IDENTIFICATION AND EXPRESSION OF THE HOLIN GENE IN SHIGA-TOXIGENIC *ESCHERICHIA COLI* SPECIFIC BACTERIOPHAGES AND THE USE OF BACTERIOPHAGE DEPOLYMERASE ON STEC BIOFILMS FORMED ON FOOD CONTACT SURFACES

By

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Abstract: Shiga-toxigenic *Escherichia coli* (STEC) are one of the most common foodborne pathogens, responsible for numerous outbreaks of foodborne illness annually. The persistence of this group of pathogens makes them an incredible challenge to the food industry. This persistence is often attributed to the strong biofilm forming capabilities of STEC. The development of new control strategies against STEC and their biofilms would have significant benefits to the food industry. Bacteriophage by-products are an attractive intervention method for STEC control, particularly on food contact surfaces. One objective of this work was to evaluate the efficacy of a bacteriophage-derived depolymerase on STEC biofilms formed on food contact surfaces. The other objective of this work was to identify and express the bacteriophage *holin* gene in anticipation of further studies using the holin protein. Bacteriophage P9 was chosen for the depolymerase study due to its high level of lytic activity against *E. coli* O45, a known former of strong biofilms. To evaluate the effectiveness of phage depolymerase as a removal agent, stainless steel and high-density polyethylene surfaces were spot inoculated and biofilms were allowed to form for 48 hours before the treatments were applied with an airbrush. The effectiveness of phage depolymerase as a preventative was evaluated by applying the depolymerase with an airbrush before spot inoculating and allowing biofilm formation over 48 hours. The study indicated that phage depolymerase is effective at both removing and inhibiting biofilm formation on both stainless steel and high-density polyethylene surfaces, causing significant ($P<05$) reduction in STEC when compared to the control. In the holin study, phage J25 was chosen due to its high level of lytic activity against *E. coli* O145. Phage J25 was shown to contain the *holin* gene, and the gene was successfully cloned, sequenced, and expressed. Results indicated that phage depolymerase could be an effective method of biofilm inhibition and removal in the food industry. Results also indicated that the *holin* gene in bacteriophages can be expressed in order to produce the holin protein, which has the potential to be an effective STEC intervention method.

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INTRODUCTION

Shiga-toxigenic *Escherichia coli* (STEC) is a group of major foodborne pathogens that are the cause of thousands of cases of foodborne illnesses every year in the United States (US). These human pathogens are commensal microorganisms for ruminant animals such as cattle, sheep, and goats *(156)*. As a result of this, these animals are most often the original source of contamination for STEC outbreaks. Enteric *E. coli* are shed in their feces, which then contaminates groundwater, runoff, and any food product it may contact such as produce or meats. Consequences of STEC infection include hemorrhagic colitis and hemolytic uremic syndrome (HUS), which often leads to kidney failure in children and elderly people *(73, 190)*. One of the major pathogenic STEC serotypes, *E. coli* O157:H7, is responsible for approximately 63,153 illnesses, 2,138 hospitalizations, and 20 deaths each year in the US *(170)*. Other STEC serotypes also known as non-O157 STEC have emerged and reported to have caused 113,000 cases of foodborne illnesses each year *(171)*. According to recent studies by the Centers for Disease Control and Prevention (CDC), non-O157 STEC serogroups collectively caused more infections than O157:H7 in the US. Despite a near-constant increase in non-O157 STEC related outbreaks *(148, 149)*, they remain underreported due to limitations in surveillance and diagnostic tools *(201)*. In the US alone from 1990 to 2017, there were 53 outbreaks confirmed to have been the result of a non-O157

STEC serotype *(121, 204)*. Overall, STEC contamination is one of the largest sources of food product recalls and foodborne outbreaks. Despite having numerous interventions in place, the food industry continues to struggle with the control of STEC contamination. One primary factor that leads to the persistence of these pathogens is their ability to produce very strong biofilms on numerous food-contact surfaces in food processing facilities, as well as on food products themselves *(124, 176)*. Biofilms consist of a large network of bacterial products such as exopolysaccharides (EPS) that protect the bacteria from harsh environmental conditions, as well as common sanitizers and antimicrobials *(69)*. It is estimated that biofilm-associated contamination costs the food industry billions of dollars each year *(177)*.

Microbes within a biofilm are capable of causing equipment damage and reduced efficiency, partially due to the biological and chemical reactions that they initiate *(108, 177, 208)*. Biofilm formation is heavily dependent on environmental factors such as nutrient availability, surface charge and hydrophobicity, temperature, and chemical composition *(108)*. Bacteria also facilitate their attachment to food contact surfaces via appendages such as pili, curli, and flagella. Studies have shown that *E. coli* produce much stronger biofilms when under nutrient-related stress conditions *(46)*. Another study showed that strong biofilms are formed on food processing equipment by both O26:H11 and O111:H8 STEC *(201)*. However, serotype is not a good indicator of biofilm forming capabilities, as this varies heavily depending on strain even within the same serotype *(201)*.

The formation of biofilms has been linked to increased resistance towards commercial sanitizers, with one study indicating that both *Salmonella* and *E. coli* are more resistant to hypochlorite in a planktonic form *(67, 95, 175)*. It has also been reported that STEC resistance to sanitizers was dependent on both strain and environmental conditions *(201)*. Mechanisms thought to be involved

in biofilm sanitizer resistance are the physiological changes in the bacterial cells, the induction of the stress response in cells, chemical and enzymatic inactivation of the sanitizers, and the diffusional resistance of the EPS matrix itself *(68)*. These characteristics of STEC biofilms makes them a serious threat to the food industry, it is critical to develop alternative sanitation methods to prevent, remove, and control STEC and their biofilms.

In recent years, bacteriophages have gained significant attention for use as a biocontrol agents against pathogens, partly due to their extreme host specificity *(47-49)*. They have been isolated from food and environmental sources, and are commensal organisms in the ruminant gastrointestinal tract *(50,51)*. Bacteriophages are viruses that target specific bacteria for reproduction, killing the host in the process. The phage uses the DNA replication and protein synthesis machinery in the host cell in order to replicate, as they lack an independent replication system. Once the phage has attached to a host and replicated inside it, the progeny produces a protein known as holin which causes pore formation in the cell membrane. Once the membrane has been perforated, the phages release an enzyme known as endolysin, completing the disintegration of the cells peptidoglycan layer, and leading to complete cell lysis *(52-55)*. The self-replicating nature of lytic bacteriophages, as well as their host-specificity, makes them a highly desirable antibacterial tool in the food industry *(48)*. Phages have been studied as antibacterial agents in humans, livestock, food products, and food contact surfaces *(5,56,57)*. Studies have indicated that phages have minimal effects on the sensory qualities of food products, particularly when compared to more traditional sanitizing agents *(58)*. Initially ignored by the scientific community, the rise of antibiotic resistance has prompted a reassessment of the use of bacteriophages *(49,59-61)*. Lytic bacteriophages have been shown to eliminate *E. coli* O157:H7

in-vitro and even in the intestinal tract of ruminant animals *(62,63)*. The efficacy of bacteriophages against STEC has also been studied in numerous produce and meat products *(64,65)*.

Phages and their by-products have shown promise as biofilm-removal agents *(69)*. Viazis et al. *(199)* tested a bacteriophage cocktail against STEC biofilms on stainless steel, ceramic tiles, and high-density polyethylene coupons, the results indicated that the phage cocktail effectively reduced the populations of pathogens in biofilm within one hour on each of the three surfaces. Another study indicated a reduction of 4.5 logs CFU/blade in STEC population when phage was used as a treatment on spinach harvesting blades *(73)*. It has also been shown that phages produce effective by-products, one such by-product being an enzyme known as phage depolymerase, which disrupts the exopolysaccharides in the biofilm, therefore exposing the bacteria for infection and lysis by the bacteriophage *(52,69)*. Phage depolymerase has been found to bind to the bacterial capsular material in order to degrade the polymer until it has reached the cell surface, where it allows the bacteriophage to bind and infect the bacterium *(69)*. Bacteria have been shown to protect themselves from this depolymerase by slightly altering the polysaccharides that are produced within the biofilm *(71)*. Bacteriophages often produce more than one depolymerase, which may allow them to degrade the altered biofilm *(69)*.

Another promising product of bacteriophages is a group of proteins known as holins. These proteins form lesions in the cytoplasmic membrane of the host cells in order to facilitate the lysis of the cell by an enzyme known as endolysin *(207)*. Holin is also responsible for the timing of lysis in lytic phages, adjusting the phage replication cycle according to environmental conditions. Without the addition of endolysin, holins cannot directly kill the host cell, but they are capable of causing eventual cell death as a result of inhibiting respiratory abilities and the depolarization of the membrane. This depolarization would allow penetration by molecules that were previously

unable to enter the cell, such as chemical sanitizers and antibacterials *(106)*. Several studies have indicated that holin is capable of inhibiting *E.coli* growth, as well as several gram-positive organisms*(152)*. Holins are believed to be nonspecific, which drastically increases their value in the food industry, as they could potentially be utilized as control methods for both pathogens and spoilage organisms.

Objective I of this study is to further analyze the effectiveness of this enzyme as a biofilm control agent, as well as a preventative measure on food contact surfaces. A previously isolated bacteriophage of high lytic activity was utilized to extract the bacteriophage depolymerase. On one set of stainless steel and high-density polyethylene coupons, enzyme was applied to food contact surfaces before the coupons were inoculated and the biofilm was allowed to grow for 48 hours. On another set of coupons, the enzyme was applied after the 48-hour biofilm formation period. Objective II of this study was to identify and express the gene responsible for the production of the *holin* gene in bacteriophages. The DNA of a previously isolated bacteriophage of high lytic activity was extracted and sequenced. The *holin* gene was identified and isolated from the genome, before being cloned and transformed into competent cells for expression.

Exploring the potential uses of phage by-products provided a better understanding of their role in biofilm reduction, as well as a better understanding of the mechanisms utilized by bacteriophages for cell lysis. The findings of these studies indicate that bacteriophage depolymerase is a promising agent for both biofilm prevention and reduction, and is effective on multiple common food contact surfaces. The expression of the holin protein could be utilized to further study the antibacterial qualities of this protein against pathogenic organisms. The implications from this research could significantly benefit the food industry. The by-products of bacteriophages show excellent biofilm-disruption capabilities, indicating they could be effective biocontrol agents, minimizing the risk of

foodborne illness. The utilization of these by-products could allow for many of the biocontrol benefits of bacteriophages, without the consumer perception issues associated with the use of viruses in the food industry.

Chapter II.

REVIEW OF LITERATURE

A. *ESCHERICHIA COLI*

1. Classification

Escherichia coli (*E. coli*) is a facultative anaerobe and gram-negative bacterium that usually exists in the digestive tract of humans, birds, and other animals *(44)*. *E. coli* is a versatile microorganism and has both commensal and pathogenic strains. Despite *E. coli* existing primarily as a beneficial gut microbe, it has acquired a series of virulent traits which enable it to survive in adverse environments while also expanding its abilities to survive in new host organisms. These adaptations occurred within the genome of *E. coli*, where the bacterium obtained genes responsible for virulence factors from other microorganisms. These new genes were what lead to the eventual rise in strains of pathogenic *E. coli.* The three most common infections caused by these pathogenic *E. coli* strains are enteric (diarrheal) disease, sepsis, and urinary tract infections. *E. coli* patho-types are categorized by their flagellar "H" antigen, and their somatic "O" antigen. There are seven well-defined pathogenic groups, and they are:

Enterotoxigenic E. coli (ETEC)

Enterotoxigenic *E. coli* cause a cholera-like acute diarrhea that is often observed in both humans and animals *(132)*. ETECs are one of the most common causes of "traveler's diarrhea", and can be fatal to young children, elderly people, or those with compromised immune systems. Another common host for this group is post-weaning piglets, which are highly susceptible to infection by ETECs. The ETEC uses colonizing factors to attach to intestinal epithelial cells *(9)*, where it produces both heat-stable (ST) and heat-labile toxin (LT) *(48, 94, 194)*. The heat-labile toxin has a structure and action similar to that of the cholera toxin *(132, 194)*.

Enteroaggregative E. coli (EAEC)

Enteroaggregative *E. coli* is the second most common source of "traveler's diarrhea" and is a major cause of diarrhea globally. The diarrhea is often watery in nature, and can contain blood or mucus *(41, 132)*. The EAEC adheres to the intestinal cells in a pattern that closely resembles a stack of bricks, which is followed by the formation of biofilms that encase the cells *(79, 80)*. Initial adhesion may be aided by adherence fimbriae types I, II, and III *(59, 138)*. There are three toxins attributed to EAEC strains and they are thought to play a role in intestinal mucus secretion *(112)*.

Neonatal meningitis E. coli (NMEC)

Neonatal meningitis *E. coli,* as the name implies, is most frequently associated with newborn meningitis infections. These infections are fatal in as many as 40% of cases, and survivors are often left with severe neurological damage *(132)*. The bacteria in this group enters the bloodstream and crosses the blood—brain barrier, eventually leading to inflammation of the meninges and an increase of white blood cells in the cerebrospinal fluid.

Enteroinvasive E. coli (EIEC)

The EIEC exhibit several features that are similar to the pathogenicity of *Shigella,* producing an illness that closely resembles dysentery, which can be fatal in young children and those with compromised immune systems *(53)*. Clinical symptoms often include bloody or watery diarrhea, fever, and vomiting *(41)*. As obligate intracellular bacteria, EIEC do not possess flagella or other adherence factors, which makes them unique compared to other *E. coli* groups. Their primary method of virulence is the Type-III Secretion System (T3SS) which is a membrane embedded protein secretion system that facilitates cell invasion, survival, and the control of apoptosis as a result of host macrophages *(137, 173)*.

Diffuse-adherence E. coli (DAEC)

A common cause of chronic diarrhea in immunocompromised individuals and malnourished children is diffuse-adherence *E. coli (132)*. The DAEC uses an adhesion protein to attach to the epithelial cells and eventually covers the entire surface in a diffuse pattern. Unlike other *E. coli* strains, the pathogenesis seems to almost exclusively be related to the adhesion protein and their relationships with the host cells *(41)*.

Enteropathogenic E. coli (EPEC)

In developing countries, enteropathogenic *E. coli* is a leading cause of infant diarrhea, which is often fatal *(93, 132)*. Infections of *E. coli* belonging to this pathotype are characterized by attaching and effacing lesions formed on the epithelial cells of the intestinal wall *(93)*. EPEC micro-colonies on the epithelium causes the elimination (effacement) of the microvilli and destabilizes actin molecules in order to lift the infecting cell, forming a distinct pedestal below the site of attachment

(93). This ability is encoded by the locus of enterocyte effacement, or LEE, which is a highly regulated chromosomal pathogenicity island *(101)*. The LEE is responsible for a Type III Secretion System (T3SS) that assists in relocating bacterial proteins into the host cells cytoplasm, allowing manipulation of the host processes by the bacterium. The LEE is presumed to have been acquired by horizontal gene transfer from another organism *(45)*. EPEC strains can be identified by their "O" and "H" serotypes.

Enterohemorrhagic E. coli (EHEC)

Enterohemorrhagic *E. coli,* composed of both O157 and non-O157 serotypes, are the most common cause of *E. coli* related illnesses. This group of *E. coli* can cause acute diarrhea and hemorrhagic colitis (bloody stools), often accompanied by a fever. These symptoms can progress to cause severe complications such as central nervous system damage and hemolytic uremic syndrome (HUS), eventually leading to kidney failure *(142)*. This group of *E. coli* produces a potent cytotoxin known as *Shigella dysenteriae*-1 (Shiga) toxin (Stx) and is capable of producing attaching and effacing lesions, such as those produced by the EPEC group *(132)*. The EHEC group has the same LEE and T3SS as the EPEC group, but the production of shiga-toxin is the differentiating factor. The production of shiga-toxin has led to this group also being called shiga-toxigenic *E. coli* (STEC).

2. Pathogenicity

The primary virulence factors associated with pathogenic *E. coli* are intimin, hemolysin, plasmid O157, the locus of enterocyte effacement, shiga-toxin, and resistance to both heat and acid.

i. Intimin

Intimin is an outer membrane protein that is associated with the intestinal epithelial cell attachment by the bacterium, an integral part of host cell colonization by shiga toxigenic *E. coli (15, 23, 135)*. The Intimin molecules initiate the attachment of the bacterium to epithelial cells, particularly to those cells of the Peyer's Patches, before the T3SS releases effector molecules *(145)*. Intimin contains two functional groups, one that inserts into the outer membrane of the bacteria (N-terminus), and one that bind to a translocated receptor (C-terminus) *(151)*. There have been as many as 17 types of Intimin identified, primarily based on sequence and antigenic variation. The sequences located at the C-terminus are the main differentiating factors among Intimin subtypes *(205)*. Intimin-γ is a tissue-specific subtype that targets follicular epithelium cells of the Peyer's Patches in the ileum *(196)*. The shiga toxigenic *E. coli* that produce *stx*2 and Intimin molecules are commonly associated with the most severe human illnesses.

ii. Hemolysin

Hemolysin is a pore forming member of the Repeats-in-Toxin (RTX) group that is secreted into the extracellular space using a type-1 secretion system. It is responsible for the lysis of red blood cells, and thus providing iron from the hemoglobin for the bacteria *(24)*. Hemolysin can cause membrane damage to multiple other cell types and is also responsible for inducing production of inflammatory cytokines *(146)*.

iii. Plasmid O157

Plasmid O157 (pO157) contains multiple genetic elements that are mobile in nature, these include insertion sequences, transposons, prophages, and parts of other plasmids. Parts of these insertion sequences are similar to the virulence plasmid found in *Shigella* spp. *(31, 123)*. This plasmid was

created by the integration of DNA fragments from different species, thus leading to many virulence factors that originated in non-*E. coli* bacteria. The pathogenesis of STEC infections involve many proteins whose genes are found on Plasmid O157, including Hemolysin.

iv. The Locus of Enterocyte Effacement (LEE)

As previously discussed, attaching and effacing (A/E) lesions are formed by STEC colonization of the intestinal mucosa. Characteristics of these lesions include adherence of the bacteria to the host cells, degeneration and removal of epithelial microvilli, and the assembly of structures such as actin beneath the bacteria in order to form a raised pedestal *(103, 128)*. The initial attachment induces multiple signal pathways, resulting in the formation of A/E lesions, bacterial ion secretion, and eventually bacterial invasion. The genes required for attaching and effacing lesions are found on the chromosomal pathogenicity island known as the LEE. The LEE contains 5 functional modules, the most important of these modules is Intimin, the Type-III secretion system (T3SS), and the LEE regulators (which are required for modification of the host cell during A/E lesion formation *(69, 202)*.

v. Shiga-Toxin (stx)

The ability to produce two groups of Shiga-like toxin (also known as verotoxin) is the most virulent trait possessed by shiga-toxigenic *E. coli*. The first group, Stx1, is highly homogenous and consists of only 3 subunits; whereas the second group, Stx2, contains 8 variants *(30, 128, 129, 146, 195)*. Both groups share a common receptor and have the same mode of action, though they only share 56% of their amino acid sequences *(203)*. Stx1 is very similar to *Shigella dysenteriae* type 1 *(135)*. A study conducted by Schmidt et al. demonstrated that the ability to cause HUS is linked to Stx2a, Stx2c, and Stx2d, where milder symptoms were linked to the remaining Stx1 and Stx2 subtypes

(172). Stx genes are inserted within the STEC genome by lambda-like phages through the process of lysogeny *(132, 168)*. Phages play an important role in the diversification of bacterial genomes, as well as for horizontal gene transfer due. Expression of stx genes has been linked to the lytic cycle of bacteriophages *(203)*. Once the stx toxins have been assembled, the final secretion is achieved via host cell lysis *(115)*.

Shiga toxins are AB toxins, consisting of an A and a B subunit. The A subunit possesses enzymes capable of cleaving adenine from the host RNA, which subsequently prevents protein synthesis and eventually leads to death of the host cell. The B pentamer is responsible for binding to the host cell membrane, which enables the toxin to target cells that express the appropriate surface-receptors *(57)*. Shiga toxin causes vascular damage within the intestinal tract, allowing toxins, lipopolysaccharides, and other inflammatory mediators into the circulatory system, which can initiate Hemolytic Uremic Syndrome *(74)*.

vi. Heat and Acid Resistance

While some STEC strains are resistant to chemical-physical stresses, others are resistant to environmental stress. The strain of *E. coli* known as O157:H7 can grow well at 44˚C, but has shown no specific heat resistant traits and is sensitive to heat compared to other mesophilic bacteria *(48)*. However, *E. coli* has been shown to survive without population loss in temperatures as low as -20˚C for up to 9 months *(51)*. Multiple studies have indicated that all STEC strains have similar D-values, the time required to kill 1 log of microorganisms at a given condition, and thus are all inactivated within the same temperature range *(58, 84, 96, 180)*. Studies have indicated that STEC have the ability to survive in moderately to extremely acidic environments *(117)*. Current research suggests that the acid-resistance is likely genotypic in nature and is highly linked to the surface

lipopolysaccharides *(16, 113)*. It has also been shown that *E. coli* had the least acid tolerance of the common foodborne pathogens, and that all non-O157 strains have a greater tolerance at lower temperatures *(29)*.

3. Epidemiology

According to the recent estimates from the Centers for Disease Control and Prevention, there are 48 million cases of foodborne illness in the United States each year *(170)*. Annually there are 3,000 deaths and 128,000 hospitalizations caused by foodborne illnesses *(140)*. The increase in STEC related outbreaks have created a worldwide public health concern. While there are an estimated 73,000 O157 related cases each year, there has been a staggering rise in non-O157 outbreaks. In 2010, a CDC report indicated that there was more non-O157 caused infections than those caused by *E. coli* O157 *(170)*. There are an estimated 287,000 cases of non-O157 infection each year, but this number seems to be increasing each year *(140)*. The largest individual non-O157 STEC outbreak was caused by *E. coli* O111:NM in 2008; this outbreak led to 341 infections, 26 cases of HUS, and 1 death *(147)*.

Between 1982 and 2006, there were 90 confirmed *E. coli* outbreaks that occurred in the UK, Ireland, Denmark, Finland, Canada, and Japan. 54.4% of these outbreaks were caused by food and dairy products, 12.2% were caused by animal contact, 7.8% from water, and 2.2% from environmental sources *(155)*. The United States Center for Disease Control and Prevention states that there were 350 outbreaks from 1982-2002 in the United States alone. A study of this information revealed that of the 8,598 cases in these outbreaks, there were 1,473 (17%) hospitalizations, 354 (4%) cases of HUS, and 40 (0.5%) deaths *(155)*. Additionally, this study

found that the transmission route of 52% of outbreaks was foodborne, with 41% being associated with ground beef and 21% associated with produce *(155)*. A 2013 study revealed that from 2002 to 2010 there were 7,694 cases of illness caused by a shiga-toxigenic *E. coli* strain. Of these, 5,688 (74%) were caused by *E. coli* O157:H7 and 2,006 (26%) were caused by Non-O157 serotypes, with O26 being the most prevalent *(70)*.

Inefficient detection methods for non-O157 STEC could indicate that foods previously associated with O157 could have also been likely to carry non-O157 *(21)*. Studies detected non-O157 STEC in a variety of common foods, such as cheese, lamb, poultry, pork, ground meat, fish, shellfish, and deli salad *(70, 130, 144, 150)*. A 1994 study revealed that 63% of veal, 48% of lamb, 23% of beef, 18% of pork, 12% of chicken, and 10% of fish were negative for O157:H7, but were positive for non-O157 strains *(63)*. Another study found that 53.9% of beef carcasses were positive for non-O157 STEC prior to processing *(12)*. This was reduced to 8.3% with process interventions such as hot water, organic acids, and steam vacuuming. The FoodNet reported that the six non-O157 serogroups accounted for 75% of the non-O157 illnesses *(88)*. Following this, the Food Safety and Inspection Service proposed the same zero tolerance policy for the big six non-O157 as the current policy for O157:H7 in the beef industry *(130)*.

4. Prevalence and Survival

Contamination of agricultural products by *E. coli* occurs through a number of vectors such as insects or wildlife; as well as through on-farm reservoirs including manure, water, and soil. While *E. coli* has been isolated from nearly all varieties of agricultural animals, cattle are most often considered to be the primary reservoir *(11, 22, 25)*. The method of intensive agriculture has been

linked with the introduction of the pathogen to previously unexposed areas such as produce farms from the original reservoirs. Animals are often contaminated through direct contact with *E. coli* in feces and contact surfaces, or through the consumption of contaminated food and water. A 1999 study found that *E. coli* is naturally found in many ruminant animals, though it is primarily commensal *(111)*. Karmali et. al found that prevalence of shiga toxigenic *E. coli* in cattle herds ranged from 10% to 28% with seasonal fluctuations *(99)*. There have been more than 435 STEC serotypes isolated from cattle *(98) E. coli*, like many enteric pathogens, is spread throughout the environment by multiplying in the intestinal tract and is shed in the feces. Humans are infected with STEC primarily through the fecal-oral route, in which they ingest materials contaminated by feces. Though only accounting for a small portion of a herd, cattle known as "super shedders" are responsible for at least 95% of STEC shed *(64)*. The concentration of shed bacteria and the length of time spent shedding are both related to the level of STEC attachment at the recto-anal junction in the bovine intestinal tract *(33)*. The use of untreated or improperly treated manure in agriculture has led to STEC being found in consumer-bought produce. Other sources of this contamination include waste water runoff, contaminated irrigation sources, and the feces of wildlife that have access to the fields *(4, 91, 92, 182)*. Contaminated fecal material can also contaminate natural bodies of water such as lakes or rivers, and eventually may contaminate improperly treated drinking water. Field workers and improperly cleaned farm equipment are also strong contributors to the spread of STEC on the farm *(19)*. The STEC group has even been known to be passed from one person to another, further emphasizing the need for proper sanitation practices in childcare and healthcare centers *(17, 143)*. Pathogen survival outside of the host organisms is dependent on a variety of factors such as pH, temperature, competing microflora, and initial bacterial concentration [71]. Organic lettuce has been found to have the highest prevalence of *E. coli* with

over 20% positive samples *(131)*. Organic farms were found to have more than 5 times the *E. coli* incidence rate when compared to conventional farms (9.7% vs 1.6% respectively). All 32 organic farms tested used aged or composted manure as fertilizer, and samples of this manure that was aged for less than 1 year was found to contain levels of *E. coli* that were 19 times greater than compost aged for longer than 1 year *(131)*. One of the biggest factors contributing to on-farm survival and survival in a processing facility is the formation of biofilms by the *E. coli*.

5. Biofilms

A biofilm is a group of bacteria that is attached to a surface and protected by an extracellular polymeric substances (EPS) matrix that is secreted by the bacteria, particularly under stress conditions *(10, 37, 38)*. This ability increases the survivability of the bacteria in harsh environments and those with low nutrient availability such as contact surfaces *(37, 38, 108)*. Biofilm formation is not surface or environment dependent, and it is triggered by environmental conditions that are specific to each bacterium. Even within STEC, there are different factors that trigger biofilm formation, though low-nutrient conditions are often one of the factors they have in common *(37, 60)*.

There are 5 primary steps to the formation of biofilms: initial attachment, irreversible attachment, microcolony formation, biofilm maturation, and dispersion (Fig 2.1). During initial attachment, the bacteria adhere along with other organic matter to form a conditioning film on the surface. This process changes the physio-chemical properties of the surface such as its hydrophobicity and electrostatic charges, which helps facilitate further bacterial attachment *(108)*. The initial attachment of bacteria is generally reversible, allowing them to return to the planktonic state if

environmental conditions improve *(8, 14, 52, 206)*. In the irreversible attachment phase, the bacteria will form permanent attachments to the surface through the use of pili, curli, or surface proteins *(47, 108, 198)*. When forming microcolonies, the attached cells will grow and aggregate using nutrients available from both the environment and the aforementioned conditioning film. This growth and aggregation develops a layer of cells, which cover the surface and begin to produce more EPS in order to help anchor the cells and protect them from the environment *(108)*. Biofilm maturation then begins, in which the cells continue to produce EPS, which in turn allows more cells to attach and release more EPS. Once many layers have been formed, the biofilm is considered to be "mature". In the final step, the bacteria residing in the biofilm may be dispersed due to changes in the environmental conditions, accumulation of a toxic byproduct, or shear stress from the biofilm itself *(85, 97, 160, 169)*. These released bacteria may then colonize a new site and form another biofilm.

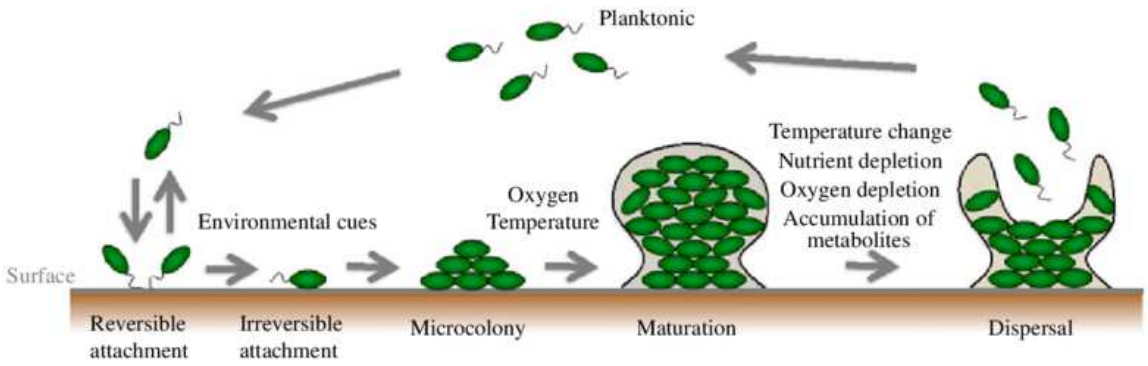

Figure 2.1. Stages of biofilm development. *Adapted from Toyofuku et. Al. (192)*

Biofilm formation can occur in less than four hours, they can also contain a bacterial population of up to 10□ cells/cm³ along with water and the EPS. 24-48 hours after initial attachment, the

bacterial population may be approaching 10^{1}□ cells/cm³ with up to 30 layers of bacteria forming the completed and mature biofilm. Biofilm formation time is highly dependent on bacterial species, as well as the environmental conditions*(139)*. The formation time can vary from a few hours to several months before a mature equilibrium is reached. When the biofilm begins to age, the bacteria will detach from the degraded biofilm in order to disperse and colonize new sites. This detachment may be large clumps of bacteria and EPS, or it may be individual bacterial cells *(47, 108)*.

The bacterial adhesion properties are primarily governed by cell surface appendages and hydrophobicity, and both of these can be controlled with the culture method. The pH of the suspension medium may also affect the surface charge of the bacterium, which also may play a role in adhesion capabilities *(26, 81, 134, 185)*. Other variables such as temperature may also play a role in the attachment phase. It has been shown that low temperatures could slow bacterial growth by changing the viscosity of the media, the physiology of the bacteria, and subsequently the ability to adhere properly to a surface in order to form a cohesive biofilm *(134)*. Another study indicated that the biofilm formation and growth patterns varied heavily between surface materials across all temperature ranges that were tested *(185)*. Rye et. al. *(164)* showed that *E. coli* produced more EPS at lower incubation temperatures and in a low-nutrient medium. Contact time is also very important in the amount of adhesion, as the number of attached bacteria increases with the contact time due to the increased likelihood of the cell making contact with the surface *(134)*.

Shiga-toxigenic *E. coli* has been associated with numerous foodborne illness outbreaks over the past few decades; understanding the mechanisms they utilize is integral in developing new

intervention strategies in the food industry *(72, 140, 154)*. Their strong biofilm forming capabilities are one of the largest contributing factors to their pervasiveness, as they are capable of forming biofilms on both abiotic and biotic surfaces such as those found in the food processing environment. The effects of temperature, anaerobic conditions, and nutrient availability were all studied to test the ability of STEC to attach and form a biofilm on stainless steel *(46)*. This study was conducted using an isolate from a 1982 national outbreak from ground beef and the results showed that the isolate was capable of forming biofilms regardless of the variable tested *(46)*. The study indicated that the biofilm developed much faster in complex media, though the biofilm had less EPS and the cells were easily dislodged. It was found that in lower nutrient environments, the biofilm was slower, but contained significantly more EPS and was much more difficult to remove from the stainless steel. This seems to indicate that stronger and more complex biofilms are developed under stress conditions, adding to the difficulties faced in a food processing facility.

Another study was conducted to simulate conditions found in a beef fabrication plant and to determine the biofilm forming capabilities of *E. coli* O157:H7 in the presence of liquid fat-lean homogenate and solid ground beef *(49)*. It was observed that surface type did not have an effect on attachment, but the beef material had a significant effect, with ground beef resulting in higher attachment levels. In another study, it was found that the presence of beef fat negated any surface effects and facilitated a similar attachment level across all surfaces *(178)*. The ability for multiple strains of STEC to co-colonize a surface can lead to product contamination by multiple strains, making traceback and recall more difficult for the responsible agencies. In the food industry, biofilm formation can occur at multiple locations, though they are primarily on floors, drains, pipes, and walls. These pose a huge post-processing contamination risk that has been seen in

virtually every type of facility from meat to produce *(108)*. Biofilms are capable of forming on nearly any surface type found in a production facility, including aluminum, polyurethane, Teflon seals, nylon, stainless steel, and even glass *(108, 176)*. Crevices in various types of equipment are particularly dangerous as they are difficult to properly clean and sanitize and thus are often a great environment for the formation of pathogenic biofilms. Biofilms can also have a drastic effect on the efficacy of common antimicrobials used on produce, carcasses, or other food products *(35, 86, 181)*.

B. INTERVENTIONS

1. Chemical Interventions

Numerous antimicrobials have been tested both alone and in combination with other products to eliminate STEC in the food industry, but unsurprisingly, none have been 100% effective *(104)*. A study completed in 2000 evaluated current interventions to determine their efficacy against *Salmonella,* non-O157 *E. coli*, and O157 *E. coli* serotypes. Results indicated that the current intervention strategies of spray water, hot water, 2% Lactic acid, 2% acetic acid, or 10% trisodium phosphate all equally reduced both O157 and non-O157 serotypes on meat surfaces. It was concluded that current interventions used to control O157 are equally effective against non-O157 STEC serotypes *(42)*. Another evaluated the effectiveness of paracetic acid (200 ppm), octanoic acid (9,000 ppm), and acidified sodium chloride (1,000 ppm). In this study, the meat surface was inoculated and dried for 30 minutes to allow for bacterial attachment, and sprayed with the aforementioned treatment solutions. This study indicated that there was no significant differences in the effect of the treatments between STEC serotypes *(32)*. These two studies indicate that STEC antimicrobial sensitivity does not vary by serotype.

Though there are many effective interventions for planktonic bacterial cells, biofilms are much more difficult to effectively sanitize. Biofilms are a huge risk for the food industry, and thus finding effective sanitizers is a primary research focus. One study compared modern chemical sanitizers against both *E. coli* and *S. aureus* biofilms on stainless steel surfaces *(13)*. This study tested three types of adhered cells: 2hr attachment, 6-day old biofilm in TSB, and a 5-day old biofilm kept in 100% humidity. These biofilms were tested with two commercial sanitizers, one chlorine-based and one alcohol-based. The alcohol sanitizer was most effective against both bacterial species, and there was no significant difference found based on the different adherence types. It was concluded that alcohol-based sanitizers may be a more effective way to control microbial contamination across multiple types of bacterial attachment *(13)*. Sanitizers have also been tested on foods to control transmission of pathogens between food products. One study found that nisin-based sanitizers were more effective than the standard chlorinated water wash, and actually reduced the pathogen population to an undetectable limit, even after enrichment *(34)*.

Biofilms have been reported to provide increased resistance to antimicrobial interventions when compared to planktonic cells. Researchers have suggested that this resistance can be attributed to the production of EPS, reduced diffusion of the antimicrobial, and the production of enzymes that degrade the antimicrobials *(65, 107)*. The EPS matrix is believed to absorb and bind to the antimicrobials, mitigating their ability to reach and affect the bacterial cells within *(27)*. The material surface that the biofilm is bound to is another factor that may play a role in sanitizer efficacy *(47)*. The properties of the surface material that may contribute to this includes the texture, composition, and charge. The penetration capabilities of the antimicrobial are directly related to

the concentration and the contact time with the biofilm, which is often difficult to control in a production environment *(191)*.

Studies have also shown that EPS production promotes STEC resistance to chlorine, without affecting the bacterial cell growth or maturation *(165)*. The production of Curli, a type of amyloid fiber used to promote community attachment, significantly enhanced biofilm production and increased chlorine resistance. Curli producing strains form a much stronger biofilm and also show a drastic increase in resistance to chlorine sanitizers when compared to non-curli producing strains. It has also been showed that older biofilms are more resistant to sanitizers, this has been speculated to be a result of the extracellular compounds interfering with the chemical sanitizers *(183)*. Lindsay et el. *(116)* revealed that multiple species may work together within a biofilm in order to shield one another, increasing their tolerance to sanitizers. This results from the physical protection of the sensitive species by the more tolerant species. In *Pseudomonas* spp., they have shown an ability to alter their EPS composition in order to increase its chemical antimicrobial tolerance *(54)*. Bacteria have shown a propensity to become tolerant of many antimicrobials, this rise in anti-microbial resistance has led to the development of biocontrol methods such as microbial molecules, bacteriophages, or associated enzymes *(69, 127)*.

2. Biocontrol

One of the oldest methods of biocontrol is the use of bacteriocins. Bacteriocins are antibacterial proteins produced by certain bacteria in order to kill or inhibit growth of competing organisms. Lactic acid bacteria (LAB) produce the highest diversity of bacteriocins, and are often found in numerous fermented foods. Bacteriocins have been categorized both biochemically and

genetically, but there are many aspects that are unknown *(36)*. Bacteriocins are incredibly popular in research because of the public push for more effective antimicrobials that are less toxic and produce fewer adverse effects compared to traditional antimicrobials such as chlorine. The production of these proteins is primarily used to minimize competition from infectious species *(133)*. Many bacteriocins act against specific organisms, and others act on a very broad spectrum of organisms. Bacteriocins utilize a few different mechanisms, but the most common is by causing cell leakage and eventual cell death through permeabilizing the membrane. Bacteriocins are equally as effective as anti-biotics and anti-microbials, but with a much lower chance of the target organism developing resistance *(36)*. One of the main limiting factors of bacteriocins is their reliance on specific environmental conditions to maximize effectiveness.

Bacteriophages (Phages), highly specialized viruses, are of great interest to the food industry due to their nearly ubiquitous nature and their minimal environmental impact. They are one of the most abundant biological particles on earth, and their exceptional specificity can ensure they are targeting the correct pathogen. Phages are easily isolated against different pathogens, and cocktails are a promising method of control in a production environment *(187)*. With the consumer push towards "natural" food and food additives, phages could hold a very important role in organic or all natural foods. Numerous studies have tested phages against their specific target species, showing great promise with phage technology. Bacteriophages can be used directly on produce and meat products. One study tested bacteriophages as a control method against *E. coli* on lettuce and cantaloupe, in this study the phages provided as high as a two log reduction on lettuce, and as much as 3 logs on cantaloupe. The phages were more effective on samples stored at refrigerator temperatures, likely due to the subdued activity of the pathogen *(141)*. Another study used

bacteriophages to control *E. coli* on steak meat, and 7 of the 9 samples showed undetectable levels of *E. coli* after enrichment. The authors of this study did indicate that phage resistant bacteria appeared, but they reverted to phage-sensitive strains within a few generations. This suggests that mutations may not limit phage technology due to the low levels of pathogen in a processing environment *(136)*. Contamination of contact surfaces is one of the largest concerns in the food industry due to the cross-contamination risks, as well as the cleaning difficulty. Bacteriophages are also a promising agent in controlling contamination on contact surfaces. One study determined that a phage cocktail effectively eliminated *E. coli* populations within an hour on stainless steel and high density polyethylene. This study was done with low levels of inoculum and at room temperature, similar to the conditions that would be found in a food processing environment *(199)*. Another study tested the pathogen reduction under high inoculum levels, and it found that bacteriophages reduced the population by less than 1 log CFU at all conditions on all surfaces, which is significantly less effective than modern sanitizers *(199)*. Bacteriophages combined with chemical sanitizers can drastically increase the effectiveness of the treatment, and can be an integral part of a multi-hurdle intervention strategy. One potential problem with the use of bacteriophages is their high level of specificity, this prevents them from targeting any other spoilage organisms or pathogens aside from the intended one. They are also ineffective against biofilms, as they cannot penetrate the EPS to target the bacterial cells.

One new, but promising, technology is the use of bacteriophage-derived products. One of these products is known as phage depolymerase. In initial studies it has been extremely effective in removing bacterial biofilms, as it helps to degrade the EPS layers *(189)*. Another promising product is holin, a type of small protein that is integral in the lysis of host cells. Holin is a

nonspecific membrane protein that rapidly degrades the host cells membrane, leading to lysis and cell death. Holin proteins have been utilized as an effective antimicrobial against *Staphylococcus aureus* and *Listeria monocytogenes* at standard incubation temperatures *(184)*. These proteins have also showed great promise in fields other than Food Safety, such as oncology *(106)*.

C. BACTERIOPHAGES

1. History

Bacteriophages are viruses that infect bacteria; they are only capable of self-replication within their host organism. Phages are one of the largest reservoirs of genetic diversity on Earth, and it is estimated that they kill up to 50% of the bacteria produced every day *(188)*. Phages were first documented by a British bacteriologist named Ernest Hankin, he discovered them in 1896 while studying *Vibrio cholerae* in rivers in India *(186)*. He suggested the existence of an unidentified organism that would pass through filters and would limit the spread of cholera *(186)*. Gamalea, a Russian bacteriologist, made similar observations two years later regarding an organism that affected *Bacillus subtilis*, though his observations were not further examined until a man named Fredrick Twort began researching these organisms *(50)*. Twort theorized that the phenomenon was the result of a virus, but this hypothesis was not tested further *(50)*. Two years later, a Canadian microbiologist by the name of Felix d'Herelle observed small, clear plaques on an agar medium containing stool samples from dysentery patients *(100, 187)*. The name bacteriophages was coined by d'Herelle, with the name being a combination of 'bacteria' and the Greek 'phagein', meaning 'to eat'. The term 'plaque' was used by d'Helle to describe the circular zone of clearing caused by a singular phage on an agar plate. It was soon discovered that phage titer increased in ill patients, indicating potential therapeutic benefits *(2)*. The study of phages has led to some of the most

important scientific discoveries, from deciphering of the genetic code, to development of molecular recombinant technology, and even the discovery of DNA as the genetic material*(82)*. Phages are currently used in both the food industry and the medical industry, as well as other biological science fields *(120, 174)*.

2. Classification

Bacteriophages are best defined as intracellular bacterial parasites, and like all viruses, they lack an independent metabolism. Phage genome size varies from a few thousand base pairs up to 498 kilobases *(76)*. At 498 kilobases, *Bacillus* Phage G is the largest known bacteriophage, with a genome approaching that of an average bacterium. A bacteriophage particle, known as a virion, consists of a DNA or RNA molecule, encapsulated inside a shell made of protein known as the capsid. The accepted classification method for bacteriophages is by the use of virion morphology and the genomic data to segregate them into 13 distinct families. Of all phages described, 95% of them belong to the order *Caudovirales*, which is also known as the tailed double stranded DNA phages. Within the *Caudovirales* order, there are two main families that are distinguished by their tail morphology: *Siphoviridae* and *Myoviridae*. *Siphoviridae* makes up 60% of the studied phages, and they are classified by their long, flexible tails. *Myoviridae* makes up 25% of studied phages, and are classified by a short, double layered, contractile tail *(61)* (Fig. 2.2). One family of phages, *Tectiviridae*, are very small tail-less double stranded DNA phages that contain a pseudo tail and a lipid layer (Fig. 2.3). These tail-less phages are oddly distributed and are often found to be associated with enterobacteria and their relatives such as *Bacillus* and *Thermus* bacteria *(3)*. Virions with tails are composed of proteins and double stranded DNA in a 1:1 ratio, and the condensed chromosome represents up to 50% of the virions mass packed within the head *(55)*. The

condensed form of the chromosome is important in the phage life cycle, as the pressure from this is what allows the capsid to inject its DNA into the host cell during infection. *Caudovirales* phages have an icosahedral head, with 20 sides and 12 vertices. The size of the packaged genome correlates directly to the size of the phage head. The corners of the icosahedral structure are made up of pentamers of capsid proteins, while the rest of the sides are generally made of hexamers of similar capsid proteins *(76)*.

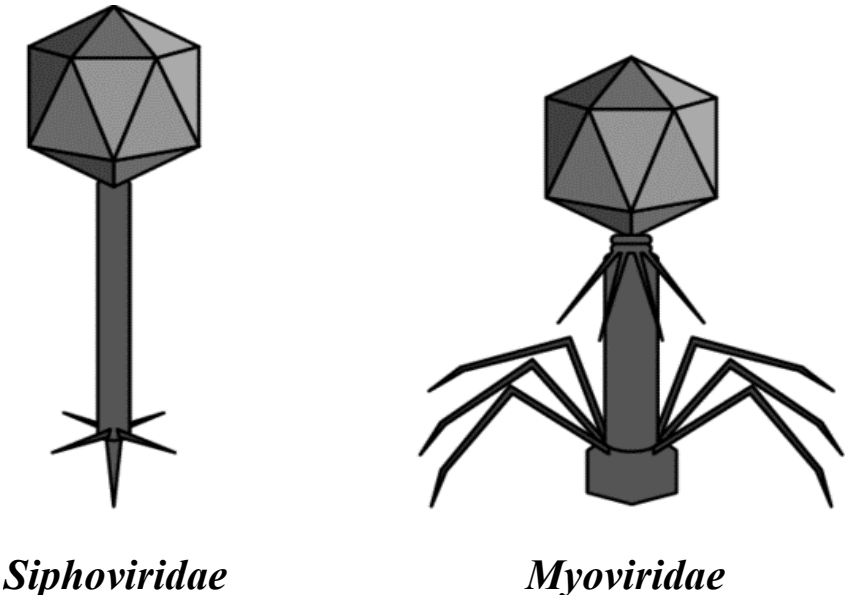

Figure 2.2. Basic morphology of the two primary families of the order *Caudovirales*. *Adapted from Cota (40).*

The proteins that connect the head and tail structures, known as the portal proteins, are instrumental in the infection cycle of the bacteriophage. This protein controls the entry of the DNA, as well as the assembly of the tail to the immature head during the development of the new bacteriophage particles. During attachment and infection, this protein goes through a confirmation change in order to allow the phage DNA to be injected into the host bacterial cell *(197)*. The DNA is injected

through the tail, which helps in attachment as well as injection. The bacteriophage tail shafts are composed of helical subunits, and have either a three-fold or six-fold symmetry. In phage families such as *Myoviridae* (e.g. phage T4), the tails are contractile, while in *Siphoviridae*, the tails are non-contractile. Phages with a long tail possess a large gene that is responsible for a 'tape measure' protein that determines the phage tail length. The non-contractile tails are most often composed as multiple copies of a single protein, known as major tail protein. This causes the overall shape of the non-contractile tail to be maintained throughout infection. Contractile tails, on the other hand, are composed of a central tail tube protein surrounded by a tail sheath protein. On infection, the sheath is contracted, causing the tail tube protein to penetrate the host cell, allowing for DNA injection *(114)*.

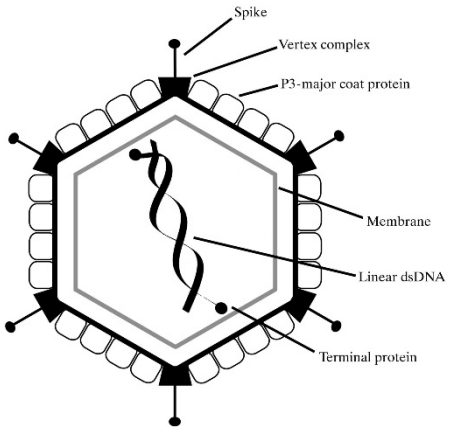

Figure 2.3. Basic morphology of *Tectiviridae. Adapted from Benson et al. (18)*

Tectiviridae (e.g. PRD1) virions have an icosahedral capsid with an underlying membrane to enclose the genome. This membrane is locked with cementing proteins, and spike complexes that form at the vertices *(163)*. The machinery responsible for DNA packaging is contained within a unique vertex, and is anchored into the membrane with the help of integral membrane proteins.

The phage membrane contains proteins and lytic enzymes which play a crucial role in the infection process *(71, 161, 162)*. During the infection process, this membrane structure is rearranged in order to form a tubular structure that protrudes from one vertex. This newly formed tubular structure then penetrates the host cell wall, forming a channel by which the phage genome is injected into the cell *(43, 71)*.

3. Mode of Action

Bacteriophages mode of action for bacterial disruption is a required part of their lifecycle. Phages have two different lifecycles, the lytic and the lysogenic (Fig. 2.4). The first step to either lifecycle is the attachment process, which starts with the phage recognizing specific receptors on the host cell in order to bind to the correct host *(125)*. The phage receptor binding protein and the host cell surface interact upon binding, which begins the processes necessary for genome injection *(125)*. Some studies have found that tailed phages will contract their tail after attachment in order to bring the genome-containing capsid closer before injection *(39)*. Once the genome has been injected, the empty virion is left attached to the outside of the host cell *(20)*. This attachment and injection process is similar across all phages, but the genomes are internalized by different mechanisms *(153)*.

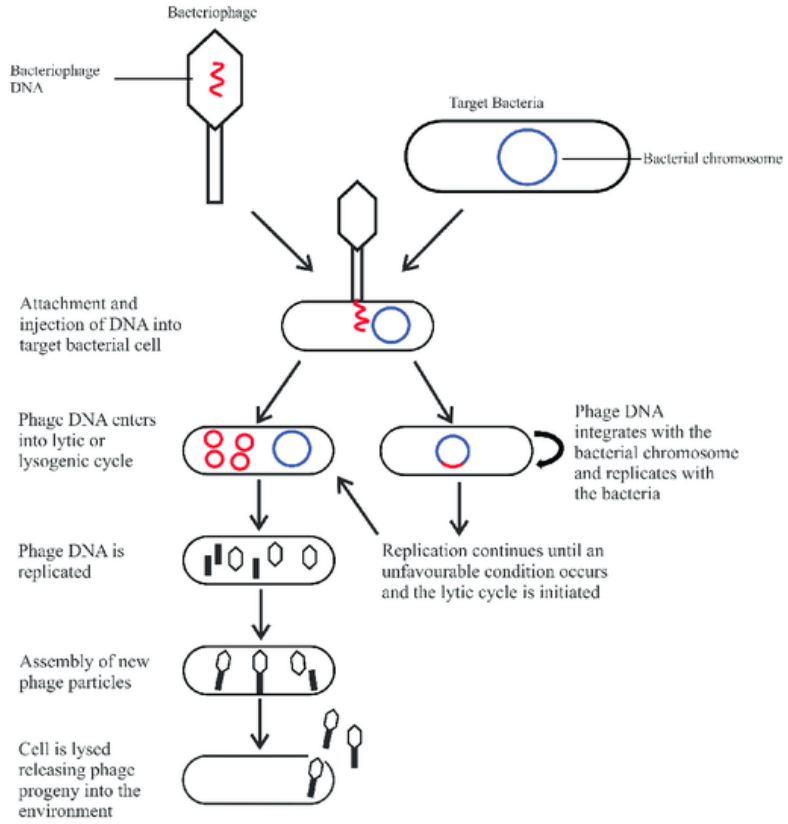

Figure 2.4. Bacteriophage lifecycles. See text for details. *Adapted from Gray et al. (1)*

The lytic lifecycle is the more traditional method of bacteriophage replication, and always results in the death of the host. Much like the lysogenic lifecycle, the bacteriophage attaches to the host cell via specific receptors before injecting its genome. After genome injection, the viral genome takes control of the hosts metabolic processes and machinery in order to replicate the components needed to assemble more viruses *(56, 78)*. The synthesis of new phages involves the creation of proteins used for the encapsulation of genetic material, the assembly of the capsid, and the tail structure *(56)*. After these components are assembled into new bacteriophages, the bacteriophages signal for the proteins known as holin to be released, causing small pores to be formed in the host cell membrane. Once the host cell membrane has been permeated, the newly created phages release an enzyme called endolysin, which degrades the peptidoglycan layer, causing them to rupture in a

process known as lysis *(126)*. The lysis of the host cell releases the new phages, where they will contact more host cells and repeat the process. This replication method allows for rapid increases in phage population in the presence of the proper host cells, making lytic phages an excellent candidate for use as a biocontrol agent.

The lysogenic lifecycle is a form of replication where the lytic functions of the virus are not used, thus allowing survival of the bacteriophage genome in the host cell *(56)*. Phages that are replicating in this manner are known as temperate phages *(78)*. The lysogenic lifecycle differs from the lytic cycle in that it is replication with the host cell, not just within it. The phage DNA will integrate into the host DNA, or will exist as an episome within the cytoplasm *(126)*. The integration process utilizes recombination at specific regions of the genomes of the host cell as well as the invading phage genome *(126)*. Once the genome has integrated with the bacterial cell, that cell becomes known as a lysogenic bacterial cell *(78)*. Since the phage genome is effectively acting as part of the host DNA, the phage DNA is replicated each time the host cell replicates, with these replicated sequences being known as prophages *(78)*. Temperate phages alter the host cell wall, removing or altering receptor sites in order to prevent invasion by other bacteriophages *(110)*. Prophages can reproduce this way indefinitely, but the introduction of stress conditions to the host cell can trigger the lytic cycle, which leads to cell lysis and the release of phage *(157)*. The released phages are lower in number than a traditional lytic cycle due to the temperate phages only replicating during stress conditions *(122)*. This is the primary disadvantage for the use of temperate phages as biocontrol *(126)*.

4. Bacteriophage Enzymes

The primary enzymes associated with bacteriophages are lysins, or enzymes related to the lytic process. These enzymes can withstand internal cytoplasmic pressures, and are integral to replication of lytic bacteriophages *(5, 78)*. Lysins are responsible for degrading the peptidoglycan layer of the cell wall, which eventually leads to cell lysis. Research using endolysins as an antimicrobial has shown promise, particularly against antibiotic resistant problem organisms such as methicillin-resistant *Staph aureus*, showing significant antimicrobial activity at concentrations as low as 1μg/ml *(77)*. While effective, the extraction process for endolysins is extremely time consuming, and only yields 12mg/L of cells grown *(77)*. This makes it an expensive option for commercial use as extraction is incredibly inefficient.

Another enzyme produced by bacteriophages is an EPS degrading enzyme known as phage depolymerase. This enzyme disrupts the EPS in biofilms, allowing the bacteria to be exposed to bacteriophages that could not previously access them *(5, 90)*. In electron micrographs, phage depolymerases are often observed as tail spike proteins that are attached to the baseplate. These depolymerases bind to EPS and degrade it until they reach the cell surface, where they bind and allow phage-mediated cell lysis *(90)*. Modifications of the EPS by the bacteria may be enough to lessen the effectiveness of the depolymerase, though phages often possess depolymerases that are specific to their target organism, allowing them to degrade the EPS. One study found that bacteriophages are capable of reducing the population of STEC in biofilms on multiple surface types within an hour of treatment *(199)*. Another study demonstrated the effectiveness of phages on STEC biofilms attached to produce harvesting equipment, showing a reduction of 4.5 logs CFU/surface *(66)*. These phage depolymerases are a promising new technology, as biofilms are

one of the leading causes of contamination in the food industry, as well as a major cause of infections in healthcare settings. Phage depolymerase is not believed to cause damage to the bacterial cells themselves, so alternative phage products could be added for a more effective multi-hurdle approach to sanitation. One such combination, active bacteriophage with extracted depolymerase, will be discussed further in the following research.

5. Bacteriophage Holin

Holins are a small group of membrane proteins that are capable of forming lesions within the cytoplasmic membrane of the host cells *(200)*. These lesions allow ~500 kDa protein complexes to pass through, one of these complexes is a muralytic enzyme known as endolysin. Endolysin passes through the lesions and is then free to degrade and digest the peptidoglycan layer *(200)*. Holin also controls the time of the phage replication cycle, shortening or lengthening it as a result of missense mutations within the *holin* gene itself. Holin is considered to be a molecular clock for lytic phages, as it is almost entirely responsible for proper lysis *(75)*. Holin has been studied heavily in recent years, with focuses on its biochemical, physiology, and microbial characteristics and effects. One of the results of this focus was the discovery of the holin lesion formation. During lesion formation, holin protomers accumulate in the cytoplasmic membrane and aggregate into large two dimensional protein 'death rafts' *(200)*. These 'death rafts' exclude lipid molecules which opens an aqueous channel within the membrane, which is followed by a change in holin conformation and rapid depolarization of the membrane. Holins alone are unable to cause cell lysis, since endolysin is needed to destroy the peptidoglycan layer. However, they are capable of killing cells by causing a loss of respiration abilities and the depolarization of the cytoplasmic membrane, causing the cells to become susceptible to normally impermeant molecules *(158)*. One

study found that holin was capable of strongly inhibiting *E.coli* lawns, producing visible plaques, this same study also found that holin was inhibitory towards several gram-positive contaminants *(152)*. Through deletion, cloning, and random mutagenesis of the *holin* gene, multiple holin-like proteins with antibacterial activity were derived in another study *(152)*. Holins and Endolysins are believed to be non-specific, making them far more valuable to the food industry than bacteriophages alone. Bacteriophage by-products also have a distinct advantage in food as they easily avoid the potential consumer stigma against associating the term virus with their food. In order to further study holin as a possible antimicrobial agent, it is necessary to identify the responsible genes in a lytic phage and express these genes in order to determine feasibility. Identification, cloning, sequencing, and expression of the *holin* gene is the focus of the following research.

Chapter III.

EVALUATE THE EFFICACY OF PHAGE DEPOLYMERASE AGAINST STEC BIOFILM FORMATION ON FOOD CONTACT SURFACES

A. MATERIALS AND METHODS

The biofilm disrupting capabilities of a bacteriophage depolymerase enzyme extracted from phage P9 was evaluated in comparison to treatments consisting of P9 alone and P9 combined with the depolymerase extract. The efficacy as both a preventative and a treatment was tested against *E. coli* O45 biofilms formed on both stainless steel (SS) and high-density polyethylene (HDPE) coupons over a 48-hour period.

1. Bacterial Cultures

The culture used for this study was *E. coli* O45 (CDC 3039), this strain was chosen due to its strong biofilm forming capabilities. This wild type isolate was retrieved from the Jaroni laboratory culture collection, which was originally isolated from a beef cattle farm environment*(119)*. The overnight culture of this STEC was prepared in tryptic soy broth (TSB), by incubating at 37˚ for 18 hours. For the enzyme extraction, 9 ml of the above culture was used to inoculate 90 ml of TSB and incubated for 16 hours at 37˚C in order to reach mid-log phase (9 logs CFU/ml).

2. Bacteriophage Culture

Previously isolated bacteriophage P9 *(118)*, an O45-specific phage, was used in this study for enzyme extraction. Bacteriophage P9 was chosen due to its high level of lytic activity as indicated by Litt et al *(118)*. In the study by Litt et al, high lytic activity was defined as complete clearing when analyzed using a spot-on-lawn assay. Briefly, phage was propagated by suspension of 100 µL of host bacterium overnight cultures in molten NZ-amine casamino acid yeast extract sodium chloride magnesium sulfate (NZCYM) agar, then plating via the double-layer agar method. The propagated phage was eluted in phosphate buffered saline (PBS) and centrifuged at 13,000 rpm for 10 minutes. A 0.22 µm syringe filter (EMD Millipore Millex™) was used to filter the resulting supernatant before the addition of 0.1% chloroform (Fisher Scientific, NJ) and storage at 4˚C until use. Prior to each experiment repetition, bacteriophage titer was determined as plaque forming units (PFU) per ml, by serial dilution of the phage stock in PBS and performing a plaque assay as described by Adams et al *(5)*. All experiments were performed with bacteriophage populations of 10□ PFU/ml.

3. Crude Depolymerase Enzyme Extraction

Crude enzyme was extracted by the method described by Hughes et al *(89)*. 10 ml of high titer phage, as prepared above, was added to 90 ml of the *E. coli* O45 culture, described above, and incubated for 6 hours at 37˚C. After incubation, the suspension was centrifuged for 20 minutes at 20,000*g* in order to separate the phages from the bacterial cell debris. The supernatant was dialyzed against PBS for 48 hours at 4˚C to remove materials with a low molecular weight, before being concentrated against polyethylene glycol (PEG, MW 6000) by surrounding the dialysis membrane in powdered PEG for 16 hours. The concentrated dialysate was re-centrifuged at 20,000*g,* then

passed through Millipore Vivacell 70 membrane filters (<100 kDA cut-off size) to separate the soluble phage enzyme from the phage particles. The filtrate was re-filtered through a 0.22 µm Millipore filter to ensure no phage remained. The presence of the enzyme in the crude extract was determined using a spot-on-lawn assay, which is performed by spotting 10 µl of the extract on a lawn of the host bacteria. These plates were incubated at 37˚C for 22-24 hours, and the spots were examined for the presence of halos in order to determine the presence of de-capsulated bacteria.

4. Inhibition of STEC Biofilms on Food Contact Surfaces

The use of the extracted bacteriophage depolymerase as a prevention method against STEC biofilms on food contact surfaces was studied in the following experiment. In the experiment, the selected treatments were applied to SS and HDPE coupons for 8 hours before *E. coli* O45 was applied in order to form a biofilm.

i. Preparation of Coupons

The coupons used in both this experiment and the following were 2 cm x 5 cm, were made of stainless steel (304 finish, type 4) and HDPE, and were prepared as described by Hood and Zootala *(87)*. The coupons were soaked in acetone for 30 minutes, before being rinsed with distilled water, and soaked in 1 M NaOH (Fisher Scientific, NJ) for 1 hour in order to remove greasiness. Coupons were rinsed again in distilled water and sonicated for 1 hour at 40 kHz. After sonication, coupons were rinsed in distilled water and dried before being sterilized for use.

ii. Prevention of STEC Biofilms using Phage Depolymerase

5 coupons of each material were used in total, one for each treatment (phage P9, phage depolymerase, P9 + depolymerase) and one for each control (positive control and negative control). Each coupon was sprayed with 500 µl of the respective treatments, using an airbrush (Badger Air-Brush Co., IL), resulting in a delivered volume of 6.34 log PFU/cm² of phage treatment. The treated coupons were allowed to dry for 8 hours at 25˚C in the biosafety hood before being spot inoculated with 50 µl (8-10 droplets) of the overnight culture described previously. The coupons were incubated at 25˚C for 48 hours in order to facilitate cellular adhesion and biofilm formation on the coupon surface. After incubation, the coupons were gently rinsed in 30ml of sterile distilled water to remove unattached bacterial cells from the surface. A sample of wash water from each sample was set aside for further analysis. The coupons were added to 30ml of PBS before being sonicated for 5 minutes at 40 kHz in order to dislodge the bacterial cells from the surface. Immediately after sonication, 3 g of glass beads (Genlantis, CA) were added and the coupons were vortexed for 1 minute to remove any remaining attached cells from the coupon *(66)*. The surviving bacterial population was determined by serial dilutions in buffered peptone water (BPW; Bacto, MD) and spread plating on TSA or MacConkey agar (MAC). Plates were incubated for 18-24 hours at 37˚C before bacterial colonies (CFU per cm²) were counted.

iii. Disruption of STEC Biofilms using Phage Depolymerase

In this study, the treatments were used after the biofilm had formed, and the disruption capabilities were studied. 5 coupons of each material were used in total, with the same treatments as the previous study. Each coupon was spot inoculated with 50 µl (8-10 droplets) of the overnight culture as previously described. The coupons were incubated at 25˚C for 48 hours in order to allow

for sufficient biofilm formation to form on the surface. The coupons were sprayed with 500 µl of their respective treatments using the same airbrush described previously, resulting in a delivered volume of 6.34 log PFU/cm² of phage treatment. The treated coupons were allowed to dry for 8 hours at 25˚C in a biosafety hood, before being gently rinsed in 30 ml of sterile distilled water to remove unattached cells. A sample of wash water from each treatment was set aside for further analysis. The coupons were added to 30 ml of PBS before being sonicated for 5 minutes at 40 kHz in order to dislodge cells from the surface. Immediately following sonication, 3 g of glass beads were added and the coupons were vortexed for 1 minute to remove any remaining attached cells *(66)*. The surviving bacterial population was determined by serial dilutions in BPW and spread plating on TSA or MAC agar. Plates were incubated for 18-24 hours at 37˚C before bacterial colonies (CFU per cm²) were counted.

5. Statistical analysis

Both experiments were repeated 3 times. Surviving *E. coli* populations, recovered after treatments, were converted to log CFU/cm² and the averages of the 3 repetitions were calculated. Data was analyzed using PROC GLM (SAS v.9.3 software; SAS Inst., NC) to determine the analysis of variance (ANOVA) for each treatment. Significant differences were separated by least significant difference (LSD) at $P< 0.05$.

B. RESULTS AND DISCUSSION

This study compared the biofilm inhibition capabilities of phage depolymerase on food contact surfaces as both a preventative measure and a treatment. Phage depolymerase was tested alone and in combination with bacteriophages on two types of food contact surfaces: SS and HDPE. Results

indicated that depolymerase was an effective preventative measure and treatment method, but was most effective when combined with bacteriophages.

1. Prevention of STEC Biofilms using Phage Depolymerase

Bacteriophage depolymerase showed significant reduction ($P<0.05$) in STEC biofilms compared to the positive control when applied to the coupon before the inoculum (Fig. 3.1). The initial *E. coli* O45 population for the positive control was 5.89 logs CFU/cm² on SS and 5.74 logs CFU/cm² on HDPE. The depolymerase treatment reduced *E. coli* O45 populations by 1.28 logs CFU/cm² on SS and by 1.50 logs CFU/cm² on HDPE. The bacteriophage treatment showed a reduction of 0.96 logs CFU/cm² on the SS coupons and a reduction of 0.74 logs CFU/cm² on the HDPE coupons. The most effective treatment was the combination of depolymerase and bacteriophage, this treatment produced a reduction of 1.61 logs CFU/cm² on SS and a reduction of 1.81 CFU/cm² on HDPE. All treatments showed a significant difference from one another. The depolymerase treatment seemed to reduce the population on the surfaces by preventing sufficient biofilm formation, allowing them to be removed during the gentle wash step. This is supported by the significant increase in residual bacteria in the wash water of the depolymerase treatment (6.41 logs CFU on SS and 5.45 logs CFU on HDPE) when compared to the wash water from the positive control (5.89 logs CFU on SS and 4.80 logs CFU on HDPE). Though all treatments showed a significant reduction in the population of *E. coli* O45, the bacteriophage treatment was the least effective, presumably due to the protection provided to the bacteria by their biofilm formation. There is a wide variation in biofilm composition (namely exopolysaccharides) among *E. coli*

serotypes, potentially providing the bacteria in biofilms increased resistance to phage depolymerase and bacteriophages *(38)*.

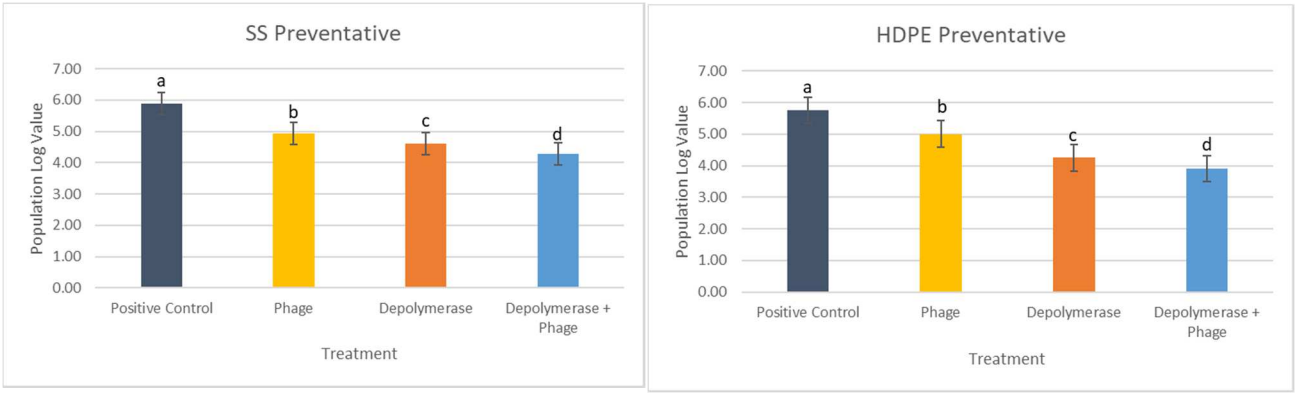

Figure 3.1 Surviving STEC population (log CFU/cm²) in the *E. coli* O45 biofilm. Values represent the average of three replications ± standard deviation. Different letters *a,b* etc. represent significant difference ($P<0.05$) between control and treatment for the same surface material.

2. Disruption of STEC Biofilms using Phage Depolymerase

Bacteriophage depolymerase also showed a significant reduction ($P<0.05$) in STEC biofilms compared to the positive control when applied to the coupon after the biofilm was allowed to form (Fig. 3.2). The initial *E. coli* O45 population for the positive control was 5.73 logs CFU/cm² on SS and 5.78 logs CFU/cm² on HDPE. The depolymerase treatment reduced O45 populations by 1.88 logs CFU/cm² on the SS coupons and by 0.68 logs CFU/cm² on HDPE. The bacteriophage treatment showed a reduction of 0.84 logs CFU/cm² on SS and a reduction of 1.16 logs CFU/cm² on the HDPE coupons. The bacteriophage treatment was not significantly different from the depolymerase treatment when applied to the HDPE surfaces. When applied to a pre-formed biofilm, the combination treatment was again the most effective, reducing the bacterial population by 3.1 logs CFU/cm² on SS and 1.49 logs CFU/cm² on HDPE. Similar to the preventative study, the depolymerase treatment seems to be disrupting the biofilm directly, rather than the cells within

it. This is again supported by the significant increase in residual bacteria in the wash water of the depolymerase treatment (5.15 logs CFU on SS and 5.08 logs CFU on HDPE) compared to the wash water of the positive control (4.63 logs CFU on SS and 4.68 logs CFU on HDPE). On the SS coupons, the bacteriophage treatment was the least effective on the formed biofilms, though the depolymerase treatment was the least effective on the HDPE coupons. This difference is likely due to a higher number of micro-abrasions on HDPE, protecting the biofilms from the depolymerase and preventing its effects *(49, 159)*. Another possible explanation for the increased attachment on HDPE surfaces could be due to variations within the polymer surface such as its smoothness, charge, zeta potential (charge developed at the interface of a solid surface and a liquid medium), and active chemical groups *(193)*. These chemical groups released by HDPE include things such as aldehydes, aromatic hydrocarbons, ketones, phenols, and quinones; all of these groups could be utilized by bacteria as a carbon source, allowing for stronger bacterial attachment *(28, 105, 179)*. All of the aforementioned reasons could be contributors to the higher bacterial attachment on HDPE, as well as the increased difficulty in removal when compared to the SS surfaces. Depolymerase treatments were visualized on Stainless Steel with Scanning Electron Microscopy (SEM) at 25,000x magnification (Fig. 3.3).

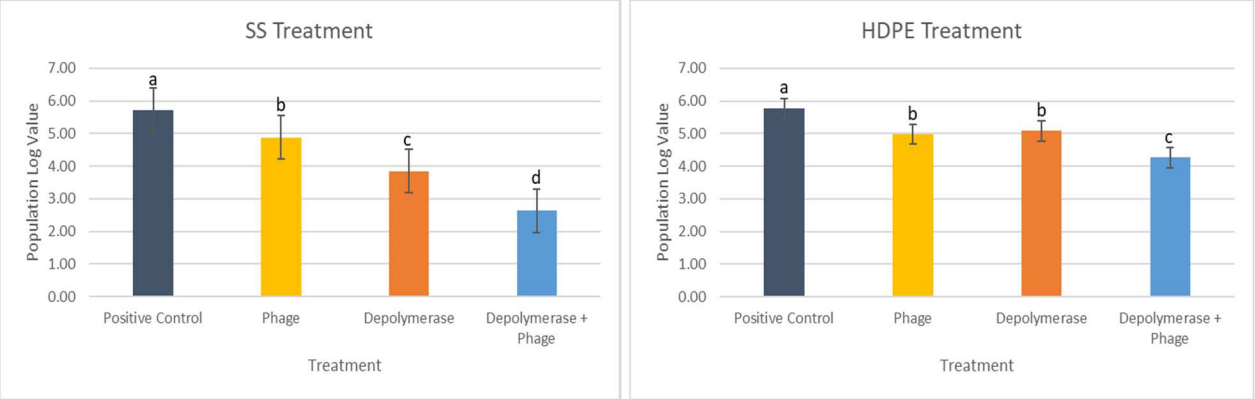

Figure 3.2 Surviving STEC population (log CFU/cm²) in the E. coli O45 biofilm. Values represent the average of three replications ± standard deviation. Different letters *a,b* etc. represent significant difference (P<0.05) between control and treatment for the same surface material. The bars with the same letters indicate that there is no significant difference (P<0.05) between the control and the treatment for the same surface material.

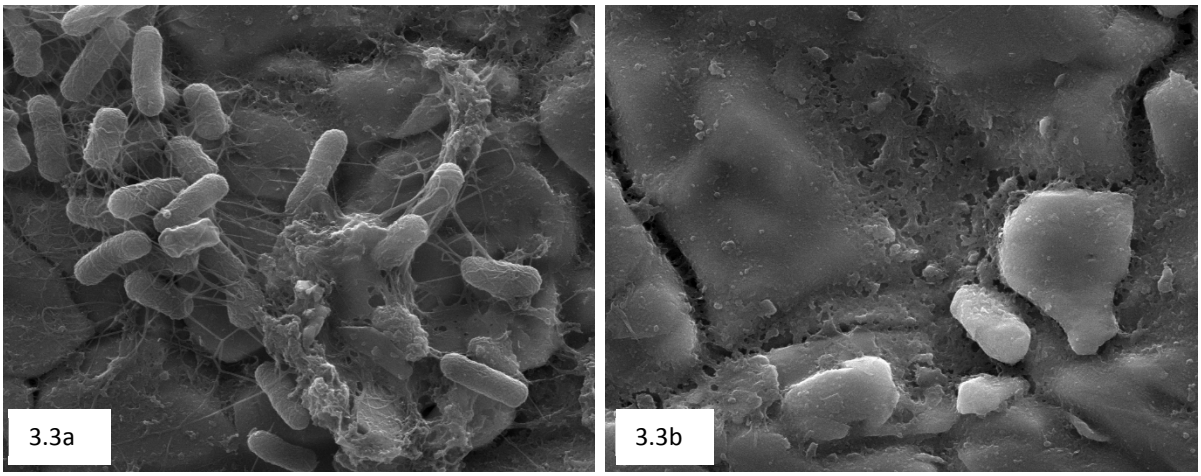

Figure 3.3 (a) Scanning Electron Microscopy of *E. coli* O45 biofilm on Stainless Steel surface. (b) Scanning Electron Microscopy of *E. coli* O45 biofilm on Stainless Steel after phage depolymerase application. (25,000x magnification)

C. CONCLUSIONS

In conclusion, this study has shown the potential for phage depolymerase as both a preventative for *E. coli* biofilm formation, as well as an *E. coli* biofilm disrupting treatment on food contact surfaces. Statistics indicated that there was no significant difference between the surface type and the application as a preventative or a corrective ($P>0.05$). Future studies should be conducted to purify the phage depolymerase extract in order to identify and remove other potential active enzymes before further experiments are carried out. The preliminary results from this study are very promising, showing that phage depolymerase could work in tangent with current sanitizers in the food industry in order to more efficiently remove biofilms formed on these surfaces.

Chapter IV.

IDENTIFICATION, CLONING, SEQUENCING, AND EXPRESSION OF THE HOLIN GENE IN O145-SPECIFIC BACTERIOPHAGE J25

A. MATERIALS AND METHODS

DNA sequencing was performed on previously isolated phage J25, which is an *E. coli* O145 infecting bacteriophage, in order to identify, clone, sequence, and express the gene responsible for producing the protein known as holin. Phylogenetic analysis was performed on J25 and the *holin* gene in order to further understand the evolutionary relationship of phage J25 and the *holin* gene.

1. Bacterial Culture Preparation

Escherichia coli O145:NM (CDC 99-3311) was used in this study. Prior to the experiment, one isolated colony from the TSA stock plate was picked and inoculated into 9 ml TSB and incubated for 37˚C for 22-24 hours. After incubation, 1 ml of the resulting culture was transferred to 9 ml TSB and incubated at 37˚C for 18 hours.

2. Bacteriophage Culture Preparation

Previously isolated bacteriophage J25 *(118)*, an O145-specific phage, was used in this study for *holin* isolation, cloning, and sequencing. J25 was chosen due to its high level of lytic activity as indicated by Litt et al *(118)*. Phage was propagated by suspension of 100 µL of host bacterium overnight cultures in NZCYM agar, then plating via the double-layer agar method. The propagated phage was eluted in phosphate buffered saline (PBS) and centrifuged at 13,000 rpm for 10 minutes.

A 0.22 μm syringe filter (EMD Millipore Millex™) was used to filter the resulting supernatant. The filtrate (phage working-stock) was directly used for the DNA extraction as described below.

3. Bacteriophage DNA Extraction

Bacteriophage DNA was prepared as described by Sambrook et al. *(167)*. A 1.8 ml volume of bacteriophage working stock was transferred to a clean microcentrifuge tube and treated with DNase I (Sigma, MO) at mg/ml and RNase A (Sigma, MO) at 12.5 mg/ml for 30 minutes at 37˚C, to remove any genomic contaminants from the preparation. Following this, 20% SDS (Fisher Scientific, NJ) and proteinase K (Sigma, MO) were added at 10 mg/ml before the mixture was incubated at 37˚C for 30 minutes, allowing for lysis of the phage capsid and the release of the phage DNA into the solution. After incubation, the solution was deproteinated by the addition of an equal volume of phenol:chloroform:isoamyl alcohol solution prepared in a 25:24:1 ratio (Fisher Scientific, NJ). The mixture was centrifuged at 3,500 rpm for 5 minutes and the supernatant was moved to a centrifuge tube containing an equal volume of chloroform:isoamyl alcohol solution (24:1) (Fisher Scientific, NJ). This mixture was centrifuged at 7,500 rpm for 5 minutes. The supernatant was again moved to a new centrifuge tube where the DNA was precipitated by the addition of 1/10 volume of 3 M sodium acetate (pH 5.2) (Sigma, MO) and an equal volume of cold isopropanol (Fisher Scientific, NJ), and incubated for 20 minutes at 24˚C. After incubation, the solution was centrifuged for 20 minutes at 14,000rpm and the supernatant was discarded. The remaining DNA pellet was washed twice with 70% ethanol and air-dried before being re-suspended in 20 μl of TE buffer (10mM Tris-HCl, pH 8.0; MP Biomedicals, CA; 1 mM EDTA, pH 8.0; Fisher Scientific, NJ).

4. Cloning Genomic Fragments of J25 for Sequencing and Identification

After extraction, digests were attempted with restriction enzymes. The digests failed to properly fragment the DNA, so the DNA was sonicated in order to create fragments for cloning. The extracted DNA diluted in 10X TM buffer (50mM Tris-HCL and 2.5mM MgCl□) and sterile ddH□O to reach a 35 µl total volume. The dilution was then sonicated using a Fisher Scientific 550 Sonic Dismembrator with cup attachment 3 times in 30-second bursts, cooling the samples in an ice bath for 1 minute between each burst. After sonication, the samples were centrifuged to reclaim the condensation before 2 µl of 0.25 mM dNTPs, 3 µl of T4 DNA polymerase (New England Biolabs), and 2 µl Klenow DNA polymerase (New England Biolabs) were added. These enzymes resulted in the filling of the DNA overhangs in a process known as "blunting", in order to be cloned into a blunt ended vector. The mixture was incubated at room temperature for 30 minutes before adding 5 µl of agarose gel loading dye was added and the mixture was added to a 1% low melting temperature agarose gel. This electrophoresis was ran for 60 minutes at 100-120 mA. After gel electrophoresis, the DNA bands were visualized under low-intensity UV light and cut from the gel and purified using the following method in order to isolate fragments. 300 µl of DNA extraction buffer was added to each gel piece and the mixture was heated at 50˚C for 10 minutes to dissolve the gel. The mixture was transferred to a microspin cup that was seated in a 2ml receptacle tube and centrifuged in a microcentrifuge for 30 seconds at maximum speed.

After centrifuging, the microspin cup was removed in order to discard the liquid in the tube, before being placed back into the tube. 750 µl of wash buffer (25ml 100% Ethanol and 25ml 2x wash buffer) was added to the microspin cup and centrifuged again at maximum speed for 30 seconds. The wash buffer was removed and the microspin cup was centrifuged at maximum speed for 30

seconds to ensure complete removal of the wash buffer. The microspin cup was transferred to a fresh 1.5 ml microcentrifuge tube and the 2ml receptacle tube was discarded. 50 µl of elution buffer (10mM) was added to each tube and the tubes were incubated at room temperature for 5 minutes. After incubation, the samples were centrifuged for 30 seconds at maximum speed to elute the DNA from the fiber matrix.

After elution, the DNA was dried and re-suspended in 36 µl of sterile ddH□O and 4 µl of 10X denaturing buffer was added. The mixture was incubated at 70˚C for 10 minutes and then placed in an ice bath before adding 1 µl of 10mM rATP, 5 µl of 10X kinase buffer, and 1 µl of T4 polynucleotide kinase (United States Biochemicals) and incubated at 37˚C for 30 minutes. One volume of phenol:chloroform was added to each sample and the tubes were vortexed. After vortexing the samples, they were centrifuged at 14,000 rpm for 5 minutes to separate the organic and aqueous layers before removing the aqueous layer to a clean tube. 0.5 volumes of 7.5M ammonium acetate was added to the tube in order to help the precipitation of the DNA, then two volumes of 100% ETOH was added and the tube was incubated for 15 minutes at -80˚C to precipitate the DNA. The tube was centrifuged at 14,000rpm for 15 minutes and the supernatant was removed, leaving the DNA pellet in the tube. 0.5 ml of 70% ETOH was added to the pellet and the tube was inverted twice before centrifuging at 14,000 rpm for 5 minutes again. The supernatant was removed and the DNA was air-dried before being re-suspended in 20 µl of TE.

The purified DNA fragments were ligated into a cloning vector, pBluescript II SK (+) (Invitrogen, CA), and digested with *Sma*I using standard techniques *(167)*. The ligations were transformed into XL1-Blue chemically competent cells. After ligation, the DNA sample volume was adjusted

to 50 µl using High Performance Liquid Chromatography (HPLC) water (Fisher Scientific, NJ), and placed on ice. The frozen XL1-Blue cells were quickly thawed before adding 3 µl of dimethyl sulfoxide (DMSO) (Fisher Scientific, NJ) and mixing. The 50 µl of competent cells were added to each tube of ligated DNA and vector before being mixed and incubated for 30 minutes on ice. The transformation mixtures were heat-shocked by placing the tube at 42˚C for 120 seconds, after which 400 µl of 2YT broth (8 g/l NaCl, 16 g/l tryptone, and 10 g/l yeast extract; G Biosciences, MO) was added, and the mixture was incubated for 45 minutes at 37˚C in order to allow the expression of the ampicillin resistance gene. The transformation mixture was plated on 2YT agar plates containing 100 µg/ml ampicillin + X-Gal (AMP; Sigma, MO) using a sterile glass "hockey stick" and incubated at 37˚C for 18 hours until colonies appeared.

Plates were observed for white colonies, which contained recombinant plasmid DNA. The single colonies were picked and grown overnight in 2 ml 2YT in a water bath with shaking. Minipreps were performed using the standard protocol by Sambrook et al *(167)* and re-suspended in 100 µl of TE. Clones were digested with *Pvu*II, electrophoresed on agarose gel, and visualized under UV light. The 2 µl DNA sample containing 6X loading dye (Sigma, MO) was loaded into 1 mm wells and electrophoresed at 80 volts to separate DNA fragments. The gel was stained in EtBr solution (0.01 µg/µl) for 10 minutes before being rinsed and visualized under UV. Clones potentially containing phage DNA were then sent to the Oklahoma State University Recombinant DNA/Protein Resource Facility (CORE) for sequencing.

5. Holin-Gene Analysis, Mutagenesis, and Cloning

After sequencing, the resultant DNA sequence was entered into the Basic Local Alignment Search Tool (BLAST) to identify similar DNA sequences in GenBank *(6)*, where it was found to be 98% similar to a bacteriophage known as RB68 (Figure 5.1). The RB68 *holin* gene was identified in the NCBI database and its sequence was located in the RB68 genome using EditSeq (DNAStar, Madison, WI). Using the known RB68 *holin* sequence, primers were designed using PrimerSelect (DNAStar, Madison, WI). The forward and reverse primers were designated RB68-01 and RB68-02 respectively (Appendix II).

The purified and unsonicated J25 DNA, prepared for shotgun cloning, was used in the following PCR reaction. 2.5 µl of DNA was added to 25 µl of Bio-X-Act Short Master Mix (Bioline, TN), along with 2.5 µl of RB68-01, 2.5 µl of RB68-02, and 17.5 µl of ddH□O the PCR was carried out under the appropriate cycling parameters. The PCR product was then TOPO cloned into pTOPO 2.1 (Invitrogen, CA) using the manufacturer's protocol. 1 µl salt solution (200mM NaCl, 10mM MgCl□), 1 µl ddH□O, and 1 µl were each added to the fresh PCR product. The reaction was mixed gently and incubated for 5 minutes at room temperature before being placed on ice. 2 µl of the aforementioned reaction was added to a vial of One Shot® Chemically Competent *E. coli* (Invitrogen, CA), mixed gently, and placed on ice for 30 minutes. The cells were heat shocked at 42˚C for 30 seconds and immediately transferred to ice. 250 µl of Super Optimal Broth with Catabolite repression (S.O.C.) was added and the tube was shaken horizontally for 1 hour at 37˚C. The samples were plated on LB plates with ampicillin and incubated at 37˚C for 12 hours. White colonies were picked and the plasmid DNA was prepared for sequencing at the CORE facility.

The *holin* gene sequence of J25 was set up again in a PCR reaction with the mutagenesis primers TK05 and TK06 (Appendix II) before TOPO cloning into pTOPO 2.1. The mutagenesis primers were designed using PrimerSelect in order to insert specific restriction enzyme sites into the sequence, allowing the sequence to be subcloned into specific sites on the pQE-9 expression vector (Qiagen, Hilden, Germany). DNA sample 9982 with the addition of the TK05 and TK06 mutagenic primers was used to complete PCR clone and TOPO cloning as described above. The resulting DNA was again prepared for sequencing at the CORE facility to ensure correct mutagenesis and introduced mutations.

6. Holin-Gene Expression

Following sequencing, the clone was confirmed to contain the J25 *holin* mutagenesis product in pTOPO 2.1 (10,188) and vector PQE-9 were digested with *Bam*HI and *Hind*III. The *Bam*H1-*Hind*III digested *holin* gene was electrophoresed and gel purified as previously described. The purified holin gene was ligated into the *Bam*H1-*Hind*III cut pQE-9 expression vector using T4 ligase and standard techniques *(167)*. Using the previously described heat shock method, the pQE-9 vector containing the J25 *holin* gene was transformed into Top10 F' competent cells and plated on 2YT + Ampicillin agar plates before incubating for 12 hours at 37˚C. Isolated colonies were picked and added to 2 ml of 2YT with 100 µg/ml Ampicillin and incubated with shaking at 37˚C overnight. This was diluted 1:50 in 2YT and incubated at 37˚C with shaking for 3 hours. Protein expression was induced by the addition of IPTG and grown with vigorous shaking, 0.2 ml samples were taken every hour as further samples and stored on ice. The samples were centrifuged at 14,000 rpm for 10 seconds before the supernatant was discarded and the pellets were re-suspended in 500 µl of PBS in order to wash the bacterial cells. The samples were centrifuged at 14,000 rpm for 10

seconds, again removing the supernatant. The pellets were suspended in 100 µl of PBS and frozen at -80˚C in order to rupture the cells. After freezing, the samples were incubated at 37˚C for 1 minute to thaw completely before adding 1 volume of 2X Laemmli's sample buffer. This mixture was boiled for 4 minutes before being loaded onto a large 12% SDS-PAGE gel and electrophoresed in order to screen for proper protein expression.

B. RESULTS AND DISCUSSION

In this study, DNA fragments from bacteriophage J25, an *E. coli* O145 infecting phage, were successfully cloned and sequenced. Phylogenetic analysis was performed in order to identify potentially related phages, which were then used to identify and locate the *holin* gene. The sonicated DNA fragments were sequenced and used in a BLAST search to find similar sequences. These sequences were used to create a phylogenetic tree (Figure 4.1). Based on the initial nucleotide BLAST search *(7)* of the DNA fragments, phage J25 was most closely related to *Enterobacteria* phage RB68 with over 98% homology. Bacteriophage J25 was placed in a clade with other enteric pathogen infecting bacteriophages such as *Shigella* phage Sf23 and *Escherichia coli* O157 typing phage 6. The BLAST comparison of phage J25 showed 90-98% homology with other phages in the same clade. Lab results have indicated that J25 is an *Escherichia coli* infecting bacteriophage *(119)*, which further explains the close relation that J25 has with both *Shigella* and *Escherichia* infecting bacteriophages.

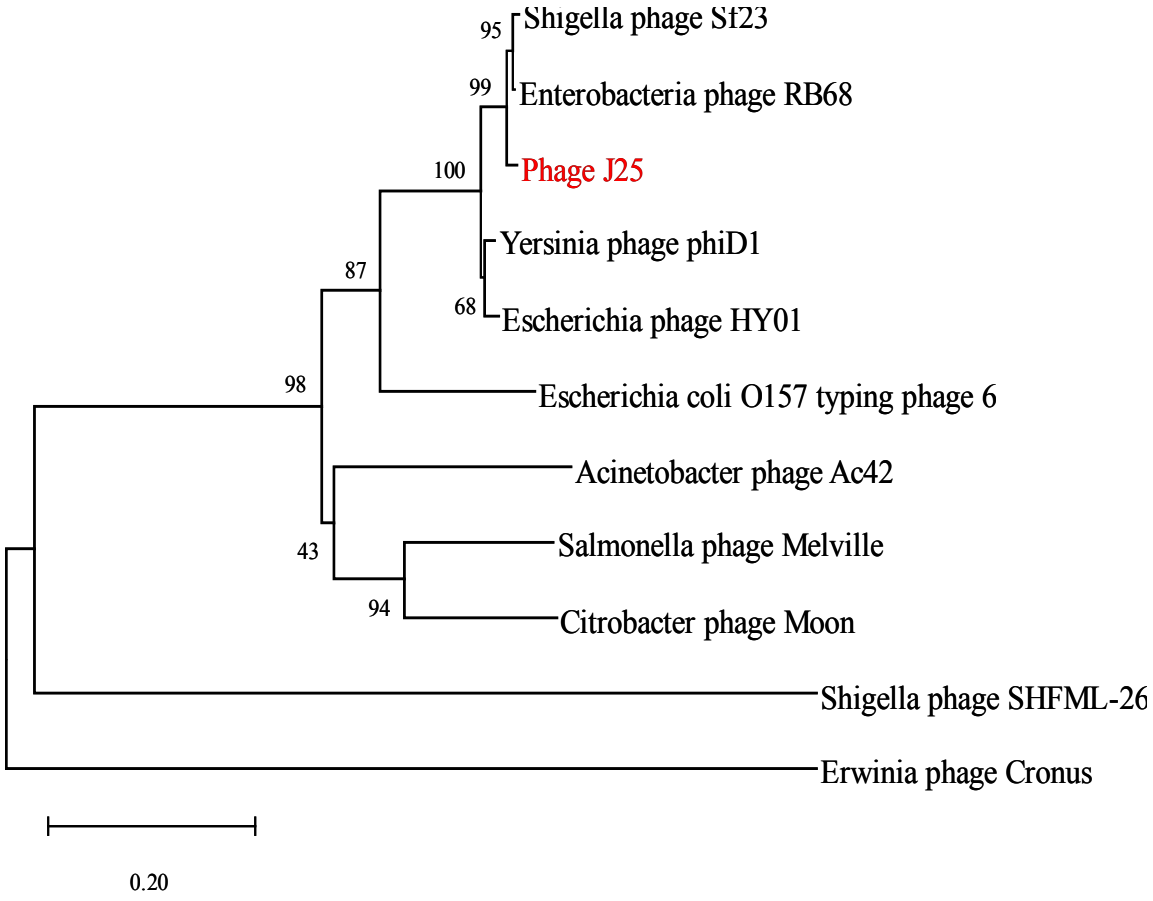

Figure 4.1. The evolutionary history was inferred using the Neighbor-Joining method *(166)*. The optimal tree with the sum of branch length = 2.82726911 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches *(62)*. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method *(102)* and are in the units of the number of base substitutions per site. This analysis involved 11 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 898 positions in the final dataset. Evolutionary analyses were conducted in MEGA 7 *(109)*.

The *holin* gene, once isolated from the J25 genome, was sent to the Oklahoma State University CORE facility for sequencing. The CORE facility sequence results indicated that the *holin* gene had been correctly isolated. The J25 *holin* sequence was used for a nucleotide BLAST search, and the matches were used for phylogenetic analysis. The J25 *holin* gene was aligned with 10 of the most similar phages using the CLUSTAL format *(83)*. The aligned protein sequences were used to create a phylogenetic tree (Figure 4.2). The J25 *holin* gene was placed in a clade with phages infecting bacteria from the genera *Escherichia, Enterobacteria,* and *Shigella.* The J25 *holin* gene showed 95-100% homology with every other similar sequence, indicating that *holin* is a highly conserved gene. The *holin* phylogenetic tree was similar to the J25 tree, in that both were most homologous to *Shigella* phage sf23 and *Enterobacter* phage RB68, further indicating that the J25 phage is closely related to those phages. This is consistent with current knowledge regarding bacteriophages, as holin production and release is an integral step in the bacteriophage lytic cycle.

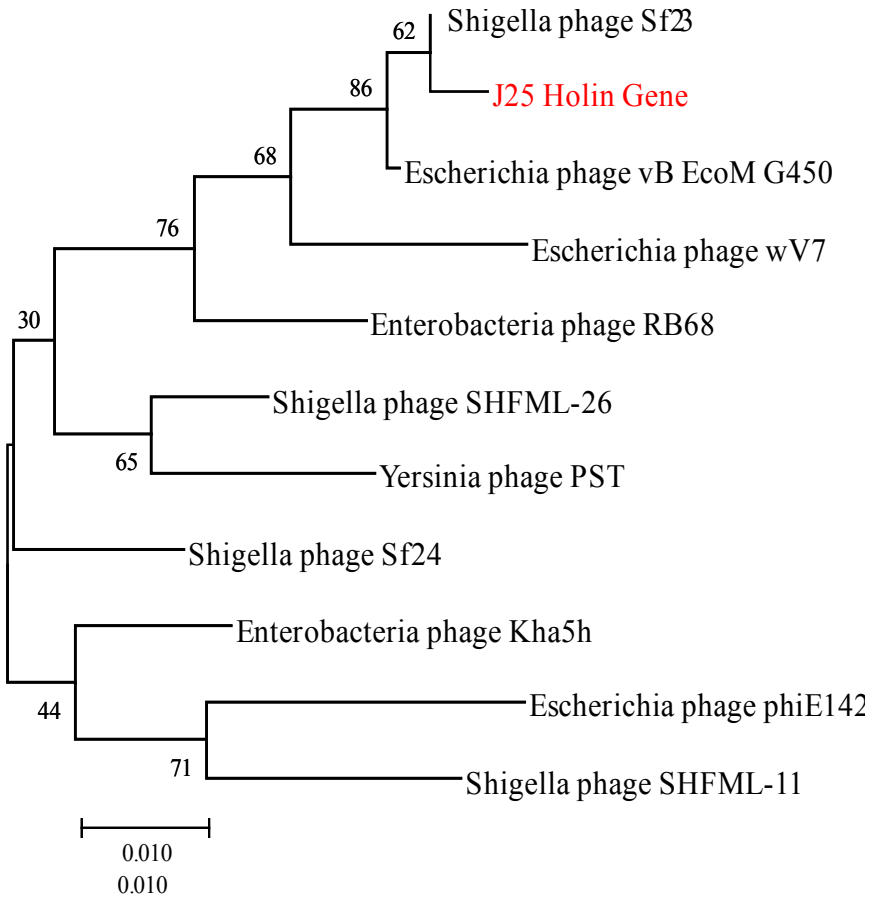

Figure 4.2. The evolutionary history was inferred using the Neighbor-Joining method *(166)*. The optimal tree with the sum of branch length = 0.19142005 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches *(62)*. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method *(209)* and are in the units of the number of amino acid substitutions per site. This analysis involved 11 amino acid sequences. The coding data was translated assuming a Standard genetic code table. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 227 positions in the final dataset. Evolutionary analyses were conducted in MEGA 7 *(109)*.

The *holin* gene was ligated into the pQE-9 expression vector and transformed into Top10 F' competent cells and expressed. The expression of the *holin* gene was verified by loading it onto an SDS-Page gel and electrophoresing. This process showed that a protein of approximately 200 amino acids was expressed from the isolated gene. This size corresponds with the approximate size of *holin*, indicating that *holin* was successfully identified, cloned, sequenced, and expressed from bacteriophage J25.

C. CONCLUSION

In conclusion, this study has shown the relation of J25 to other enteric pathogen infecting bacteriophages, as well as the conserved nature of the *holin* gene, and the potential for holin to be expressed outside of a bacteriophage. Further studies should be conducted to purify the holin protein for further analysis and potential application assays. The conserved nature of the *holin* gene indicates that it can be isolated from a large variety of bacteriophages, which may allow for the identification of bacteriophages that produce holin more efficiently or in larger volumes. The preliminary results from this study may be used to explore the use of holin as a potential intervention method in the food industry.

Chapter V.

SUMMARY

Shiga-toxigenic *E. coli* is a persistent group of foodborne pathogens that has been linked to numerous outbreaks of foodborne illness. They are strong biofilm formers and are often difficult to remove from food processing facilities. Bacteriophages and their by-products could be a natural and effective biocontrol method to minimize the persistence of STEC and the biofilms they form. The first objective of this study was to analyze the efficacy of a bacteriophage depolymerase against STEC biofilms on stainless steel and high-density polyethylene, two common food contact surfaces. The second objective of this study was to isolate, clone, sequence, and express the bacteriophage *holin* gene for further study as a potential STEC intervention.

In the first study, bacteriophage depolymerase was extracted from previously isolated phages, chosen by their level of lytic activity against *E. coli* O45. The depolymerase was used as a biofilm preventative on one set of surfaces, and as a treatment on another. When applied as a preventative, the depolymerase was shown to reduce the *E. coli* population in biofilms by 1.28 to 1.50 logs CFU/cm². As a treatment applied after biofilm formation, the depolymerase was shown to cause a reduction of 0.68 to 1.88 logs CFU/cm². The depolymerase effectiveness was also examined when combined with bacteriophages, where it showed a 1.61 to 1.83 log CFU/cm² reduction as a preventative. This combination also showed a 1.50 to 3.09 logs CFU/cm² reduction

when utilized as a treatment. Both the depolymerase alone and the combination treatments were shown to be more effective as a preventative on the high-density polyethylene surfaces, reducing populations by 1.50 to 1.83 logs CFU/cm² versus the 1.28 to 1.61 logs CFU/cm² reduction on stainless steel. However, as a treatment, the depolymerase and combination were both more effective onstainless steel surfaces, reducing the populations by 1.88 to 3.09 logs CFU/cm² vs the 0.68 to 1.50 logs CFU/cm² on high-density polyethylene.

In the second study, previously isolated bacteriophage 'J25' was chosen due to its high level of lytic activity. The phage DNA was extracted, cloned, and sequenced to locate the gene responsible for the holin protein. A phylogenetic tree was also created using the J25 DNA sequence in order to identify potential relationships with other bacteriophages. Once this gene was located, primers were designed to isolate the gene. The gene was cloned into pTopo 2.1 and sequenced to identify the J25 *holin* sequence. Mutagenesis primers were designed to allow cloning of the gene into an expression vector. The gene was amplified and cloned into pTopo and sequenced for further verification. This clone was digested with *Bam*HI and *Hind*III before being subcloned into the pQE-9 expression vector and the protein was expressed. Protein expression was verified using a 12% SDS page gel. The *holin* gene was used for phylogenetic analysis, which indicated that *holin* is a highly conserved gene amongst bacteriophages.

The findings from this study have shown that bacteriophage by-products are very effective in both the removal and the inhibition of STEC biofilms on food contact surfaces. Future studies are required to analyze the complete composition of the crude depolymerase extract, as well as to purify the depolymerase for further application studies. The highly conserved nature of the *holin*

gene could be utilized to identify other bacteriophages that may produce holin in higher quantities, which could lead to their use as a more effective biocontrol method within the food industry. The expression of the *holin* gene opens the door for its potential use in the food industry as a part of the multi-hurdle intervention strategies to reduce the incidence of foodborne illness outbreaks. These results could lead to the isolation and application of more bacteriophage protein and enzyme by-products in order to create new methods of both pathogenic and spoilage organism control in the food industry.

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APPENDICES

Appendix-I

Scanning Electron Microscopy of Stainless Steel Surface Treated with Phage Depolymerase

The following procedure was used to prepare Stainless Steel coupons for imaging under the scanning electron microscope (SEM). Coupons were cut into 1cm x 1cm squares using an angle grinder, and were then prepared, inoculated, and treated as described in Chapter III. The coupon was inoculated with *E. coli* O45 and allowed to form a biofilm for 48 hours before being treated with the phage depolymerase that was extracted as described in Chapter III.

The treated coupons were transferred to a tissue culture plate (Corning, NY) before being fixed in 1ml 2% glutaraldehyde, prepared in 0.2M Sodium Cacodylate (Fisher Scientific, NJ) solution, for 2 hours. After fixation, the glutaraldehyde was removed and the coupon was washed 3 times, for 15 minutes each, in wash-buffer (0.2M Sodium Cacodylate, 12.3g Sucrose; Fisher Scientific, NJ). The coupons were dehydrated with 50,70,90, and 95% ethanol in sequence for 15 minutes each before 3 treatments in 100% ethanol for 15 minutes each. Ethanol was carefully removed using vacuum suction, and the sample was washed 2 times, for five minutes each, in Hexamethyldisilazane (HMDS; Sigma Aldrich, MO). The coupons were dried overnight before mounting. After drying, the coupons were mounted onto the stub with double-sided tape before being coated with Gold/Palladium (Au-Pd) using a sputter coating system. The coupons were visualized under the scanning electron microscope (Quanta 600 FEG SEM, FEI) at the

Oklahoma State University Microscopy lab located at the Oklahoma Technology and Research Park in Stillwater, OK.

Appendix-II

Primer	Sequence
RB68-01	CTT AAA AGG AGG GTC TAT G
RB68-02	TCA AGA AGA TAA GAA AAC CCT CAT
TK05	TTG GAT CCC GAA TTC AAC GAG G
TK06	ACA AGC TTA GGA GGG TCT ATG GC

VITA

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