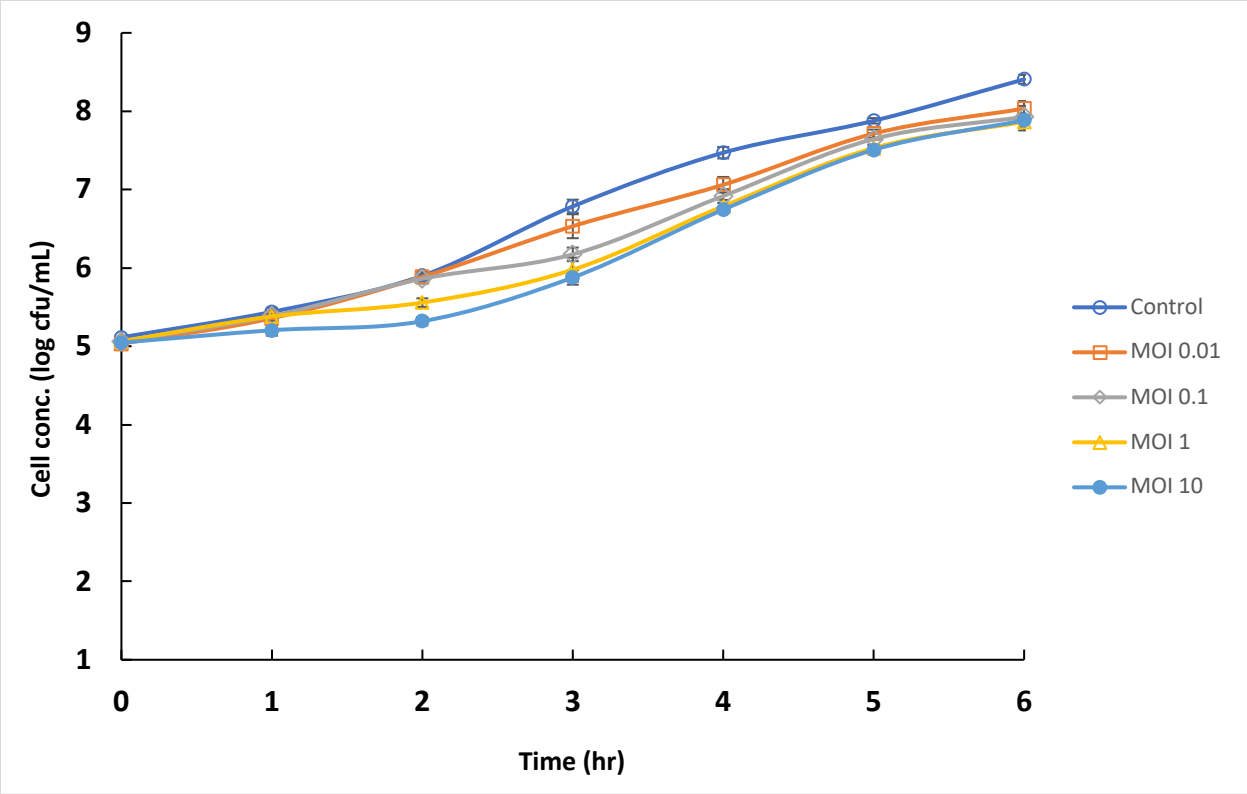

Fig. 17. Effectiveness of Φ107E-p1 against *E. cloacae* 107E in a cucumber juice at 37°C over a 6 hr time period.

The effectiveness of phages Φ107E-p1 and Φ115E-p2 against their hosts was determined in a model food system of cucumber juice. Cucumber juice was used as a model food system due to the fact that it is a simpler system to work with rather than whole cucumbers but is chemically similar. Host and phage at various MOIs were added into cucumber juice and incubated for 6 hr. Samples were taken every hour during this time period starting with time 0 to measure how bacterial concentration changes over time when infected by the phages. A control containing just the host in cucumber juice was used for comparison. Bacterial concentration was determined by enumerating healthy colonies. Healthy colonies were those that appeared to be normal shape and size using control colonies as a comparison. Colonies that were smaller in size or

misshapen when compared to the control colonies were considered to be unhealthy and not included in concentration calculations.

For Φ115E-p2, seen in figure 16, the control and both treatments had a host concentration of around 6.6 log cfu/mL at hour 3. By hour 4, host concentration decreased by approximately 4-log at MOI 0.1. By hour 5, host concentration at MOI 0.01 had decreased by approximately 4-log. Control host concentration was approximately 5.5-log higher than both MOI 0.1 and MOI 0.01 at hour 5. By hour 6, both MOIs had an increase in host concentration that is likely due to phage resistance in the host. However, at hour 6, there was still a 5-log difference between the control and the phage treated hosts at MOIs 0.1 and 0.01. This indicates that Φ115E-p2 is effective at reducing the host organism.

For Φ107E-p1, seen in figure 17, the host was not killed off. Instead, host growth was slowed at MOIs of 0.01, 0.1, 1, and 10. Higher MOIs were used for this phage to determine if phage concentration was the reason this result was seen. Higher MOIs showed similar results as when the phage was added at a lower initial concentration. This slowed growth could be seen as early as hour 2 for MOIs 1 and 10 and seen as early as hour 3 for MOIs 0.01 and 0.1. However, all MOIs had host concentration around 7.9-log by hour 6. This concentration is only about a half log less than the concentration of the control. This suggests that Φ107E-p1 is not effective at killing the host organism. As a result, this phage would not be considered as a candidate for the control of its host in an industrial fermentation setting despite having some desirable characteristics (i.e. broad host range, short latent period, etc.).

Both Φ115E-p2 and Φ107E-p1 have very similar growth kinetics, but their ability to effectively control their hosts is very different. Φ115E-p2 is more effective at rapidly killing the host organism while Φ107E-p1 only slows the growth of the host organism. It is currently unknown why Φ107E-p1 was unable to kill the host. A possible indication of this result can be seen by examining the clarity of the plaques. Φ107E-p1 had plaques that were considered clear but were slightly less clear than Φ115E-p2 plaques. This difference required careful observation, and it is unknown if this is an indicator of the results that were seen during the effectiveness experiments.

More studies would need to be done to see how both phages effect the host over long periods of time, especially since the host treated with Φ115E-p2 showed a slight recovery. This slight recovery indicates phage resistance in the host which is not uncommon. These future studies would include extending the sampling time to see how the host responds to the phages over longer periods of time to examine how the development of phage resistance in the host can impact host concentration over a period of days to weeks to help establish potential efficacy in an industrial setting.

In a study by Jamal et al. (2018), phage phi MJ2 against *E. cloacae* was tested for its effectiveness at slowly and preventing the growth of biofilms on stainless steel plates containing TSB and incubated at 37°C. MOIs of 0.1, 0.5, 1, and 5 were used in the study in addition to a control containing just the host organism. All MOIs showed an ability to reduce the host concentration after 1 hour. However, MOIs 0.1 and 0.5 showed a more gradual decline whereas the other 2 MOIs showed a more rapid decline in host concentration. There was no host recovery observed over a 5-hour testing period. A 4-hour phage treatment was done, and the biofilms were allowed to grow with media

being refreshed. After 24 hours, there was a 2.8-log reduction in host concentration. Comparing Φ115E-p2 to this phage, both phages were able to effectively decrease host concentration at a MOI of 0.1. However, Φ115E-p2 did not cause a decrease in host concentration as rapidly as phi MJ2. Φ107E-p1 only slowed the growth of the host as opposed to killing the host unlike Φ115E-p2 and phi MJ2.

Conclusions

This research isolated two phages, Φ107E-p1 and Φ115E-p2, against 2 different strains of *E. cloacae*. TEM images suggest that both phages belong to the *Siphoviridae* family based on virion morphology. The host range study revealed that Φ107E-p1 has a broad host range. In contrast, Φ115E-p2 has a narrow host range. The two phages had similar growth kinetics. Restriction analysis of phage DNA revealed that the phages are genetically different, and protein analysis of the two phages suggest that the two phages have different major structural proteins. Φ115E-p2 showed the ability to rapidly reduce its host's concentration in cucumber juice (a model food system). In contrast, Φ107E-p1 could only slightly inhibit host growth. This data suggests that only Φ115E-p2 is a potential candidate for use in industrial fermentation to prevent the incidence of bloater defect. More research will need to be done in order to evaluate the efficacy of Φ115E-p2 in industrial use.

Future research should evaluate the stability of Φ115E-p2 in the pH range similar to that during cucumber fermentations. Additionally, thermal stability experiments focusing on a range of environmental temperatures the fermentation tanks may be exposed to would also be needed. Eventually, the phage should be tested in fermentation tanks to evaluate its effectiveness to reduce bloater defect by killing its host during cucumber fermentations.

Statement of Integration

This research integrated the techniques on many levels of biology. The original objectives on this research were to isolate, characterize, and determine the effectiveness of phages against *E. cloacae.* While the primary field of interest was microbiology, there were many molecular biology methods used to help characterize the isolated phages (i.e. the techniques used for DNA analysis and protein analysis). Additionally, there was a food science aspect to this research due to the fact that the results can be used to develop biocontrol methods in the food industry. Conclusions drawn from this research provide information that could lead to the industrial use of phages for biocontrol of undesired bacteria.

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