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CHARACTERIZATION OF LYTIC SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* BACTERIOPHAGE ISOLATED FROM BOVINE FECES

By

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CHARACTERIZATION OF LYTIC SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* BACTERIOPHAGE ISOLATED FROM BOVINE FECES

Thesis Abstract – Idaho State University (2017)

In the last few decades Shiga toxin-producing *Eschericha coli* have become a major cause of food-borne infections. These strains of *E. coli* can result in the life-threatening illnesses hemorrhagic colitis and hemolytic uremic syndrome. Infections from these strains commonly arise from ingesting contaminated meat, fresh produce and unpasteurized beverages. Current decontamination treatments must often be combined to achieve adequate *E. coli* reduction and can adversely affect food quality. Research into more effective and targeted decontamination methods has turned to bacteriophage, viruses that infect and kill bacteria. Bacteriophage are highly specific and considered safe for human consumption. This study examined eight different bacteriophage isolated from cow manure from a cattle ranch and dairy farm. These bacteriophage were characterized by examining their host range specificity against different strains of Shiga toxin-producing *E. coli*, observing their lytic characteristics in broth and determining the type of nucleic acids composing their genomes.

CHAPTER I

Pathogenic *Escherichia coli* Groups

In 1947, Kauffmann suggested typing of the "Coli group" using the O, H and K antigens. The term "Coli group" encompassed non-slimy colonies of Gram-negative, non-spore forming rods that are citrate negative, VP negative, MR positive, and do not liquefy gelatin [75]. The O antigen is a lipopolysaccharide endotoxin, while the H antigens are flagellar antigens and the K antigens are made up of the L, A and B antigens that were described as envelope antigens [34] [75].

Myron M. Levine places diarrheagenic *E. coli* in four major categories: enterotoxigenic (ETEC), enteroinvasive (EIEC), enteropathogenic (EPEC) and enterohemorrhagic (EHEC). A fifth category of enteroadherent (EAEC) has been suggested, but is less well defined [89].

EPEC strains typically lack the production of a heat-stable toxin referred to as ST, and heat-labile toxin referred to as LT, as well as the ability to invade epithelial cells. The EPEC category consists of two classes. Strains that fall into class I exhibit localized adherence to Hep-2 cells due to EPEC adherence factor (EAF). Strains that fall into class II exhibit either no adherence or diffuse adherence to Hep-2 cells due to a lack of EAF. Strains that fall under the EPEC category may often be referred to as attaching and effacing *E. coli* (AEEC). The classic EPEC strains are the O55, O111 and O127 serogroups which fall under Class I. Additionally, O serogroups associated with EPEC class I include: O26, O86, O119, O125, O128ab and O142. O serogroups associated with EPEC class II include: O18, O44, O112 and O114 [89].

In developing countries, ETEC strains are a major cause of infant diarrhea, as well as traveler's diarrhea. Infections by these strains are considered rare in developed countries. Infections are generally acquired by ingestion of contaminated food or water. Subsequently, the bacteria colonize the small intestine and produce heat-labile enterotoxin (LT) or heat-stabile enterotoxin (ST). O serogroups associated with ETEC are: O6, O8, O15, O20, O25, O27, O63, O78, O80, O85, O115, O128ac, O139, O148, O153, O159, and O167 [89].

Strains of *E. coli* that cause an invasive, dysenteric form of diarrhea form the EIEC category. These strains closely resemble *Shigella* by invading epithelial cells and subsequently causing cell death. Like *Shigella*, EIEC strains are non-motile and there are many cross-reactions between the O antigens of EIEC strains with *Shigella*. Similar to *Shigella*, the invasiveness of EIEC strains is the result of outer membrane proteins that are encoded on large plasmids. O serogroups associated with EIEC are: O28ac, O29, O124, O136, O143, O144, O152, O164 and O167 [89].

EHEC strains cause a unique diarrheal disease that distinguishes it from *Shigella*, EIEC or classic dysentery. Patients are typically afebrile, with bloody, copious diarrhea. Notably, fecal leukocytes are absent. Additionally, EHEC strains have been incriminated as a cause of hemolytic uremic syndrome (HUS). These strains produce endotoxins, encoded on phage, against Vero cells and HeLa cells. Shiga-like toxin I is identical to the cytotoxin produced by *S. dysenteriae* type I. Shiga-like toxin II is antigenically distinct from Shiga toxin. Similar to EPEC, EHEC strains attach and efface enterocytes, destroying the microvilli. However, in piglets, EPEC infections involve the entire

intestine whereas EHEC infections only involve the cecum and colon. O serogroups typically associated with EHEC are O157, O26 and O111 [89].

EAEC strains of *E.* coli are EAF negative, but capable of adhering to Hep-2 cells. They do not produce LT, ST or elevated levels of SLT, nor are they capable of invading epithelial cells. These strains cause mild diarrhea without blood or fecal leukocytes. Currently the O serogroups of EAEC are not defined [89].

In 1977, Konowalchuk *et al.*, investigated toxins produced by *E. coli* strains. *E. coli* strains were capable of producing three different toxins, a heat-stable toxin referred to as ST, and heat-labile toxin referred to as LT and a toxin against Vero cells referred to by Konowalchuk *et al.* as Verotoxin or VT. Vero cells were a line of kidney cells that came from African Green Monkeys [74]. Strains that produced the LT generally did not produce the VT and no tested strain that produced ST produced VT [81].

Konowalchuk *et al.* found a significant difference in the cellular response between VT and LT in Vero cells. Vero cells affected by LT were enlarged, thick-walled, and refractile with filamentous tendrils. The Vero cells affected by VT appeared shriveled and round, with many free-floating cells. The effect of VT on cells increased with time, with 50% affected cells within 24 hr and maximum titers after four days. Within three days, cells affected by LT appeared normal, whereas there was no recovery for cells affected by VT [81].

LT was known to cause diarrhea through the stimulation of cyclic adenosine 5'-monophosphate. Unlike LT, VT did not stimulate cyclic adenosine 5'-monophosphate, but was not ruled out from contributing to diarrhea. Konowalchuk *et al.* suggested the cytotoxic effect of VT on intestinal cells may result in fluid accumulation within the

intestines and may contribute to diarrhea in human infants [81]. Cantey & Blake demonstrated in 1977 that *E. coli* can produce diarrhea in rabbits even when it is unable to invade the mucosa or synthesize enterotoxins [18].

In 1980, Scotland *et al.* examined strains of enteropathogenic serogroups (EPEC) for production of VT. Of the 253 EPEC strains tested, 25 strains produced VT whereas none produced LT or ST. Of the 25 VT positive strains, 23 belonged to the serogroup O26 and the other two belonged to the serogroup O128. The VT positive strains from the serogroup O26 either had the H11 (20 strains) or H^- (3 strains) antigens. The strain that was studied by Konowalchuck *et al.* that was VT positive was H19, an O26:H11 strain. The VT positive strains from the serogroup O128 both had the H2 antigen [117].

In 1983, Smith *et al.* tested 519 different strains of *E. coli* for the production of toxins acting on Vero cells. 68 of the tested strains were positive for VT. Most of the VT positive strains were from weaned pigs and belonged to the serogroups O141:K85, 88, O141:K85, O138:K81 and O139:K82. Six of the VT positive strains came from human babies and included the strains O26 and O128. They found that the VTs from the human strains of *E. coli* were antigenically different from the VTs from the pig strains [122].

Smith *et al.* demonstrated the toxic effects of VT by transferring the VT genes from the human *E. coli* strains into *E. coli* K12 to be expressed. Ligated segments of rabbit intestines were then inoculated with the culture extracts and found to cause fluid accumulation. This effect was not seen with the extract from the parent K12 strain. When the extracts were administered intravenously in mice, they were found to be lethal within 2 to 6 days following administration [122].

The first suggestion that HUS may be linked to *E. coli* was a 1968 article from South Africa. The authors, Kibel and Barnard, suggested the disease may be caused by a strain of *E. coli*, mutated by a bacteriophage [76]. In 1986, Strockbine *et al.* investigated two different phage, 993J and 993W, from *E. coli* O157:H7 strain 933. The two phage produced toxins that were antigenically distinct, despite the phage having homologous nucleotide sequences. They found that the 933J toxin was neutralized by the monoclonal antibody against the B subunit of Shiga-like toxin and by anti-Shiga toxin. However, it was not neutralized by anti-933W toxin. In contrast the 933W toxin was neutralized by the anti-933W toxin, but not by the monoclonal antibody against the B subunit of Shiga-like toxin or by anti-Shiga toxin. Because the two toxins were not cross-neutralized, it was determined that they were antigenically distinct [134].

Strockbine *et al.* proposed that 933J toxin be named Shiga-like toxin I (SLT-I) and 933W toxin be named Shiga-like toxin II (SLT-II). The previously identified toxins verotoxin 1 (VT1) and verotoxin 2 (VT2) were alternate names for SLT-I (VT1) and SLT-II (VT2). Given the similar cytotoxicity of the two toxins to Shiga toxin, the name Shiga-like toxin was felt to be the more descriptive term for the previously identified verotoxins [134]. While many researchers have adopted the names SLT-I and SLT-II, others continue to refer to the toxins as VT1 and VT2 with no clear consensus.

In 1985, Karmali *et al.* established a link between HUS and Verotoxin-producing *E. coli* (VTEC) infection. HUS is characterized by three features: acute renal failure, thrombocytopenia and microangiopathic hemolytic anemia. In idiopathic HUS, these features are believed to be a result of endothelial damage and local intravascular coagulation. Karmali *et al.* studied 40 pediatric patients with idiopathic HUS. All 40

experienced diarrhea, with 34 experiencing bloody stools, 24 reported abdominal pain, 22 had mild vomiting, 18 had fever and 8 had upper-respiratory-tract symptoms. Abdominal pain could not be assessed in children younger than 2 years of age. Diagnosis of HUS occurred within two to 14 days of onset of symptoms [74].

Of the 40 patients studied, VTEC and/or free VT were detected in 24 patients, whereas none of the 40 control patients tested positive for either VTEC or free VT. All patients that tested positive for VTEC also tested positive for free VT, with the exception of three patients that had insufficient stool samples. 12 patients were culture negative for VTEC, but tested positive for free VT. The free VT titers were typically higher in stool samples that were received during the early stages of illness but had no correlation to the severity of the illness [74].

From the study, Karmali *et al.* isolated 12 different strains of VTEC. One isolate of O26:K60:H11, one isolate of O111:K58:H8, two isolates of O111:K58:H^-, two isolates of O113:K75:H21, one isolate of O121:H19, one isolate of O145:H^-, three isolates of O157:H7 and one isolate of O(rough):H-. All 12 isolates were negative for both the LT and ST. From this study, 60% of the patients with idiopathic HUS showed evidence of an infection with VTEC. While the strains varied significantly, the production of free VT was a common factor suggesting a causal relationship [74].

In 1986, Marques *et al.* studied 418 different strains of *E. coli* and found that all strains of *E. coli*, regardless of their classification make cell-associated cytotoxins. The level of cytotoxin production varied from low, moderate and high. All strains of O157:H7 produced moderate or high levels of cytotoxins. 48 out of 49 strains of *E. coli* that produced elevated levels of cytotoxins were from cases of diarrhea, hemorrhagic colitis

(HC) or HUS. The strains that were low-level cytotoxin producers were isolated from humans without reported disease. These findings suggested that the level of cytotoxin production plays a role in the development of disease [97].

Johnson and Lior investigated 11 human isolates of *E. coli* O113:H21 in 1987 for VT production. Of the 11 strains tested, 6 were found to produce VT. Of the 11 strains tested, none of them produced ST or LT enterotoxins. Antitoxin against O157 VT only partially neutralized the verotoxin from O113 strains, indicating expression of both VT1 and VT2 [67].

In 1996 Calderwood *et al.* recommended changing the SLT/VT nomenclature to Stx1/Stx2. Shiga toxin, SLT-I and SLT-II all share DNA sequence homology and operon structure, AB_5 subunit stoichiometry, identical enzymatic activity of the A subunit, Gb3 membrane receptor, and biological cytotoxicity. With these shared properties, Calderwood *et al.* argued that the toxins all belonged to the same family, and should therefore share the same nomenclature. Shiga toxin nomenclature had already been previously established with *stx* as the gene designation and Stx as the protein designation. They proposed that the gene designation for SLT-I/VT1 should be stx_1 and the protein designation should be Stx1. Similarly, they proposed that the gene designation for SLT-II/VT2 should be stx_2 and the protein designation should be Stx2 [16].

The recommendation by Calderwood *et al.* was met with resistance. That same year, Karmali *et al.* wrote that the terms Verotoxin and Shiga-like toxin had been widely used in the literature for over two decades. They argued that due to precedence, VT should be used to describe these toxins in *E. coli*. However, they also argued that the extensive usage of SLT in the literature should make it equally interchangeable.

Confusion surrounding these different names had already been cleared up and they saw no reason to change the name of the toxins yet again. Karmali *et al.* argued the name change would only exacerbate confusion, rather than alleviate it [73]. While most researchers have adopted the Stx nomenclature, including the CDC, many still prefer the VT nomenclature [71] [95].

The Shiga toxins, Stx/Stx1 and Stx2, are approximately 70 kDa AB5 protein toxins that bind to Globotriaosylceramide (Gb3), a glycosphingolipid membrane receptor [4] [100]. In sensitive eukaryotic cells, they inhibit protein synthesis by targeting the ribosome. The pentamer of B subunits bind to the Gb3 receptor on cell membranes. Receptor mediated endocytosis leads to internalization of the toxins and transport to the Golgi apparatus. The toxins then travel to the endoplasmic reticulum, where the N-glycosidase activity of the A subunit removes an adenine residue from the 28S rRNA of the 60S ribosome subunit [95] [100]. This prevents binding of aminoacyl-tRNA to ribosomes resulting in inhibition of protein synthesis, eventually leading to cell death [95]. In addition to inhibiting protein synthesis, the damaged ribosome induces "ribotoxic stress response" and an unfolded protein response due to stress on the ER [65] [87]. These responses result in inflammation and apoptosis of sensitive cells [100]. Known target cells for Stx are endothelial cells in the kidneys, brain, and intestinal submucosa, however, any cell with sufficient Gb3 receptors may be a target cell [95]. Stx can also bind to the Pk antigen on red blood cells. Arvidsson *et al.* demonstrated that Stx2 induces hemolysis by activating complement on RBC through the terminal complement pathway [4].

In vitro, both Stx1 and Stx2 show equivalent enzymatic activites/ng protein, which does not match observed cellular effects. Stx1 has been found to be about 10-fold more cytotoxic to Vero cells than Stx2; however, in mice, Stx1 is about 100-400-fold less lethal to mice than Stx2. Ostroff *et al.* showed that clinical isolates of O157:H7 carrying only stx_2 were 6.8 times more likely to cause severe disease in patients than strains carrying stx_1, or both stx_1 and stx_2 [106]. These differences may arise from the type of cells being targeted [100]. Louise and Obrig obtained renal microvascular endothelial cells from human glomeruli and found these cells to be about 100-fold more sensitive to Stx2 than Stx1 [92].

The A subunit of Stx/Stx1a is 293 amino acids in length, whereas the A subunit of Stx2a has an additional 4 amino acids at the C terminus. The B subunit of Stx/Stx1a is 69 amino acids in length while the B subunit of Stx2a is 71 amino acids in length [100].

There have been no reported variants of Stx produced by *Shigella*, however, there are three subtypes of Stx1; Stx1a, Stx1c and Stx1d. Stx1a is the most commonly encountered subtype in disease, with Stx1c and Stx1d only associated with mild disease. There are 7 different subtypes of Stx2; Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f and Stx2g. The first four subtypes, Stx2a-d have all been associated with human cases of STEC, while Stx2e-f have only been associated with animal strains of Shiga toxin-producing *E. coli* (STEC). Stx2a is the most commonly encountered subtype in disease, however, Stx2c and Stx2d have also been associated with disease. Both Stx2c and Stx2d have reduced cytotoxicity for Vero cells than Stx2a. When Stx2c is injection into mice it is less toxic than Stx2a while Stx2d is shows similar toxicity as Stx2a. Stx2b is only

associated with mild disease, while Stx2e is the only animal associated subtype that has been implicated in animal disease causing edema disease of swine [100].

The STEC center divides *E. coli* strains that carry Stx genes into four classes; EHEC 1, EHEC 2, STEC 1 and STEC 2. The class EHEC 1 includes the serotypes O157:H7, O157:H- and the non-shigatoxin producing serotype O55:H7. EHEC 2 includes the serotypes O111:H8, O111:H11, O111:H-, and O26:H11 [128]. Strains from both EHEC 1 and EHEC 2 have been recovered from patients with HC or HUS except for the O55:H7 serogroup. EHEC 1 and EHEC 2 were determined to be genetically divergent by Whittam and McGraw[150]. STEC 1 includes the serotypes O113:H21, O104:H21, O146:H21, O103:H6, O15:H27 OX3:H21 and O91:H21. Theses strains are often isolated from both human and bovine cases of disease. The last class, STEC 2 includes O103:H2, O103:H6 and O45:H2 [128]. Because the strains in STEC 1 and STEC 2 are not usually associated with intestinal disorders, they are referred to as "Shigatoxigenic *E. coli*" or STEC [95].

More precise grouping, based on genetic relatedness is through multilocus sequence typing (MLST). MLST can be used to determine the genetic relatedness of bacterial strains by comparing housekeeping genes. These genes usually have between 10 to 36 different alleles, and each unique combination of alleles is referred to as a sequence type (ST) [94]. There are currently three different MLST schemes used for pathogenic *E. coli*; st2, st7 and st15. The most commonly performed MLST schemes is st7, which looks for the loci: *aspC, clpX, fadD, icdA, lysP, mdh, and uidA*. Each of the four classes of Shiga toxin-producing *E. coli* have defined STs associated with them [39].

Class EHEC 1 is in clonal group 11 for pathogenic *E. coli*. The reference strain for EHEC-1 in EcMLST is isolate Sakai with accession number TW08264. Sakai was isolated in Japan in 1996. Its serogroup is O157:H7 and is ST-66 with st7. MLST sequence types using st7 that correspond to EHEC 1 are: 57, 63, 64, 65, 66, 67, 69, 71, 73, 74, 75, 76, 77, 237, 257, 275, 350, 355, 556, 567, 680, 809, 819, 821 and 822 [38].

Genetically the *E. coli* strains O157:H7, O157:H- and O55:H7 are very closely related. This has been determined by comparing the EAE genes in numerous strains of pathogenic *E. coli*. Due to the close genetic relationship between O55:H7 and O157:H7, Whittam *et al.* hypothesized that an O55:H7 strain that had already acquired the EAE gene acquired shigatoxin genes through horizontal gene transfer to eventually give rise to the O157:H7 strain [151].

Two examples of EHEC 1 strains that have been implicated in disease are EDL933 and 8257. Both strains are from the O157:H7 serogroup, producing both stx1 and stx2. *E. coli* strain EDL933 was isolated in 1982 from ground beef in Michigan [86]. EDL-933 has accession number TW02302 in EcMLST and ST-66 with st7 [131]. *E. coli* strain 8257 was a clinical isolate obtained from the Idaho Department of Health from a human infection in 2004 [86]. There is no st7 information for strain 8257 because it is not included in the EcMLST database.

Of these two strains, EDL933 has been the most thoroughly investigated, and is considered a reference strain for the O157:H7 serotype [84]. In 2001, Perna *et al.* sequenced and published the full genome of EDL933. EDL933 has 18 different prophages and prophage-like elements encoded in its genome. Prophage CP-933V,

referred to as phage 933J by Strockbine *et al.*, encodes Stx1 while prophage BP-933W encodes Stx2 [108].

Class EHEC 2 is in clonal group 14 for pathogenic *E. coli*. The references strain for EHEC-2 in EcMLST is DEC8B, with accession number TW00970. DEC8B was isolated in Idaho in 1986 from a patient diagnosed with HC. Its serogroup is O111:H8 and is ST-106 with st7. MLST sequence types using st7 that correspond to EHEC 2 are: -36, 98, 104, 106, 107, 108, 109, 111, 115, 310, 432, 532, 533, 561, and 681 [38].

Class STEC 1 is in clonal group 34 for pathogenic *E. coli.* The reference strain for STEC-1 is B2F1, with accession number TW01393. B2F1 was isolated in Canada in 1985 from a patient diagnosed with HUS. Its serogroup is O91:H21 and is ST-89 with st7. MLST sequence types using st7 that correspond to STEC 1 are: 89, 90, 610 and 693 [38].

Class STEC 2 is in clonal group 30 for pathogenic *E. coli.* The reference strain for STEC-2 is CL-3, with accession number TW01391. CL-3 was isolated in Canada in 1980 from a patient diagnosed with HUS. Its serogroup is O113:H21 and is ST-231 with st7. MLST sequence types using st7 that correspond to STEC 2 are: -2, 134, 223, 230, 231, 234, 268, 314, 388, 392, 406, 408, 413, 417, 422, 424, 425, 429, 435, 436, 439, 455, 483, 488, 496, 498, 508, 510, 584, 637, 648, 657, 719, 721, 742, 749, 769, 777, 817, and 820 [38].

Three examples of STEC 2 strains that have been clinically isolated are DEC16A, EK33 and DA-33. Strain DEC16A, also referred to as TW02918, was isolated from an adult in Bangkok, Thailand, sometime in the 1980s, who experienced diarrhea for less than 48 hours. The strain is positive for stx_2 but negative for stx_1 [130]. The serotype of

the strain is O113:H21, placing it in the STEC 1 group according the STEC Center. However, multilocus secquence typing (MLST) with st7 shows that it is ST-233, placing it in the STEC 2 clonal group [43]. DEC16A has no defined ST on EcMLST and may be the reason it is listed as a STEC-1 strain by the STEC Center [130].

Strain EK33 was islolated from a female in Washington state who experienced diarrhea. The serotype of the strain is O103:H2, placing it in the STEC 2 group according the STEC Center. The strain is positive for *stx*$_1$ but negative for *stx*$_2$ [132]. EK33 has accession number TW08641 and is ST-119 with st7, which places it in clonal group 17 [38] [132]. Clonal group 17 makes up class EPEC 2 according to EcMLST [38].

Strain DA-33 was islolated from a 4 year-old female in Ohio state in 1998. Clinical symptoms of the patient are unknown. The serotype of the strain is O103:NM, placing it in the STEC 2 group according the STEC Center based on serotype. The strain is positive for *stx*$_1$, but negative for *stx*$_2$. DA-33 has accession number TW07959 and has no defined ST [129].

Epidemiology

In 1983, Riley *et al.*, investigated two outbreaks of gastrointestinal illness in Oregon and Michigan. The illness was characterized by severe crampy abdominal pain, watery diarrhea followed by grossly bloody diarrhea and little or no fever. This illness was called hemorrhagic colitis (HC) and was associated with undercooked beef from a meat lot in Michigan. Stool cultures from these patients isolated *Escherihia coli* O157:H7, a rare strain of *E. coli* [115]. In 1985, Karmali *et al.*, reported an association between cytotoxin producing *E. coli* and hemolytic uremic syndrome, linking the serotypes O26, O111, O113, O121, O145 and O157:H7 [74].

Prior to 1982, O157:H7 strains were extremely rare in the United States, United Kingdom and Canada. Only one O157:H7 strain out of 3,000 *E. coli* strains was clinically isolated between 1973 and 1983 in the US. Between 1978 and 1982 only one out of 15,000 *E. coli* strains was typed O157:H7 in the UK. Canada found six O157:H7 strains out of 2,000 *E. coli* strains isolated from patients between 1978 and 1982. In 1998, the CDC estimated there to be more than 20,000 O157:H7 infections and approximately 250 deaths each year, with a much lower incidence of EHEC in developing countries [102].

Animal reservoirs for EHEC commonly include cattle, sheep, goats, pigs, cats, dogs, chickens and gulls. Cattle are the greatest source of human infection, due to high rates of colonization. While the colonization rate in bovine herds is typically between 10-25%, they have been found as high as 60%. Most animals are asymptomatic, possibly experiencing brief diarrhea in young animals. Due to cross-contamination of foods in grocery stores, EHEC has also been isolated from raw fish and shellfish. Most cases of EHEC infection are from ingesting contaminated foods, but can also be transmitted from person to person and through contaminated water. EHEC has a relatively low infectious dose, requiring only 100 to 200 organisms to cause infections. This dose is similar to *Shigella* infections, and is consistent with waterborne and person-to-person transmission [102].

The largest outbreak in North America occurred between December 1992 and February 1993 and was linked to hamburgers from a fast-food restaurant chain. A total of 732 individuals were infected in Washington, Idaho, Nevada and California. Of the 732 affected, 195 were hospitalized and four individuals died [11]. Between December 1992 and January 1993, an outbreak in Las Vegas, Nevada infected 58 people, resulting in

three cases of HUS. The Nevada outbreak was only recognized in retrospect, after the reported outbreak in Washington state. In contrast to Washington state, *E. coli* O157:H7 was not routinely cultured for by any local Las Vegas laboratories, even when the stools were bloody. The public announcement came on January 18, 1993, which alerted the public to the outbreak. Affected patients contacted their health district after a press release on January 22, 1993. By this time, onset of symptoms for most patients had occurred over two weeks prior. Due to the rapid shedding of *E. coli* O157:H7, only one affected Las Vegas patient's stool recovered the pathogen [25].

This case not only raised public awareness of pathogenic *E. coli* in undercooked beef, it also raised concerns about routine testing and mandatory reporting. While mandatory reporting of *E. coli* O157:H7 was required in Nevada, no laboratories cultured for it. In response to the outbreak, laboratory protocols were changed to screen for *E. coli* O157:H7 with sorbitol-MacConkey agar. Additionally, at the time of the outbreak, Nevada did not required reporting of cases of HUS. Mandatory reporting of HUS may have caught the outbreak earlier [25].

In 1996, Japan experienced the largest recorded outbreak of *E. coli* O157:H7 involving approximately 10,000 people with 14 separate clusters, resulting in 11 deaths. In one cluster of the outbreak, over 6,000 school children were involved. Uncooked white radish sprouts from the same farm were linked to the illness [148].

Fresh sprouts have caused several EHEC outbreaks in the United States. In Michigan and Virginia, two outbreaks of *E. coli* O157:H7 were linked to alfalfa sprouts in 1997. A total of 60 people were infected in Michigan and 48 people were infected in Virginia. In Michigan, 25 people were hospitalized, with two developing HUS. In

Virginia, 11 people were hospitalized and there were no cases of HUS. In these two cases, the seeds came from the same distributor, rather than the same farm. It was believed that the seeds were the source of contamination [23].

In 2006, 199 people were infected in 26 different states with a strain of *E. coli* O157:H7 that was linked to fresh spinach. Among the ill, 102 were hospitalized and 31 developed HUS. There were three deaths associated with the outbreak, a 2-year-old child from Idaho and two elderly women from Nebraska and Wisconsin [19].

In addition to animal meat, O157:H7 infections have been linked to other food items such as mayonnaise, unpasteurized apple juice, fermented hard salami and raw vegetables. *E. coli* O157:H7 is capable of growth at low pH levels and can survive for days at pH 3.4 [102]. Unpasteurized apple juice was linked to an outbreak of *E. coli* O157:H7 in Washington State, during October 1996. This outbreak infected 70 people, resulting in 25 hospitalization, 14 cases of HUS and one death. Previous apple juice outbreaks had been linked to unwashed apples that were picked up from the ground. This led to the CDC recommending apples be washed and brushed before pressing. While processing plants requested only hand-picked apples, there was no way to enforce this requirement. After investing the outbreak, no source of contamination could be found within the plant, leading investigators to suspect the source of contamination was the apples coming into the plant. Like cattle, deer can harbor *E. coli* O157:H7, and often enter apple orchards where they defecate. Apples that fall on the ground can then become contaminated with deer feces [27].

Ingestion of contaminated water, including recreational water, well water and even municipal water has led to outbreaks. An improperly repaired water system in

Missouri allowed unchlorinated water to be distributed through a municipal water system causing four deaths and 243 infections with *E. coli* O157 between December 1989 and January 1990. Freezing temperatures caused two water mains to break. During repairs, water was drawn from a second well to accommodate water demands. It is thought that the less used well may have had locations of unchlorinated static water which served as a source of contamination [135].

One of the largest outbreaks of *E. coli* O157:H7 associated with lake water occurred in Clark Country, Washington in August 1999. A total of 37 patients were infected with *E. coli* O157:H7 in August 1999 with all patients reporting diarrhea. Of the 37 patients, 29 had visited Battle Ground Lake in Clark County, Washington and were identified as primary cases. The other 8 patients all had close contact with one of the 29 patients that had visited the lake and were identified as secondary cases. Of the 37 infected patients, *E. coli* O157:H7 was isolated from 35 patients. Prior to the outbreak, the lake water was not routinely monitored. Testing of the lake water did not find elevated levels of fecal coliforms, however, a strain of *E. coli* O157:H7 was isolated. All 35 patient strains had identical DNA fingerprints that matched the environmental isolate. Since the outbreak, the EPA has established guidelines for monitoring recreational fresh water for *E. coli* and enterococci [15].

Every year, the CDC investigates outbreaks of Shiga toxin-producing *E. coli*. In 2016 alone, there were three different outbreaks. The first outbreak began in December 2015 and continued through September 2016. The multistate outbreak infected 63 people and involved *E. coli* O121 and O26 from contaminated flour. While no deaths were reported, 17 people were hospitalized and one patient developed HUS. Due to the long

shelf-life of flour, more infections are likely from this outbreak, despite a recall [20]. The second outbreak occurred between January and February 2016, where 11 people in Minnesota and Wisconsin became ill due to alfalfa sprouts contaminated with a strain of *E. coli* O157. There were no deaths or cases of HUS, but two people were hospitalized [21]. The third outbreak occurred in September, 2016. A slaughterhouse recalled beef, veal and bison products after 11 people were infected with a strain of *E. coli* O157:H7 in five different states. While no deaths were reported, 7 people were hospitalized and one patient developed HUS [22].

While most deaths have been associated with *E. coli* O157:H7, other non-O157:H7 STEC strains can also be fatal. In April 1993, a 15-month-old male died due to an infection of *E. coli* O26:H11. The infant was admitted to a hospital with symptoms of HUS, and died only 50 hours after admission. It is unclear if treatment with the antibiotics gentamicin and ampicillin exacerbated his condition. The source of the infection was not determined, however, the case did draw attention to the potential severity of non-O157 *E. coli* strains [114].

Risks of foodborne bacterial infections can be minimized by proper food preparation and storage both at home and in commercial settings. Ground meat must be cooked thoroughly, hands and cooking surfaces that handle raw meat must be cleaned. The contamination of meats usually occurs at the slaughter-houses and meat-processing plants. However, cases have also occurred due to cross-contamination at grocery stores. This is a risk that often occurs in the home as well. The areas at greatest risk for spreading contamination are meat grinders and packing areas. Great care needs to be

taken in order to disinfect all working surfaces. Warm summer months are particularly prone to infections, often due to poor refrigeration camping and picnicking outdoors [91].

Decontamination

CATTLE

Studies with non-pathogenic *E. coli* O157:H7 have shown that it can survive in the environment for months. Ma *et al.* in 2011 showed that the survival of *E. coli* O157:H7 does not appear to be dependent on pathogenic genes (*stx*$_1$, *stx*$_2$, and *Eae*), however, soil type does affect survivability. *E. coli* O157:H7 EDL933 had the longest survival in silty clay soil (110 days) and the shortest survival in loamy sand soil (32 days). It took 110 days for the *E. coli* concentrations to decrease to 100 cfu/g soil in silty clay soil and only 32 days in loamy sand soil [93].

One strategy to reduce food and water contamination with pathogenic *E. coli* is to target the animal reservoirs pre-slaughter. Approaches include the administration of probiotics, antibiotics, vaccines and even diet modification [17]. Cray & Moon studied fecal shedding of *E. coli* O157:H7 by inoculating both calves and adult cattle with *E. coli* O157:H7 strain 3081. They found that while calves shed *E. coli* for longer and in greater numbers than adults, individuals from each group continued to shed for months [28]. Similarly, Jonsson *et al.* found that calves inoculated with *E. coli* O157:H7 shed the organism for approximately two weeks, however, one calf continued to shed the organism for 45 days [69].

Probiotics are harmless or beneficial bacteria which either compete with or are found to be antagonistic to pathogenic bacteria. In competitive exclusion, nonpathogenic

bacteria are introduced to the intestinal tract which limit populations of pathogenic bacteria. This can occur through competition for limited nutrients, competition for binding sites within the gut epithelium, or through the production of toxic compounds [17].

Probiotic bacterial strains tested in cattle include organisms within the *Bifidobacteria* group, *Paenibacillus polymyxa,* species in the genus *Lactobacillus* and even non-pathogenic strains of *E. coli*. Zhao *et al.* studied non-pathogenic strains of *E. coli* that produced colicins, which are inhibitory to diarrheagenic *E. coli*. When these cultures were administered to adult cattle, they displaced *E. coli* O157:H7 from the rumen of the animals [158]. Stanford *et al.* studied a mixture of *Lactobacillus casei, Lactobacillus lactis,* and *Paenibacillus polymyxa* in cattle feed and found a reduction in shed *E. coli* O157:H7 in feces. *P. polymyxa* produces several broad-spectrum lipopeptide antibiotics that target several Gram-negative organisms such as *E. coli*. *L. lactis* inhibits the growth of Gram-negative organisms through the production of lactic and acetic acids [127]. Tahamtan *et al.* studied the inhibitory effects of four different strains of *Bifidobacteria* on *E. coli* O157:H7. They found that when the *Bifidobacteria* were applied to Vero cells, they were able to neutralize Stx2 cytotoxicity and reduce the adhesion of *E. coli* O157:H7 to the Vero cells [136].

Commercial implementation of probiotics has been met with some difficulty, mostly with cost and effectiveness in "real-world" settings. Cost for long-term administration can be a deterrent for many farms, leading research to focus on treatments that are only administered during the weeks leading up to slaughter [7] [127]. The use of antibiotics to promote growth can interfere with the effectiveness of probiotics. For

probiotics to gain significant use, farms must first move away from prophylactic antibiotic use [17].

Antibiotics have also been investigated as a tool for eliminating STEC in cattle, with neomycin showing promise. In multiple studies, neomycin sulfate has been shown to decrease fecal shedding of *E. coli* O157:H7 in cattle [153]. Neomycin sulfate is an aminoglycoside that is commonly found in topical ointments and creams. It is often prescribed orally to reduce the risk of enteric infection during bowel surgery [112]. Other aminoglycosides include streptomycin, gentamicin, kanamycin and vancomycin, which are used in human medicine to treat serious infections [64]. Neomycin use in cattle does pose a risk for the development of resistance and cross-resistance [17].

Vaccination as a control method has been difficult due to the lack of pathogenesis of STEC in cattle [40]. While *E. coli* O157:H7 has been implicated in cases of diarrhea in calves, Cray & Moon found no evidence of disease for calves or adult cattle. Necropsies from the inoculated animals in their study revealed normal histologic sections of intestines with no evidence of the organism spreading to the liver, spleen or kidneys [28]. Several studies have shown that vaccines against *E. coli* O157:H7 can reduce shedding in cattle, but have been unsuccessful at preventing reinfection [99] [110] [144].

Calves are more likely to carry *E. coli* O157:H7 than adult cattle. While they are not typically part of the food supply, their close quarters with adult cattle allow for cross-contamination of their feces on to the hides and hooves of cattle that are sent to processing plants. To minimize the prevalence of STEC on farms, research has focused on minimizing the carrier risk of calves. One approach is through immunizing adult cows and relying on passive immunization through colostrum and milk to transfer antibodies to

newborn calves. Rabinovitz *et al.* demonstrated that immunizing pregnant cows with EspA, EspB, γ-intimin C280, or inactivated Stx2 produced high levels of antibodies against these proteins in the colostrum and milk of the immunized cows and in the serum of the calves. While these results are promising, testing still needs to be done to determine if immunization reduces shedding time or the infection rate [111].

A high-grain diet is fed to most cattle to increase growth. Several studies have shown that switching from a high-grain diet to hay can dramatically decrease fecal shedding of *E. coli* O157:H7 [31] [52]. The decrease in fecal shedding is not seen when cattle are fed hay as a long-term diet [17].

Antimicrobial interventions in beef processing plants varies for each plant. These interventions include trimming, steam vacuuming, hot water washing, chemical sanitizers, organic acid washing, steam pasteurization and irradiation[3][40]. Carcass contamination typically comes from the animal itself during the hide removal process, bung tying, and evisceration. Carcass contamination can come from sources other than the animal, including but not limited to: walls, floors, air, personnel, knives, protective garments and even other carcasses during chilling [40] [98].

The slaughter process begins first with stunning the animal, either mechanically, electrically or chemically. The next step is exsanguination by cutting the carotid arteries and jugular vein. This results in 85-95% of blood removal. An automated hide puller is generally used to remove the hide and the carcass is ready for evisceration. To prevent leakage and contamination, both the esophagus and bung must be tied off. All organs in the abdominal and thoracic cavities are removed. Once evisceration is complete, the

carcass is inspected and decontaminated. The final steps include weighing the carcass, assigning an identification tag, and storage in a chill room [40].

Dust, dirt and fecal material that are present on the hides of cattle can contaminate the carcass during the removal process [40]. Barkocy-Gallagher *et al.* found that during the spring, summer and fall, *E. coli* O157:H7 can be found on approximately 70% of all hides. During the winter, however, the rate dropped to only 30% [9]. Similarly, a study in 2004 by Arthur *et al.* found that 76% of tested cattle hides carried *E. coli* O157:H7 when entering the processing plants from the end of September to the beginning of November [2].

Decontamination of the hide, prior to hide removal can has been shown to reduce the chance of contaminating the carcass during the removal process. Bosilevac *et al.* found that washing the hides with an electric pressurized sprayer reduced *E. coli* O157:H7 by 1.5 log cfu/100 cm2. Vacuuming off residual water from the hides further reduced *E. coli* by an additional 1.3 log cfu/100 cm^2 [13]. Nou *et al.* found that chemical dehairing using sodium sulfide can reduce the prevalence of *E. coli* O157:H7 on carcasses by about 2 logs [104]. However, the use of sodium sulfide is an environmental and health hazard, which creates new problems for the processing plants to address [40].

The Bung tying process involves cutting loose the anus, followed by bagging the bung. It is secured with a tie or clip and then pushed through to the abdominal cavity. The bung and internal organs are removed during evisceration [40]. Tools as well as personnel can cross-contaminate the carcass during the bung-tying process [98]. Automated systems have been found to reduce this risk of cross-contamination [119].

After slaughter, the stomach and intestines begin to swell. Swelling increases the potential of puncturing or rupturing the intestines. Pressure on the gallbladder from swelling can push bile into the liver and surrounding tissue causing green discoloration. To reduce the risk of swelling, the gut must be removed within 30 minutes after slaughter [40].

Visible contamination of carcasses is generally removed physically by trimming off areas of contamination. This can result in cross-contamination by contaminated knives when the borders of contamination are not well defined [54]. Steam vacuuming uses steam to kill bacteria while also removing visible fecal contamination and has been found to be just as effective, if not more than trimming [40].

Washing the carcasses with sanitizers such as organic acids can remove contamination that is not visible, however, there are concerns that washing may result in the spread of contamination from liquid runoff. Hardin *et al.* investigated the use of lactic acid and acetic acid to determine the effectiveness of the acid washes as well as the risk of spreading bacterial contamination [54].

Hardin *et al.* found the organic acids were more effective on regions covered by subcutaneous fat than they were on lean muscle. Water washes did result in spreading contamination to previously uncontaminated areas, and resulted in only minimal reduction of bacteria from contaminated areas. Both acid washes were found to reduce contamination levels to levels achieved through trimming. While there was some spreading associated with the acid washes, the contamination was found to be below minimum detection levels [54]. In 1997, Dorsa *et al.* found that 12% trisodium phosphate, 1.5% lactic acid and 1.5% acetic acid washes were effective spray washes for

decontaminating beef carcasses [33].

Kalchayanad *et al.* investigated the use of hypobromous acid, neutral acidified sodium chlorite and two commercial citric acid-based antimicrobial compounds, Citrilow and FreshFx, on beef flanks against seven different STEC strains. They found that while all four compounds reduced the concentrations of STEC, none of them were capable of completely eliminating the pathogens. These results are similar to studies with other compounds, indicating that multiple approaches are necessary for the elimination of STEC [70].

Common preservation additives used in the meat industry include citric acid, sodium citrate and phosphates. Lenzi *et al.* investigated the *in vitro* effect of citric acid, lactic acid, acetic acid, sodium citrate and disodium phosphate on the growth of STEC, production of stx-phage and Stx. They found that lactic acid, acetic acid and citric acid inhibited bacterial growth without increasing production of stx-phages or Stx. Disodium phosphate and sodium citrate were not very effective at reducing growth of STEC and 2.5% sodium citrate increased the production of Stx [88].

Title 9, Chapter III, CFR 310.25 addresses federal requirements for testing for microbiological contamination of animal products. Samples must be collected from all animal carcasses after their final wash, prior to chilling. For cattle, samples must be collected from the flank, brisket and rump. For swine, samples must be collected from the ham, belly and jowl. Samples may be collected either by sponge or tissue excision [140].

Surveys done at 4 different beef processing plants in the United States in 1999 found a sample of non-O157 STEC in 54% of carcasses tested prior to processing. After antimicrobial interventions were applied, non-O157 STEC was recovered in 8% of

carcasses sampled [3]. That same year, Elder *et al.* surveyed 4 different beef processing plants in the United States for *E. coli* O157:H7 and O157:nonmotile. They found 43% of preevisceration carcasses were positive for *E. coli* O157. After antimicrobial interventions, *E. coli* O157 was recovered from only 2% of sampled carcasses [41]. Current antimicrobial interventions are significantly reducing contamination levels, but not completely [3] [70].

PRODUCE

After the spinach outbreak in 2006, the CDC recommended blanching fresh spinach in 71.1^{o}C (160^{o}F) water for 15 seconds to reduce the risk of STEC [19]. While this recommendation may eliminate the risk of infection from STEC, it also adversely affects the quality of the spinach by loss of nutrients, firmness, color and weight of the leaves. In order to maintain color and firmness of the leaves, Kim *et al.* demonstrated that 45 seconds at 61.9^{o}C with 0.52% $Ca(OH)_2$ could reduce STEC as much as the CDC method. The lower temperature helps preserve the color of the leaves while the calcium ions from $Ca(OH)_2$ can enhance the firmness of the leaves by forming cross-link complexes with free pectic acids in the spinach leaves. This method may help preserve the quality of cooked spinach, it is still impractical for produce that is consumed raw [77].

Common decontamination methods for fresh produce include chemical sanitizers, organic acids, essential oils, hydrosols, ultraviolet light, ozonation, ultrasound, high pressure, mild heat (40 to 50^{o}C) and ionizing irradiation [37] [139]. Chemical sanitizers are currently the most common method for decontaminating fresh produce [139].

Unfortunately, on leafy greens most chemical sanitizers result in <2 log reduction of *E. coli* O157:H7, leading to increased research on more effective treatments [36]. Combination treatments are much more effective. When UV light, mild heat and acidified sodium hypochlorite were combined, a >5 log reduction was found on green onions and *E. coli* O157:H7 was reduced below the detection limit on spinach [37].

Ganesh *et al*. demonstrated that the organic acids malic acid, tartaric acid, lactic acid and grape seed extract when applied with an electrostatic sprayer inhibited *E. coli* O157:H7 on spinach and iceberg lettuce. Electrostatic spraying with the inorganic acid phosphoric acid resulted in even greater *E. coli* inhibition, however, it resulted in discoloration of the produce. Electrostatic spraying results in an even distribution of antimicrobials onto produce. During storage, the antimicrobial action of all four organic solutions increased with time [45].

Essential oils and their components (EOCs) are aromatic, organic compounds derived from plant tissues. Many EOCs have demonstrated antimicrobial properties against common pathogens and may serve as a safe and effective method for decontaminating food surfaces and fresh produce. Three EOCs that have demonstrated antimicrobial activity against *E. coli* O157:H7 are eugenol, carvacrol and thymol. Eugenol is an EOC from clove, carvacrol is an EOC from oregano, and thymol is an EOC from thyme. Due to their hydrophobicity, large concentrations are required for use in foodborne pathogen reduction. Microencapsulation in surfactant micelles offers a mode of delivery that reduces the required concentrations. When tested on spinach leaves, eugenol in SDS or CytoGuard® LA 20 micelles, was the most effective at reducing *E. coli* O157:H7, reducing *E. coli* O157:H7 by 4 log CFU/cm^2 [116].

Vaporized ethyl pyruvate, a lipophilic ester derivative of pyruvate, has been investigated for fresh produce decontamination against *E. coli* O157:H7 with promising results. In 2012, Durak *et al.* published their results using vaporized ethyl pyruvate on green onions and baby spinach leaves. At 420 mg/liter of EP, >4.7 log CFU/g reduction of *E. coli* O157:H7 was observed on green onions at after 7 days at 4^{o}C. At the same concentration, a 4.3 log CFU/g reduction of *E. coli* O157:H7 was observed on baby spinach after 7 days at 4^{o}C. Treating green onions with vaporized EP did not significantly change the color, odor or texture according to panelists. However, in baby spinach leaves, vaporized EP caused yellowing in addition to decreased scores for odor and texture [36]. In 2015, Tornuk and Durak published their results using vaporized ethyl pyruvate on parsley leaves. At 1000 µl, vaporized ethyl pyruvate completely inhibited growth of *E. coli* O157:H7 in addition to slowing decay. However, it also caused drying, and yellowing of the parsley leaves [139].

Current FDA guidelines for sprouts recommend treating seeds with 20,000 ppm calcium hypochlorite and periodically testing sprouts and spent irrigation water for enteric pathogens [44]. Treating seeds with 20,000 ppm calcium hypochlorite has been shown to reduce enteric pathogens by 2.2 logs. This reduction can be increased at higher concentrations of calcium hypochlorite, or through the use of concentrated acids, high temperature or bleaches. However, these treatments significantly reduce the number of germinating seeds and are not considered practical [47].

Gamma irradiating seeds has been shown to dramatically reduce pathogenic bacteria, however, this approach remains unpopular due to the expense of irradiating machinery and the public concern about irradiated foods. Many consumers believe that

irradiated foods become radioactive during the process. While this is untrue, public education would be required to ease consumer concerns [47].

A promising new method for decontaminating sprout seeds uses heat and relative humidity. At 65ºC, with 45% relative humidity, Kim *et al.* obtained a 7 log CFU/g reduction in *E. coli* O157:H7 on radish seeds without a significant effect on germination rate. This method appears both practical and effective for growers that use growth chambers [78].

Berries are another type of produce that is at risk for *E. coli* contamination. Xu *et al.* investigated the effects of washing raspberries with two different sanitizers and treating them with pulsed light on the survival of *E. coli* O157:H7. The first sanitizer, citric acid plus sodium dodecyl sulfate, reduced *E. coli* by 4.1 log CFU/g. The second sanitizer, citric acid plus thymol, reduced *E. coli* by 3.7 log CFU/g. Treatment with pulsed light reduced *E. coli* by 3.3 log CFU/g. Raspberries treated with pulsed light had increased redness, higher phenolic and anthocyanin content, in addition to decreased *E. coli* [156].

Pasteurization is the standard method for decontaminating liquid foods, however, the high temperature required for pasteurization can reduce both the quality and nutrition of liquid foods. One alternative approach that is currently under investigation is the use of pulsed electric fields (PEF). Through electroporation, PEF can form pores in bacterial membranes resulting in the loss of intracellular contents [147].

Walking-Ribeiro *et al.* found that heat-assisted PEF was more efficient than PEF at reducing populations of *E. coli* O157:H7. Heat-assisted PEF reduced *E. coli* by >3.4 log while PEF resulted in less than a 1 log reduction. Heat-assisted PEF treatment

includes heating the sample to 52^{o}C while applying the electric field, whereas PEF samples range from 30 to 45^{o}C while applying the electric field [147].

Chlorination is the most common method for decontaminating water supplies. While effective for most pathogens, halogenated by-products are a concern. Additionally, it is ineffective against chlorine-resistant parasites such as *Cryptosporidium parvum* and *Giardia lamblia*. Both UV and ozone treatments are currently under investigation due to their effectiveness and minimal by-products. Fang *et al.* found that treating water with UV between 11.4-34.2 mJ/cm^2 along with 0.05 mg/L ozone exposure effectively inactivated *E. coli* in water samples [42].

SURFACES

The surface type used in food preparation determines appropriate disinfectants to use. Stainless steel is the easiest to sanitize, followed by plastics, with wood being the most difficult [49]. The porous nature of wood makes it more difficult to sanitize, however, it is generally considered safe for use in the food industry. Some researchers have found the rough surface of wood creates unfavorable growth conditions for microorganisms [5]. Other researchers have found wooden surfaces to be almost impossible to disinfect [49]. The type of wood used and direction it was cut can greatly affect the growth of microorganisms. Many wood types contain natural antimicrobial components that can inhibit bacterial growth. Scots pine, for example, has a demonstrated antibacterial effect on *E. coli* and *Enterococcus faecium.* Many plastic surfaces have been shown to hold bacterial populations steady, or even increase populations with time. Wood

surfaces, on the other hand, have been shown to reduce bacterial populations over time [5].

It is recommended that separate cutting boards and knives are used when handling raw meat and ready-to-eat foods. The most effective decontamination method for cutting boards and knives is mechanical washing with water and detergent. However, if insufficient cleaning is performed bacterial transfer may remain a risk. This risk can be mitigated by allowing cleaned items to dry before reuse. Wet surfaces and knives are more likely to transfer bacteria than when dry. This is thought to result from decreased bacterial attachment to wet cutting boards and knives [49].

In the home setting, there are some common misconceptions regarding cleaning and disinfection. Many people are unaware that surfaces should be cleaned first before disinfection. Because microorganisms cannot be seen, it is difficult to know when disinfection is needed or to what extent it is achieved. Additionally, concerns about 'chemicals' have led many to seek 'natural' or 'green' cleaning products and 'do-it-yourself'(DIY) recipes that have not been tested. Goodyear *et al.* tested three different cleaning products on ceramic and stainless steel surfaces to determine their effectiveness at cleaning and disinfecting. They compared a conventional cleaning product, Clorox® bleach, to an environmentally preferable product containing 0.05% thymol, and a DIY recipe consisting of club soda, distilled vinegar and tea tree oil. They found that both commercial products, bleach and thymol, were effective at cleaning stainless steel whereas the DIY mixture was not. None of the three tested products effectively cleaned the ceramic surface, although the DIY mixture achieved the greatest removal of soil. Both commercial products reduced *E. coli* and *Staphylococcus aureus* by >5.00 log on

both ceramic and stainless steel. The DIY solution reduced *E. coli* by 3.97 log on ceramic and >5.00 log on stainless steel. The DIY solution was less effective against *S. aureus,* with only 1.90 log reduction on stainless steel and 2.71 log reduction on ceramic [51].

STEC strains capable of forming biofilms (matrices composed of proteins, nucleic acids and extracellular polysaccharides) pose an increased risk of food contamination. Biofilms provide physical, mechanical and biological protection to the bacterial cells that secrete them. Controlling the formation of biofilms on equipment surfaces is of great concern to the food industry. Park and Chen compared the effectiveness of four different sanitizers on their ability to reduce biofilms on stainless steel and polystyrene. They compared acetic acid, lactic acid, a commercial alkaline sanitizer (pH 13) and a commercial acidic sanitizer (pH 1.0 to 1.5). All four sanitizers reduced the amount of accumulated biofilm, but the two commercial sanitizers were more effective than the organic acids. Unfortunately, none of the treatments were capable of completely removing the formed biofilms emphasizing the importance of finding more effective sanitizers and treatment conditions [107].

Ethyl pyruvate is currently under research as a surface disinfectant and as a systemic therapy for infections. It has been demonstrated to prevent the formation of biofilms in addition to promoting the breakdown of existing biofilms. EP inhibits glyoxalase 1 and pyruvate kinase, causing a depletion of cellular ATP. This mechanism allows EP to inhibit the growth of both pathogenic fungi and drug resistant pathogenic bacteria [30].

Patient Therapy

Diagnosis of STEC infection is through detection of fecal Stx, detection of STEC antigens in patient serum or through isolation of *E. coli* O157:H7 or other Stx-producing strains from patient stool samples. Isolation of STEC requires culturing early from time of infection. Within the first six days of disease, the recovery rate through culture is 91.7%. After six days, the recovery rate drops to 33.3%. Most patients don't seek treatment for illness until symptoms of HUS develop which is usually around six days after they experience diarrhea. The majority of patients with HUS (~2/3) will not culture positive for STEC [137].

E. coli O157:H7 has an incubation period of 1 to 4 days with symptoms lasting 5 to 10 days. The most common symptom of infection is watery diarrhea, which often goes unreported unless illness worsens. Death due to infection occurs in about 2 to 5% of HUS cases, with children and elderly at greatest risk[91]. Death due to HUS is often caused by cerebral bleeds or strokes. Other causes include congestive heart failure, pulmonary hemorrhage and hyperkalemia [12].

Suspected cases of STEC infection should be referred to a hospital for diagnosis and appropriate treatment [12]. Care must be taken to reduce the risk of person-to-person transmission of STEC. It is recommended that patients with confirmed, or suspected, cases of STEC should be isolated. Good hand hygiene is essential in reducing the spread [138].

Treatment of STEC is generally limited to supportive care. Supportive measures may include peritoneal dialysis, antiplatelet and thrombolytic agents and thrombin

inhibitors, antimicrobials, probiotics, toxin neutralizers, and antibodies. Blood transfusions may be indicated due to anemia resulting from hemolysis [50].

Due to vomiting and diarrhea, patient dehydration may occur, but must be carefully treated to prevent overhydration or the development of hyponatremia. Patient serum is monitored for sodium, potassium, phosphate and pH concentrations to watch for hyponatremia, hyperkalemia, hyperphosphatemia and metabolic acidosis. Intravenous administration of hypertonic saline may be required to correct hyponatremia. Hyperkalemia, hyperphosphatemia and metabolic acidosis are corrected through dialysis [72].

In cases of severe HUS, patients may experience seizures and extensive renal damage can lead to anuria and hypertension [72]. Acute renal failure as a result of HUS causes fluid and electrolyte imbalances [50]. Dialysis is often indicated to correct severe electrolyte or acid-base imbalances due to kidney damage. It may also be used if the patient is experiencing fluid overload and is not responding to diuretics [12].

In addition to renal cells, red blood cells and islet cells of the pancreas are targeted by Stx. Packed red blood cell transfusions are indicated if patient hemoglobin drops below 6 g/dl. Blood transfusions increase the risk of volume overload and must be administered slowly while monitoring blood pressure changes. Damaged islet cells may result in hyperglycemia, ketonemia and acidosis. Treatment with insulin can ameliorate these symptoms, but will leave the patient insulin-dependent [72].

Determining the effectiveness of antibiotics and other drugs to treat STEC infections and prevent development of HUS is often difficult. Most patients are not

enrolled in trials until they have already presented with symptoms of infection, which indicates that Stx has already been absorbed by the gut [138].

The administration of most antibiotics for STEC infections is contraindicated [26]. Between April 1997 and August 1999, Wong *et al.* studied 71 children infected with *E. coli* O157:H7 and concluded that antibiotic treatment placed children at a significantly higher risk for developing HUS. Only 5 of the 62 children who were not given antibiotics developed HUS, compared to 5 of the 9 children given antibiotics. Antibiotics used for treatment included trimethoprim-sulfamethoxazole, cephalosporin and amoxicillin [154].

Herold *et al.* demonstrated that administration of norfloxacin, a quinolone antibiotic, induces phage genes. In *E. coli* EDL933 late region genes in BP-933W were the most strongly affected. These genes include stx_2, which saw more than a 150-fold increase in production [55]. Antibiotics also reduce the normal intestinal flora which is thought to result in increased toxin absorption [26].

Some researchers have found classes of antibiotics that seem to decrease the risk of HUS. Geerdes-Fenge *et al.* found that the antibiotic ciprofloxacin reduced the risk of developing HUS from *E. coli* O104:H4 infections. Only 40% of patients treated with ciprofloxacin developed HUS compared to 89% of patients who did not receive ciprofloxacin [46].

Neutralizing antibodies are currently being researched for treating STEC infections. Monoclonal antibodies against Stx have shown promise in both mouse models and piglets [101]. Skinner *et al.* found that the combined therapy of tigecycline and antibodies to Stx1 and Stx2 fully protected Vero cells [120]. Hemolysis and complement activation disrupt the clotting cascade, resulting in increased risk of thrombocytopenia

and stroke [50]. A humanized monoclonal antibody against complement, Eculizumab, has been developed and tested. Unfortunately, by the time patients are admitted due to infections, it is often too late to administer eculizumab to inhibit complement-mediated hemolysis since it has likely already occurred [4].

Probiotics are currently being investigated as a treatment option for STEC infections. Dini *et al.* investigated a probiotic mixture of bacteria and yeast isolated from kefir grains. The mixture included *Lactobacillus plantarum, Lactococcus lactis, Lactobacillus kefir, Kluyveromyces marxianus* and *Saccharomyces cerevisiae*. Treating *E. coli* O157:H7 infected Hep-2 cells with the microbial mixture was found to reduce cell detachment [32].

In addition to treating Hep-2 cells with probiotics, Dini *et al.* tested a bacteriophage. Unlike the microbial mixture, the bacteriophage treatment did not reduce cell detachment, rather increased it. This effect may be caused by the burst of toxins upon lysis of phage infected cells. When the microbial mixture was combined with the bacteriophage, cell detachment was reduced to the same levels as the microbial mixture alone. Additionally, the phage combined with the microbial mixture reduced the adhesion of *E. coli* O157:H7 to Hep-2 cells by 1.15 log. The microbial mixture alone only reduced the adhesion of *E. coli* O157:H7 to Hep-2 cells by 0.31 log. The lyophilized phage when used alone only reduced the adhesion of *E. coli* O157:H7 to Hep-2 cells by 0.62 log. These results indicate that the combined treatment of both antimicrobial agents may be an effective treatment option for STEC infections [32].

Types of Bacteriophage

Bacteriophage are small viruses that only infect specific strains of bacteria[53]. Bacterial viruses, or bacteriophages, are classified based on viral morphology and type of nucleic acid into 10 families; *Myoviridae, Siphoviridae, Podoviridae, Tectiviridae, Corticoviridae, Plasmaviridae, Microviridae, Inoviridae, Cystoviridae,* and *Leviviridae* [83].

The order *Caudovirales* is formed by the families *Myoviridae*, *Siphoviridae,* and *Podoviridae*. *Caudovirales* are tailed bacteriophages with icosahedral shaped protein capsids packed with double stranded linear DNA. The length of DNA varies significantly from as little as 11.6 kb, as seen in podovirus P1, to as much as 497.5 kb, as seen in myovirus G. Myoviruses possess a contractile tail while siphoviruses possess long, non-contractile tails and podoviruses possess short, non-contractile tails. The tailed viruses are the most extensively studied, and make up the vast majority of sequenced bacteriophage. When infecting a cell, they inject their DNA into the cell and the virion proteins are left attached to the outside of the cell. Many of the tailed viruses are temperate bacteriophage that may be integrated into the host chromosome or exist as extra-chromosomal plasmids [83].

The *Myoviridae* family is composed 8 genera; I3-like, Mu-like, P1-like, P2-like, PhiH-like, PhiKZ-like, SPO1-like and T4-like. Bacteriophage within the 8 genera possess long, thick and ridged contractile tails with lengths from 80 to 455nm and widths between 16 and 20nm. The tails are surrounded by a contractile sheath, which shorten during contraction. This shortening results in the tail core making contact with the bacterial plasma membrane, an essential step in infection [60]. The T4-like viruses make

up the largest group of bacteriophage and the type species, Enterobacterial phage T4, has been the most studied [85].

The family *Siphoviridae* is composed of 9 different genera; c2-like, l5-like, Lambda-like, N15-like, PhiC31-like, PsiM1-like, SPbeta-like, T1-like and T-5-like. All bacteriophage within *Siphoviridae* have long, non-contractile tails 65-570 nm in length and 7-10nm thick. They are known to infect both Gram-positive and Gram-negative bacteria [62].

The family *Podoviridae* is composed of two subfamilies; *Autographivirinae* and *Picovirinae*. An additional 6 genera fall under *Podoviridae* that are not included in the two subfamilies; BPP-1-like, Epsilon15-like, LUZ24-like, N4-like, P22-like, and Phieco32-like. All bacteriophage within *Podoviridae* have short, non-contractile tails around 20nm in length and 8nm thick. Bacterial targets include both Gram-positive and Gram-negative organisms [61].

Tectiviruses have linear double stranded DNA genomes around 15 kb packed inside a protein-rich membrane vesicle, surrounded by an icosahedral protein capsid. The genomes of tectiviruses that can infect Gram-negative bacteria share very little in common with the genomes of tectiviruses that can infect Gram-positive bacteria. For this reason, they are grouped based on the type of bacteria they infect. Within groups, their genomes share significant sequence identity [83]. The most well studied tectivirus is PRD1, which infects *E. coli* and *Salmonella typhimurium*. PRD1 is an IncP-type plasmid which requires a mating pair formation (Mpf) receptor on the host cell membrane which is responsible for transporting viral DNA into the host [29]. After virus replication and

assembly, the new bacteriophage exit the cell by initiating cell lysis. There are no observed tectiviruses that exhibit host genome integration [8].

There is only one identified corticovirus, PM2, which infects marine *Pseudoalteromonas*. PM2 has a supercoiled circular double stranded DNA genome around 10 kb. Similar to tectiviruses, the genome is packed inside a protein-rich membrane vesicle and surrounded by an icosahedral protein capsid [83]. The capsid is approximately 55 nm across between facets, and approximately 60 nm across between vertices. Trimers of the major capsid protein P2, make up the facets of the capsid, with spike protein P1 associated with the capsid vertices. The spike protein is responsible for receptor binding. The lipid core, or internal membrane vesicle, is a lipid bilayer with associated proteins P3 to P9, with protein P4 interacting with the viral DNA [80].

L2 is the only identified plasmavirus, which has a circular, double stranded DNA genome with a pleomorphic capsid and an envelope [83]. The L2 genome is 11.97 kb, with 15 identified ORFs. It infects members of mycoplasma by fusing its envelope membrane with the host cell membrane, and leaves the infected cell through budding from the cell membrane. L2 is capable of lysogeny within the host genome [96].

The family *Microviridae* has two subfamilies; *Bullavirinae* and *Gokushovirinae*. They are capable of infecting enterobacteria and the intracellular parasitic bacteria *Bdellovibrio, Spiroplasma,* and *Chlamydia* [83]. The subfamily *Gokushovirinae* is made up of the parasitic bacteria viruses while *Bullavirinae* is made up of the enterobacteria bacteriophage[59]. Viruses in this family are small, non-enveloped, icosahedral viruses with circular, positive sense single stranded DNA genomes. The genomes of the two groups share very little sequence similarity [83]. Gokushovirus genomes are between 4.4

and 4.9 kb, while bullavirus genomes are larger, between 5.3 and 6.1 kb. This difference in size is due to the inclusion of genes for major spike and external scaffolding proteins which are missing in gokushoviruses [59].

In 2011, the International Committee on Taxonomy of Viruses (ICTV) published their 9th report. The ICTV 9th report identifies two genera within *Inoviridae*; *Inovirus* and *Plectrovirus*. Inoviruses are long, flexible filamentous virions between 700 and 900nm in length and around 7nm in diameter. Inoviruses are capable of infecting both Gram-negative and Gram-positive bacteria. Plectroviruses are short rods between 70 and 280nm in length and around 15 nm in diameter. Plectroviruses infect *Acholeplasma* and *Sprioplasma* [57]. This family of viruses has helical symmetry and small, circular, positive sense, single stranded DNA genomes between 4.5 to 9.0 kb [83]. Once a cell is infected, the virus can exist in a latent prophage state or a virulent productive state [57]. Unlike most other bacteriophage, inoviruses do not lyse their host cells, rather, they exit the host cell by extrusion during virion assembly [83].

Cystoviruses are the only known double stranded RNA viruses that infect bacteria, with their genome composed of three separate segments [142]. The genome is packed in a double-layered icosahedral capsid that is surrounded by a membrane envelope. Hosts of cystoviruses are phytopathogenic pseudomonads [83]. An RNA-dependent RNA polymerase resides in the inner capsid and performs both replication and transcription within the inner capsid. RNA for protein synthesis or viral progeny is then secreted into the host cell for translation or packaging [142].

The family *Levivridae* is composed of two genera, *Allolevivirus* and *Levivirus.* Both genera are spherical, icosahedral non-enveloped viruses around 26 nm in diameter.

They are positive sense single stranded RNA viruses with genomes of 3466-4276 nucleotides. Both genera target Enterobacteria, with Enterobacteria phage MS2 as the type species for *Levivirus* and Enterobacteria phage Qbeta as the type species for *Allolevivirus.* Leviviruses differ from Alloleviviruses in genome length, cell lysis and Host Factors. The genomes of Alloleviviruses are longer than Levivirus genomes. Leviruses have a lysis gene whereas cell lysis in Alloleviviruses is a secondary function of the A-protein. Both genera produce the A-protein which is responsible for virion maturation and pilus attachment [58].

Bacteriophage Life Cycle

The different hosts that a bacteriophage can infect are called the 'host range'. Some bacteriophage are only capable of infecting a few strains of one bacterial species, while others may infect several different species within a genus. Several factors determine the host range of a bacteriophage such as the presence of cell surface receptors, the accessibility of those receptors, and the ability to survive within the host. Bacteria have several different mechanisms to combat infection, including restriction-modification systems, abortive infection mechanisms and CRISPR [48].

Bacteriophage are brought into contact with susceptible hosts through diffusion [82]. Bacteriophage infections begin with adsorption to the bacterial cell through recognition of receptors [149]. The high degree of specificity required for receptor recognition is primarily responsible for the limited host range of each bacteriophage. Receptors on Gram-negative cells are typically found on the outer membrane, while receptors on Gram-positive cells are found typically found on the cell wall. In some

cases, conical holes in the cell wall can result in the cytoplasmic layer protruding through the cell wall, allowing receptors to be located on the cytoplasmic membrane in Gram-positive cells. The capsular layer, flagella, and pili can also serve as receptor locations for both Gram-negative and Gram-positive cells [90].

Once the bacteriophage attaches to the cell, the viral genetic material must be transferred into the cell. This requires penetrating the cell wall through enzymes in the tail or capsid [149]. The mechanisms for injecting viral DNA into the host varies significantly by the type of bacteriophage involved [83]. Once the bacteriophage genome is inside the cell, it can either be incorporated into the host genome or remain in the cytoplasm [149].

The latent period of infection is the time between adsorption and release of new bacteriophage particles [149]. The latent period is further divided into the eclipse and post-eclipse periods. During the eclipse period, the bacteriophage genome is replicated in addition to production of viral particles, however, no mature viral particles can be detected in the cell. The viral particles are fully assembled in the post-eclipse period [56]. The release of mature viral particles from the cell signals the beginning of the rise period and the end of the latent period. Bacteriophage release can occur through cell lysis or budding [149].

Bacteriophage life cycles include lytic, lysogenic and chronic infections. In lytic infections, bacteriophage are released from the infected cell by lysing the host. Endolysins and holins, produced by lytic bacteriophage, cause lysis by breaking down the cell wall and damaging the plasma membrane resulting in host cell death. In chronic infections, bacteriophage are released from infected cells by budding. This leaves the host

cell alive to continue to produce and release bacteriophage [149]. Temperate bacteriophage are capable of lysogenic infections. In a lysogenic infection, the viral genome is incorporated into the bacterial genome or exist as an extrachromosomal plasmid. During cell replication, the viral genome is replicated with the host genome until the lytic cycle is triggered [82].

The burst size of a bacteriophage infection is the number of viral particles released from a single infected cell [149]. Timed infections of *E. coli* with T4 bacteriophage found the largest burst size occurred in cells immediately before cell division. The lowest burst sizes were found in cells immediately after cell division. Analysis of intracellular resources found that RNA levels were also highest immediately before cell division. The increased RNA is the result of higher numbers of ribosomes and RNA polymerases. The replicating bacteriophage can hijack ribosomes and RNA polymerases for their own production [133].

Bacteriophage Therapy

Bacteriophage discovery is largely credited to both Frederick Twort in 1915 and Félix d'Herelle in 1917. The potential of bacteriophage to control pathogens was recognized upon their discovery by Félix d'Herelle. However, early trials often lacked reproducibility, produced inconsistent results and lacked appropriate controls. After antibiotics were discovered, the interest in utilizing bacteriophage to control infections was largely abandoned in western medicine. However, bacteriophage therapy continued to be investigated in the former USSR, Poland and India [152].

As the prevalence of antibiotic resistant bacteria grows, novel antibiotics have been slow to hit the market. The search for alternative treatments has renewed interest in bacteriophage therapy due to their high degree of specificity and limited side effects. Due to their limited host range, bacteriophage have very little impact on the microbiome. Bacteriophage do not damage mammalian cells, which also greatly reduces the side effects of bacteriophage therapy compared to antibiotics. Another benefit to bacteriophage therapy is the wide systemic distribution. Unlike many other drugs, bacteriophage are capable of penetrating the blood-brain barrier [152].

One of the earliest experiments to investigate the *in vivo* effects of bacteriophage in the United States was published in 1943. Mice were experimentally infected with *Shigella dysenteriae* intracerebrally. In untreated mice, this infection causes meningitis without generalized septicemia, and results in death within 3 to 10 days. Bacteriophage isolated from New York City sewage water, were injected intraperitoneally into infected mice. Within 1 hour of injection, bacteriophage were detected in the brain at levels considerably higher than levels circulating in the blood. Mice injected with 10^9 bacteriophage particles survived the experimental infection, while mice injected with only 10^5 bacteriophage particles did not [35].

In a similar study, Smith and Huggins isolated a bacteriophage (phage R) from sewage that was lytic against *E. coli* O18:K1:H7, a strain of *E. coli* isolated from an infant with meningitis. Using a mouse model, they found that a single dose of either intramuscular or intravenous phage R particles was sufficient to cure mice that were given lethal intramuscular or intracerebral doses of *E. coli* O18:K1:H7. A total of 8 doses of streptomycin was required to achieve a similar cure rate. Phage R was demonstrated to

be as effective as multiple doses of streptomycin, and more effective than multiple doses of ampicillin, chloramphenicol, tetracycline, or trimethoprim plus sulphafurazole [124].

One concern regarding bacteriophage therapy is the possibility of selecting for bacteriophage resistant mutants. In the laboratory setting, bacteriophage resistant mutant strains of bacteria frequently arise. Fortunately, *in vivo* studies have shown this is a rare occurrence in practice and generally results in loss of virulence [152]. Smith and Huggins investigated the *in vivo* occurrence of phage-resistant mutants in their 1982 study. They examined the inoculated muscle, uninoculated muscle, blood, spleen, liver and brain tissue of mice from their study with phage R. They only found a few phage R-resistant mutants in the mice they treated, with the mutants limited to the inoculation site only [124].

Smith and Huggins went on to study the effectiveness of phage in treating diarrhea in calves, piglets and lambs that were experimentally infected with *E. coli* strains. Like their study with mice, they demonstrated that phage isolated against the target strains of *E. coli* were capable of curing otherwise lethal oral infections. Phage-resistant mutants were found in both calves and piglets, but not any lambs. The mutants were found to have significantly decreased virulence compared to the parent strains [123].

Bacteriophage Preparation

The work of Smith and Huggins, while demonstrating the effectiveness of phage treatment, also exhibited the need for the isolation of new bacteriophage for each strain of *E. coli* tested [123]. One of the greatest limitations and greatest appeals of bacteriophage

therapy is the highly specific nature of bacteriophage. In an outbreak, it is impractical to first isolate the strain causing disease and then test against a battery of bacteriophage to determine treatment. A pool of bacteriophage, active against a large range of strains, is more practical. As outbreaks of new disease-causing strains occur, bacteriophage can be added to the pool that are active against the new strains [126].

In practice, bacteriophage therapy requires viable, highly virulent bacteriophage in preparations free from toxic impurities. Bacteriophage that are incapable of the lysogenic cycle or chronic infections are referred to as 'virulent'. Virulent bacteriophage are candidates for bacteriophage therapy, where death by host cell lysis is the desired outcome. Several factors affect the degree of virulence of bacteriophage such as rates of adsorption, extent of the latent period, burst sizes and their ability to bypass bacterial mechanisms intended to prevent infection [48]. Barrow *et al.* recommend using bacteriophage that target virulence factors such as the K1 antigen in *E. coli*. In order for bacterial strains to become resistant to K1 bacteriophage, they must either modify their K1 antigen, or lose the antigen entirely. A lost, or highly modified K1 antigen, has been shown to significantly reduce the virulence of the bacteria [10].

Bacteriophage are found in every bacteria-rich environment. When looking for new bacteriophage, most researchers look to environments where the target bacteria reside. Sewage samples, especially from hospitals, represent a rich source of novel bacteriophage active against pathogenic bacteria [24]. Similarly, soil and water samples have been used for the isolation of bacteriophage against plant pathogens, while animal feces have often been used for the isolation of bacteriophage against animal pathogens [126] [141].

After bacteriophage are isolated, they must be characterized. This involves determining morphology with transmission electron microscopy (TEM), sequencing the genome and determining growth parameters. Bacteriophage genomes must be screened for genes involved in toxin production, antibiotic resistance, and lysogeny [48]. Growth parameters such as optimal pH and temperature must be determined to ensure successful treatments [125].

Many bacteriophage are susceptible to strongly acidic conditions, which are commonly encountered in the digestive tract of animals. Smith *et al.* found higher concentrations of phage in the small intestines of calves when orally administered phage shortly after a milk feed than were found if administered 10 hours after the milk feed. The raised pH caused by the milk feed could be replicated by administering $CaCO_3$ in feed and was found to help increase the number of orally administered phage entering the small intestine of calves. However, phage-neutralizing antibodies passed through colostrum are inactivated at low pH. Smith *et al.* found that administering $CaCO_3$ with colostrum greatly reduced the number of phage in the alimentary tract of calves due to the neutralizing effect of antibodies that survived in the higher pH [125].

The virulence of most bacteriophage vary with temperature. Smith *et al.* studied the *in vitro* virulence of 21 different phages. They found that the majority of phage were the most virulent around 37^{o}C while below 20^{o}C and above 43^{o}C, most of the phage were avirulent. Bacteriophage successfully used in animal treatments may not be as effective for environmental treatments [125].

Bacteriophage morphology can provide significant information about newly isolated bacteriophage[48]. Filamentous bacteriophage often cause chronic, rather than

lytic infections while Tectiviruses have not been observed to undergo lysogeny [83]. Using chloroform to screen bacteriophage can select for bacteriophage that lack lipids in their capsids, which may help in eliminating less virulent bacteriophage [48].

Generally, temperate bacteriophage are undesirable for bacteriophage therapy for a number of reasons. First, the bacteriophage genomes may contain undesirable genes that will become expressed after lysogeny. These events are termed 'lysogenic conversion' and are responsible for the conversion of *E. coli* O55:H7 into *E. coli* O157:H7 [48] [151]. Another complication of lysogeny is conferred immunity to infection from the same bacteriophage or closely related bacteriophage. Transduction, the movement of bacterial DNA from one host to another may also occur with temperate bacteriophage. For these reasons, it is usually necessary to avoid the use of temperate bacteriophage [48].

Avoiding temperate bacteriophage can be difficult in practice. It is recommended when isolating new bacteriophage to avoid selecting plaques that are cloudy or have bacterial colonies growing within them. In addition, screening bacteriophage genomes for genes necessary for lysogeny, such as integrases and repressor genes can help identify temperate bacteriophage. Temperate bacteriophage may still play a role in bacteriophage therapy after undergoing genetic modification. It may be possible to reduce bacterial pathogenicity through lysogenic conversion [48].

Bacteriophage treatments require amplification and purification of bacteriophage. Purification is required to prevent unwanted reactions in humans or animals due cell debris and culture components. Amplification is required to ensure bacteriophage are in suitable concentrations for treatments. The most common method for bacteriophage

purification is through polyethylene glycol (PEG) precipitation followed by ultracentrifugation with a CsCl gradient. Ultracentrifugation with CsCl is time consuming, potentially dangerous due to the equipment and chemical, and limits the volume of culture that can be processed. Besides PEG precipitation, other common methods for concentrating bacteriophage include the addition of flocculating agents, tangential flow filtration, chromatography, and precipitation by combined alcohol and pH changes. Flocculating agents cause bacteriophage to aggregate into large, loose complexes called flocs, which can be collected through filtration, low-spin centrifugation, or sedimentation. Tangential flow filtration, filters a bacteriophage culture through a 0.2μm filter, followed by a 100 kDa membrane. This removes both cell debris and most of the media components allowing the bacteriophage particles to be reconstituted in a sterile buffer of choice in the desired concentration. Modifying capsid proteins with affinity tags can allow for bacteriophage purification and concentration through liquid chromatography [48].

Clinical uses of Bacteriophage Therapy

Phage banks, collections of bacteriophage with established host ranges, could be employed in bacteriophage therapy. This first requires isolation and identification of the pathogen, followed by screening the bacteria against bacteriophage in the phage bank. This would allow for customized treatments and ensure that only effective bacteriophage are used in treatment. The downside to phage banks is the amount of time required for selecting appropriate bacteriophage which will result in delayed treatment [48].

In 1987, Ślopek *et al.* summarized the results of 550 clinical cases of bacteriophage therapy between 1981 and 1986 in Poland. Out of the 550 cases, bacteriophage therapy was ineffective in only 4 cases. Patient ages ranged from 1 week to 86 years with the majority of infections (518 cases) caused by antibiotic resistant organisms. Bacterial strains were isolated from each patient and tested against available bacteriophage. Selected virulent bacteriophage were administered orally 3 times a day before meals in addition to local administration if applicable [121].

Bacteriophage cocktails, combinations of bacteriophage designed to extend the host range of the treatment, may be a more practical approach when delays are unacceptable [48]. Jikia *et al.* reported on a case study of two patients with radiation injuries that became infected with multi-drug resistant *Staphylococcus aureus*. After treatment with antibiotics failed, both patients' wounds were treated with PhagoBioDerm™, a biodegradable polymer impregnated with ciprofloxacin and a mixture of bacteriophages. The bacteriophages in PhagoBioDerm™ have lytic activity against strains of *S. aureus, E. coli, Pseudomonas aeruginosa, Proteus,* and *Streptococcus.* The *S. aureus* strains isolated from the two patients were found to be sensitive to the bacteriophage in PhagoBioDerm™. Within 7 days of treatment following application of PhagoBioDerm™, *S. aureus* was eliminated from their wounds, despite the strain being resistant to ciprofloxacin [66].

Several bacteriophage therapies are currently undergoing clinical trials such as WPP-201, a bacteriophage solution with viruses active against *S. aureus, P. aeruginosa,* and *E. coli,* which just completed phase I clinical trial in Texas with promising results [113]. Phagoburn, a cocktail of 13 bacteriophages that target *E. coli* and 12

bacteriophages that target *P. aeruginosa,* is currently undergoing phase II clinical trials in Europe [109]. AmpliPhi just completed a phase I clinical trial on their *S. aureus* bacteriophage treatment for wounds and chronic Rhinosinusitis. AmpliPhi has another bacteriophage treatment targeting *P. aeruginosa* in Cystic Fibrosis patients with phase I clinical trials expected to begin this year [1]. If these studies are successful, clinical bacteriophage therapy may become a common treatment in western medicine [79].

Other Bacteriophage Uses

Bacteriophage have been used in various industries besides clinical treatments such as food safety, agriculture, and epidemiological studies. The specificity of bacteriophage allows their use in food manufacturing to control pathogens and undesirable contamination without altering cultures in fermented products. Besides controlling pathogens, bacteriophage are also used for detecting contamination and in bacterial identification [152].

Phage typing is often used in epidemiological studies to identify and characterize outbreak strains. Compared to other epidemiological methods such as DNA-PCR fingerprinting and pulsed field gel electrophoresis, phage typing is quick, relatively simple and cost-effective [53]. Initially developed for typing *S. aureus*, phage typing systems have been developed for *E. coli* O157, *Mycobacterium tuberculosis, Listeria, Campylobacter* and *Pasteurella multocida* [103] [118].

Detecting pathogens in water supplies is of significant concern, but can be difficult due to low contamination count numbers. Tagged bacteriophage offer detection methods that don’t require conventional culturing that are relatively quick and reliable.

Waddell and Poppe modified a temperate bacteriophage with *n*-decanal-dependent bioluminescence and published their results in 2000. Infection of *E. coli* O157:H7 strain R508 with the modified bacteriophage produced measurable bioluminescence within 1 hour post infection [146].

Oda *et al.* modified a bacteriophage to express GFP on the phage capsid. Incubating the modified bacteriophage with a host strain of *E. coli* O157:H7 for 1 hour allowed the visualization of *E. coli* cells by fluorescence microscopy [105]. This bacteriophage was then further modified to inactivate lysozyme. Without active lysozyme, the assembled bacteriophage could not lyse the cell and instead accumulated within the bacterial host. This, combined with nutrient uptake analysis, allowed the researchers to evaluate the metabolic activity of infected cells to differentiate between dead cells, viable but nonculturable and cultivable cells [6].

Bacteriophage bioassays that indirectly detect bacteria by measuring out-put signals attached to bacteriophage capsids can result in false positives due to attachment to metabolically inactive bacteria. Vinay *et al.* developed a method that requires metabolically active bacteria to produce the detected GFP. Their phage-based detection method for pathogens in water samples used phage with the gene for GFP (green-fluorescent protein). Bacteria infected with the bacteriophage produce GFP and were detected using a portable flow cytometer. Incubating the water sample with the bacteriophage for 1 hour at 30^{o}C, which allowed time for infection with the bacteriophage and production of GFP, they were able to detect target bacteria at concentrations as low as 10 bacterial cells ml^{-1} [145].

Bacteriophage are currently under investigation for decontaminating fresh foods and food surfaces. Viazis *et al.* developed a bacteriophage mixture, BEC8, which is a combination of 8 different bacteriophage with activity against 123 different *E. coli* O157:H7 strains. They tested BEC8 for hard surface decontamination using stainless steel, ceramic and high density polyethylene chips and achieved a 4 log cfu/chip reduction [143] .

Several bacteriophage cocktails have already been developed and approved by the FDA for fresh produce decontamination, with many more currently under development. Three bacteriophage products are currently available from Intralytix; ListShield™, EcoShield™, and SalmoFresh™. ListShield™ targets strains of *Listeria monocytogenes,* EcoShield™ targets strains of *E. coli* O157:H7, and SalmoFresh™ targets strains of *Salmonella* [157]. Boyacioglu *et al.* demonstrated the efficiency of EcoShield™, a bacteriophage cocktail consisting of 3 different bacteriophage with activity against *E. coli* O157:H7, for decontaminating spinach, green leaf and romaine lettuce. They found that EcoShield™ was the most effective when fresh-cut greens were stored at 10^{o}C. EcoShield™ effectively reduced *E. coli* counts on all three leafy greens tested by greater than 3 log CFU/cm^{2} [14]. Woolston *et al.* demonstrated the effectiveness of SalmoFresh™ for decontaminating stainless steel and glass surfaces, with reductions between 2.1 and 4.3 log CFU/g. SalmoFresh™ has 6 different bacteriophage with activity against *Salmonella enterica* serovars *S. enterica* typhimurium, *S. enterica* Enteritidis, *S. enterica* Heidelberg, *S. enterica* Newport, and *S. enterica* Hadar [155].

One limitation of bacteriophage use out in the field is their inactivation by UV light. To minimize this, bacteriophage preparations could be sprayed onto crops late in

the day, thus extending the length of time the bacteriophage would be active [68]. Iriarte *et al.* investigated two alternative bacteriophage applications for controlling agricultural pathogens on tomato plants; bacteriophage mixed in soil and attenuated bacteria infected with bacteriophage. Both applications resulted in extended survival of bacteriophage and detectable bacteriophage within the tested tomato plants [63].

CHAPTER II

CHARACTERIZATION OF LYTIC SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* BACTERIOPHAGE ISOLATED FROM BOVINE FECES

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ABSTRACT: Shiga toxin-producing *Escherichia coli* are common food-borne pathogens that can cause hemorrhagic colitis and hemolytic uremic syndrome. Cattle represent the most common animal reservoir and are often responsible for contaminating soil and water supplies. Current methods for decontaminating raw beef and other food products include organic acid washes, heat, chemical sanitizers, pasteurization, UV light, ozonation, and irradiation. These treatments are not always effective on their own and can adversely affect food quality. Bacteriophage, viruses that infect and kill bacteria, represent a safe and specific decontamination method for food products. Cow manure from a cattle ranch and dairy farm were used to isolate eight bacteriophage. Their host range specificity was determined against 28 different strains of Shiga toxin-producing *E. coli*, and their lytic characteristics were observed in broth with five different strains of Shiga toxin-producing *E. coli.*

KEYWORDS: *Escherichia coli*, Enterohemorrhagic *E. coli*, EHEC, Shiga toxin-producing *E. coli,* STEC, *E. coli* O157:H7, bacteriophage, bacteriophage therapy, bacteriophage biocontrol

Introduction

Many strains of *Escherichia coli* have been implicated in both human and animal diseases, which are often characterized by diarrhea and can vary substantially in their severity. Among the diarrheagenic strains of *E. coli* are the Shiga-toxin producing *E. coli* (STEC) strains. These strains emerged in the 1980s as serious food-borne pathogens and are linked as the causative agent of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) [18] [22] [27]. Hemorrhagic colitis is characterized by afebrile bloody

diarrhea [30]. HUS is characterized by acute renal failure, thrombocytopenia and microangiopathic hemolytic anemia [18]. Children and the elderly are at the greatest risk of HUS, with 2 to 5% of cases resulting in death, most commonly caused by cerebral bleeds or strokes [5] [23].

STEC strains gain their pathogenicity through a lysogenic bacteriophage that produces proteins almost identical to the toxins produced by *Shigella dysenteriae* [22]. These toxins are called Shiga toxins, and they target endothelial cells in the kidneys, brain and intestinal submucosa by binding Globotriaosylceramide (Gb3), a glycosphingolipid membrane receptor [3] [26]. Shiga toxins enter cells with sufficient Gb3 receptors and inhibit protein synthesis by targeting the ribosome, which eventually leads to cell death [25] [26]. In addition to Gb3 receptors, Shiga toxins can bind to the Pk antigen on red blood cells, which induces hemolysis by activating complement [3]. Patient treatment is generally limited to supportive care, which may include dialysis, toxin neutralizers, antibodies, blood transfusions and antimicrobials [15]. Most antibiotics are contraindicated as they have been shown to make infections worse [11].

The STEC center divides *E. coli* strains that carry Shiga toxin genes based off serotypes into four classes; EHEC 1, EHEC 2, STEC 1 and STEC 2. The class EHEC 1 includes the serotypes O157:H7, O157:H- and the non-shigatoxin producing serotype O55:H7. EHEC 2 includes the serotypes O111:H8, O111:H11, O111:H-, and O26:H11. STEC 1 includes the serotypes O113:H21, O104:H21, O146:H21, O103:H6, O15:H27, OX3:H21 and O91:H21. The last class, STEC 2 includes O103:H2, O103:H6 and O45:H2. Strains from both EHEC 1 and EHEC 2 have been recovered from patients with

HC or HUS with the exception of O55:H7. STEC 1 strains are often isolated from both human and bovine cases of disease [33].

The most common reservoir for STEC strains are cattle, which shed STEC in their feces. Other animal reservoirs include deer, sheep, goats, pigs, cats, dogs, chickens and gulls [12] [14] [28]. Due to water runoff, contaminated feces can result in both water and soil contamination, which if ingested, can result in illness [25] [35]. The largest outbreak of STEC in North America between 1992 and 1993 included four states and infected 732 individuals. Hospitalization was required for 195 and four individuals died. This outbreak was linked to undercooked hamburgers from a fast-food restaurant chain that were contaminated with *E. coli* O157:H7, one of the most common and most pathogenic STEC serotypes in the United States and Canada [4] [10]. The largest recorded outbreak occurred in Japan in 1996 and involved *E. coli* O157:H7 contaminated radish sprouts. This outbreak infected almost 10,000 people, mostly school children, and resulted in 11 deaths [40]. Outbreaks have been tied to undercooked meat, raw vegetables, unpasteurized apple juice, flour, mayonnaise, recreational water and even municipal water supplies [7] [8] [12] [28] [35].

Common methods for controlling STEC contamination on food products include organic acid washes, heat, chemical sanitizers, pasteurization, UV light, ozonation, and irradiation [2] [13] [36]. While each of these methods reduce contamination levels, they are often insufficient on their own, requiring multiple interventions and can affect the quality of the food [20] [39]. Researchers continue to look for more effective decontamination methods that are safe for consumers and preserve the quality of their

products. This has led many researchers to look towards bacteriophages as decontamination methods [38] [42].

Bacteriophage are viruses that are virulent only against specific strains of bacteria [17]. Bacteriophage life cycles include lytic, lysogenic and chronic infections. In lytic infections, replicated bacteriophage are released from the infected cell by lysing the host, resulting in a burst of bacteriophage particles [15] [41]. The high degree of specificity, minimal impact on the microbiome, self-amplifying nature, and ability to cross the blood brain barrier has made bacteriophage attractive for both biocontrol and patient therapy [42]. Commercial products are currently available that use bacteriophage for controlling food-borne pathogens and for treating patients [6] [18] [43] [44]. Several more bacteriophage preparations are currently undergoing clinical trials for patient treatment [1] [21] [29] [30].

Every bacteria-rich environment is also rich in bacteriophage, with most bacteriophage residing in the same environment as their hosts. Common environmental samples used for isolating novel bacteriophage include sewage samples, soil and water [9] [33] [37]. It was hypothesized that bacteriophage active against STEC strains could be isolated from bovine feces.

Methods and Materials

Bacteriophage Isolation

Bovine feces from a dairy and a cattle ranch were donated by students at Idaho State University. Extracts from each sample were prepared by mixing 5 g of feces in 100 ml LB broth (per liter: 10 g NaCl, 10 g tryptone, 5 g yeast extract) and incubated

overnight at 37ºC while shaking. Samples were then centrifuged at 10,000xg for 10 minutes. The supernatant for each sample was then filtered through a 0.22 µm MCE filter (Fisher) and stored @4ºC.

Extracts were then enriched with *E. coli* EDL933 by mixing 5 mL filtered extract, 1mL overnight *E. coli* EDL933 culture, 40 mL LB broth and 23 µl 2M $CaCl_2$. Enrichments were incubated overnight at 37ºC while shaking. The enrichments were then centrifuged at 3,000xg for 40 minutes at 4ºC. The supernatant for each enrichment was then filtered through a 0.22 µm MCE filter (Fisher) and stored @4ºC. Overlay plates were prepared by mixing 100 µL filtered enrichment and 150 µL overnight culture of EDL933 in 2.5 mL molten soft LB agar (0.5% agar, 10mM $CaCl_2$) kept at 47ºC poured on solid LB agar plate (2% agar, 10mM CaCl2) and incubated for 18 hours at 37ºC. Plates were then observed for any plaques (areas of clearing). Plaques on plates were cored using a sterile Pasteur pipette for removal, and placed into 1.0 mL 0.085% NaCl and .05% chloroform (Fisher). Each core was vortexed for 15 seconds every 15 minutes for 1 hour. Overlay plates were prepared with the cores by mixing 300 uL plaque core solution with 300 µL overnight culture of EDL933 in 2.5 mL molten soft LB agar (0.5% agar, 10mM $CaCl_2$) kept at 47ºC poured on solid LB agar plate (2% agar, 10mM CaCl2) and incubated for 18 hours at 37ºC. Plates were then observed for amplification of plaque numbers. The coring and subsequent overlay process was repeated three more times to obtain pure phage stocks, with the fourth overlay performed in triplicate.

The fourth overlay was used to obtain a working phage stock. After the 18hr incubation, 10 mL LB broth (10mM $CaCl_2$) was poured onto the surface of each plate and incubated at ambient air temperature for 24 hours. Broth from the plates was then

collected, combined for each bacteriophage isolate and centrifuged for 20 minutes at 1,500xg at 4°C. The samples were then centrifuged for an additional 20 minutes at 3,000xg at 4°C. The supernatant for each phage stock was then filtered through a 0.22 µm MCE filters (Fisher) and stored @ 4°C. Phage stock titers were determined by serially diluting phage stocks and overlay plating 100µL diluted phage stocks mixed with 300 µL overnight EDL933 culture in 2.5 mL molten soft LB agar (0.5% agar, 10mM $CaCl_2$) kept at 47°C poured on solid LB agar plate (2% agar, 10mM CaCl2) and incubated for 18 hours at 37°C. Plaque numbers were then recorded for each dilution for each phage stock.

Host Range

Seventy-two different *E. coli* strains were obtained from Dr. Catherine Spiegel and the STEC Center. Each strain was plated to determine the presence/absence of induced prophage and degree of biofilm formation on plates. An overlay plate with each strain of *E. coli* was prepared by mixing 300 µL LB broth (100mM $CaCl_2$) and 300 µL overnight *E. coli* culture in 2.5 mL molten soft LB agar (0.5% agar, 10mM $CaCl_2$) kept at 47°C poured on solid LB agar plate (2% agar, 10mM CaCl2) and incubated for 18 hours at 37°C.

28 different *E. coli* strains with no or low numbers of induced prophage and smooth lawns were chosen for testing with phage stocks. An overlay plate with each selected strain of *E. coli* was prepared by mixing 300 µL phage stock (diluted to ~3000 plaque forming units (PFU)/mL) and 300 µL overnight *E. coli* culture in 2.5 mL molten soft LB agar (0.5% agar, 10mM $CaCl_2$) kept at 47°C poured on solid LB agar plate (2% agar, 10mM CaCl2) and incubated for 18 hours at 37°C. Plates were then observed for plaque counts.

Absorbance Assay

Five strains of *E. coli* that demonstrated significant susceptibility to the phage stocks (EDL933, 8257, DEC16A, EK33, & DA-33) were then tested to determine how the phage stocks affected growth over time. Phage stocks were diluted in 10mM $CaCl_2$ LB broth to a concentration of $1.4x10^8$ PFU/mL. *E. coli* strains were grown overnight at room temperature in 10mM $CaCl_2$ LB broth. Each phage stock was tested in triplicate, with 2.0 mL of each diluted phage stock added to a sterile test tube followed by the addition of *E. coli*. For controls, also performed in triplicate, 2.0 mL of 10mM $CaCl_2$ LB broth was added to a sterile borosilicate glass test tube (13x100mm, Fisher), followed by the addition of *E. coli*. Immediately after the addition of *E. coli*, the absorbance of the broth was measured at 600nm on a spectrophotometer (Genesys 20, ThermoSpectronic) with a target absorbance of 0.050. At the target absorbance, the initial *E. coli* concentration should have been around $5.0x10^7$ colony forming units/mL (cfu/mL). After measuring the absorbance, test tubes were placed on a shaker at room temperature until the next absorbance reading. Absorbance readings at 600 nm were taken every 2 hours, for 6 hours after briefly vortexing samples. Each experiment was repeated on four different days to produce a total of 12 different readings for each phage stock against each of the five strains of *E. coli*.

Cocktail Absorbance Assay

The five strains of *E. coli* (EDL933, 8257, DEC16A, EK33, & DA-33) were then tested against a cocktail mixture of three bacteriophage; PS1, PS7 and PS16. Phage stocks 1, 7, and 16 were mixed together in LB broth (10mM $CaCl_2$) each at a concentration of $4.7x10^7$ PFU/mL, making the total phage particles in the cocktail

$1.41x10^8$ PFU/ml. Eighteen hour cultures of each strain were diluted in 2 mL of the bacteriophage cocktail to an initial absorbance of approximately 0.030 at 600 nm (Genesys 20, ThermoSpectronic) in borosilicate glass test tubes (13x100 mm, Fisher). Absorbance readings at 600 nm were taken every 2 hours, for 6 hours after briefly vortexing samples. Each strain was tested against the bacteriophage cocktail in triplicate, with experiments repeated on two additional days.

Nucleic Acid Isolation

Nucleic acids were isolated from each bacteriophage stock using MagJET Viral DNA and RNA Kit (Thermo Scientific) following protocol B. Isolated nucleic acids were then digested with Plasmid-Safe™ ATP-Dependent DNase (Epicentre Biotechnologies) and RNase A (5 Prime). Digests and undigested nucleotides, along with λ DNA cut with Hind III, were then run on a 1% agarose gel strained with ethidium bromide at 72V for 1.5 hours. Gels were then observed under UV light using a Bio-Rad ChemiDoc Gel Imager.

Results

Bacteriophage Isolation

Eight bacteriophage with activity against *E. coli* EDL933 were isolated from bovine feces and named phage stocks (PS) 1, 2, 3, 4, 7, 8, 13 and 16. Phage stocks 1, 2, 3, 4, 7, and 8 were all isolated from cow manure from a dairy farm, while phage stocks 13 and 16 were isolated from cow manure from a cattle ranch. All eight bacteriophage demonstrated amplification upon subsequent cores, with plaque size <0.5 mm in diameter when plated with EDL933. Undiluted phage stocks were as follows: PS1contained

$1.17x10^9$ PFU/mL, PS2 contained $4.30x10^9$ PFU/mL, PS3 contained $3.5x10^9$ PFU/mL, PS4 contained $1.05x10^{10}$ PFU/mL, PS7 contained $7.10x10^9$ PFU/mL, PS8 contained $1.17x10^{10}$ PFU/mL, PS13 contained $1.65x10^{10}$ PFU/mL and PS16 contained $6.53x10^9$ PFU/mL.

Host Range Specificity

The lawn results and number of background plaques for each *E. coli* strain are shown in Table 1. Nine of the 72 strains had 10 or more background plaques when plated only with LB broth (10mM $CaCl_2$), while eight of the 72 strains produced a thick biofilm during culture that caused the lawns to be speckled with broken pieces of biofilm. Strains that demonstrated smooth lawns and limited number of prophage plaques chosen for testing against the 8 phage stocks.

The host range results against the 28 strains of *E. coli* are shown in Table 2. At ~3000 pfu/ml, confluent lysis was not observed, but sufficient phage particles were expected to result in plaque formations on susceptible strains. The strains were divided into their EHEC1, EHEC2, STEC1 and STEC2 groupings from the STEC Center [34]. Nine of the strains belong to EHEC1, with 7 strains belonging to the O157:H7 serotype and two belonging to the O55:H7 serotype. None of the eight phage stocks produced >50 plaques, the minimum plaques required to call a strain susceptible, with the two O55:H7 strains. The strain G5101 was the only O157:H7 strain that was not susceptible to any of the phage stocks. The other six O157:H7 strains were susceptible to all eight phage stocks producing plaques <0.5 mm in diameter.

There were six different EHEC2 strains tested; 928/91, DEC8C, BCL19, DEC10C, DEC8B, & 8361. None of the six tested strains were found to be susceptible to

any of the eight phage stocks. Only four STEC1 strains were tested; G5506, 90-1787, 23/67, & DEC16A. Only DEC16A, with serotype O113:H21 was susceptible to the phage stocks, with all eight phage stock producing >1000 plaques. The plaque morphology for the 8 phage stocks showed variation against DEC16A with phage stocks 1,2 & 3 producing plaques ~1.0mm in diameter, while the other five phage stocks all produced plaques <0.5 mm in diameter.

Six STEC2 strains were tested; EK33, TB154A, DA-33, RW1372, 8419, & DA-21. Only EK33 and DA-33 were sensitive, with all 8 phage stocks showing virulence producing plaques <0.5 mm in diameter. Three additional *E. coli* strains have not been assigned to any of the STEC groupings were tested: DA-69, 555932 & K-12. None of the eight phage stocks were active against these three strains.

Absorbance assay

To help determine any differences between the eight different phage stocks, their lytic characteristics in broth at room temperature were tested against 5 different strains of *Escherichia coli* that had demonstrated strong activity on agar plates. The strains chosen, highlighted in Table 2, were EDL933, 8257, DEC16A, EK33 and DA-33. Both EDL933 and 8257 belong to the EHEC1 group of Shiga toxin-producing *E. coli*, while DEC16A belongs to the STEC1 group, and EK-33 and DA-33 belong to the STEC2 group of Shiga toxin-producing *E. coli*. For each strain, the average absorbance for each experimental sample at each time point was graphed on a scatter plot. Error bars show the standard deviation for each experimental sample at each time point. Due to the impact of fluctuating room temperatures and variations in initial absorbance readings, all experiments were looked at individually for trends. These trends, rather than exact

absorbance readings were then compared to the other experiments for each strain. For all five strains of *E. coli*, the absorbance values measured for the controls was higher than the phage stocks. By removing the control data from the graphs, the phage stock trends could be examined.

The first strain to be examined was EDL933, with absorbance values recorded in Table 3. The graphed data from each experiment with EDL933 is displayed in Figures 1, 2, 3 and 4. Figure 1 is the data from the first experiment, Figure 2 is the data from the second experiment, Figure 3 is the data from the third experiment and Figure 4 is the data from the fourth experiment. In all four experiments, the final absorbance readings of the controls were around three times larger than the final absorbance of any of the phage stocks. Figures 5, 6, 7 and 8 show the data from each experiment with EDL933 without the control. Figure 5 is the data from the first experiment, Figure 6 is the data from the second experiment, Figure 7 is the data from the third experiment and Figure 8 is the data from the fourth experiment.

The second strain of *E. coli* to be tested was DEC16A, with absorbance values recorded in Table 4. The data from each experiment with DEC16A is displayed in Figures 9, 10, 11 and 12. Figure 9 is the data from the first experiment, Figure 10 is the data from the second experiment, Figure 11 is the data from the third experiment and Figure 12 is the data from the fourth experiment. In all four experiments, the final absorbance readings of the controls were between 1.7 and 2.7 times larger than the final absorbance of any of the phage stocks. Figures 13, 14, 15 and 16 show the data from each experiment with DEC16A without the control. Figure 13 is the data from the first

experiment, Figure 14 is the data from the second experiment, Figure 15 is the data from the third experiment and Figure 16 is the data from the fourth experiment.

The third strain of *E. coli* to be tested was 8257, with absorbance values recorded in Table 5. The data from each experiment with 8257 is displayed in Figures 17, 18, 19 and 20. Figure 17 is the data from the first experiment, Figure 18 is the data from the second experiment, Figure 19 is the data from the third experiment and Figure 20 is the data from the fourth experiment. In all four experiments, the final absorbance readings of the controls were around 1.7 times larger than the final absorbance of any of the phage stocks. Figures 21, 22, 23 and 24 show the data from each experiment with 8257 without the control. Figure 21 is the data from the first experiment, Figure 22 is the data from the second experiment, Figure 23 is the data from the third experiment and Figure 24 is the data from the fourth experiment.

The fourth strain of *E. coli* to be tested was DA-33, with absorbance values recorded in Table 6. The data from each experiment with DA-33 is displayed in Figures 25, 26, 27 and 28. Figure 25 is the data from the first experiment, Figure 26 is the data from the second experiment, Figure 27 is the data from the third experiment and Figure 28 is the data from the fourth experiment. In all four experiments, the final absorbance readings of the controls were around 3 times larger than the final absorbance of any of the phage stocks. Figures 29, 30, 31 and 32 show the data from each experiment with DA-33 without the control. Figure 29 is the data from the first experiment, Figure 30 is the data from the second experiment, Figure 31 is the data from the third experiment and Figure 32 is the data from the fourth experiment.

The last strain of *E. coli* to be tested was EK-33, with absorbance values recorded in Table 7. The data from each experiment with EK-33 is displayed in Figures 33, 34, 35 and 36. Figure 33 is the data from the first experiment, Figure 34 is the data from the second experiment, Figure 35 is the data from the third experiment and Figure 36 is the data from the fourth experiment. In all four experiments, the final absorbance readings of the controls were between 2 and 3 times larger than the final absorbance of any of the phage stocks. Figures 37, 38, 39 and 40 show the data from each experiment with EK-33 without the control. Figure 37 is the data from the first experiment, Figure 38 is the data from the second experiment, Figure 39 is the data from the third experiment and Figure 40 is the data from the fourth experiment.

Cocktail Absorbance Assay

To further investigate any differences between the phage stocks a cocktail was prepared by mixing PS1, PS7 and PS16, and the absorbance experiment was repeated with the cocktail against each of the 5 strains of *E. coli* in place of the pure phage stocks. The experiment was repeated on three different days to produce a total of 9 different readings against each of the five strains of *E. coli*. The absorbance readings are recorded in Table 8. The average absorbance for each experimental sample at each time point was graphed on a scatter plot. Error bars show the standard deviation for each experimental sample at each time point.

Figure 41 shows the bacteriophage cocktail results against EDL933. All three controls had a final absorbance reading between 0.33 and 0.35, while the final absorbance readings for the three cocktail experiments were between 0.21 and 0.11. The results from the cocktail against DEC16A are shown in figure 42. All three controls had a final

absorbance reading between 0.36 and 0.37. The final absorbance readings for the three cocktail experiments were all below 0.100.

Figure 43 displays the results from the cocktail against strain 8257. All three controls had a final absorbance reading between 0.26 and 0.29. The final absorbance readings for the three cocktail experiments were all between 0.22 and 0.29 with overlapping error bars with the controls. The results of the bacteriophage cocktail mixed with DA-33 are shown in Figure 44. All three controls had a final absorbance readings between 0.30 and 0.33. The final absorbance readings for the three cocktail experiments were between 0.14 and 0.17. The bacteriophage cocktail results against EK33 are shown in figure 45. All three controls had a final absorbance reading between 0.33 and 0.39. The final absorbance readings for the three cocktail experiments were between 0.13 and 0.16.

Nucleic Acid Isolation

Nucleic acid digests for phage stocks 1, 2 and 3 are displayed in Figure 46. In lanes 1 and 12, the banding pattern for λ-Hind III is clearly visible. Lane 2, which contains undigested PS1 nucleic acids, has a sharp band up around the upper band for λ-Hind III. Lane 3 contains PS1 nucleic acids digested with DNAse and has no visible band. Lane 4 contains PS1 nucleic acids digested with RNAse and has a visible band slightly higher than the band in lane 2. Lane 5, which contains undigested PS2 nucleic acids, has a sharp band up around the upper band for λ-Hind III. Lane 6 contains PS2 nucleic acids digested with DNAse and has no visible band. Lane 7 contains PS2 nucleic acids digested with RNAse and has a visible band slightly higher than the band in lane 5. Lane 8, which contains undigested PS3 nucleic acids, has a sharp band up around the upper band for λ-Hind III. Lane 9 contains PS3 nucleic acids digested with DNAse and

has no visible band. Lane 10 contains PS3 nucleic acids digested with RNAse and has a visible band slightly higher than the band in lane 8. Lane 11 has no visible bands and was not loaded with nucleic acids.

Nucleic acid digests for phage stocks 4, 7 and 8 are displayed in Figure 47. In lanes 1 and 12, the banding pattern for λ-Hind III is clearly visible. Lane 2, which contains undigested PS4 nucleic acids, has a sharp band up around the upper band for λ-Hind III. Lane 3 contains PS4 nucleic acids digested with DNAse and has no visible band. Lane 4 contains PS4 nucleic acids digested with RNAse and has a visible band slightly higher than the band in lane 2. Lane 5, which contains undigested PS7 nucleic acids, has a sharp band up around the upper band for λ-Hind III. Lane 6 contains PS7 nucleic acids digested with DNAse and has no visible band. Lane 7 contains PS7 nucleic acids digested with RNAse and has a visible band around the same height as the band in lane 5. Lane 8, which contains undigested PS8 nucleic acids, has a sharp band up around the upper band for λ-Hind III. Lane 9 contains PS8 nucleic acids digested with DNAse and has no visible band. Lane 10 contains PS8 nucleic acids digested with RNAse and has a visible band slightly higher than the band in lane 8. Lane 11 has no visible bands and was not loaded with nucleic acids.

Nucleic acid digests for phage stocks 13 and 16 are displayed in Figure 48. In lanes 1 and 12, the banding pattern for λ-Hind III is clearly visible. Lane 3, which contains undigested PS13 nucleic acids, has a sharp band up around the upper band for λ-Hind III. Lane 4 contains PS13 nucleic acids digested with DNAse and has no visible band. Lane 5 contains PS13 nucleic acids digested with RNAse and has a visible band slightly higher than the band in lane 3. Lane 7, which contains undigested PS16 nucleic

acids, has a sharp band up around the upper band for λ-Hind III. Lane 8 contains PS16 nucleic acids digested with DNAse and has no visible band. Lane 9 contains PS16 nucleic acids digested with RNAse and has a visible band slightly higher than the band in lane 7. Lanes 2, 6, 10 and 11 have no visible bands and were not loaded with nucleic acids.

Discussion

Host Range

For the host range determination, strains that produced uneven agar surfaces due to biofilm production or strains with high numbers of induced prophage were not chosen due to difficulty in obtaining accurate plaque counts. Of the 28 strains chosen for testing host range, all 8 phage stocks shared identical host range specificities, with only slight variations in plaque counts. All eight phage stocks were <0.5mm in diameter against all of the strains tested, with the exception of DEC16A. On DEC16A, phage stocks 1, 2 and 3 were ~1.0mm in diameter while phage stocks 4, 7, 8, 13 and 16 were <0.5mm in diameter. While the current host range does not show any definitive differences between the 8 phage stocks, this difference in morphology does suggest that phage stocks 1, 2, and 3 are not the same as the other five phage stocks.

EHEC1 strains showed the greatest number of susceptible strains with all 8 phage stocks showing activity against most of the O157:H7 serotypes. *E. coli* strain G5101 was the only O157:H7 strain that was not susceptible to the 8 phage stocks, suggesting that further testing against other O157:H7 serotypes may provide variations in host range between the 8 phage stocks.

While none of the tested EHEC2 strains were sensitive to the 8 phage stocks, other serotypes belonging to the EHEC2 groups were not tested such as O45:NM and O70:H11. It is currently unknown if the 8 phage stocks may have activity against any strains within these two serotypes. Other EHEC2 strains not tested may also be sensitive to the phage stocks. As demonstrated by G5101, serotype alone is not a predictor of sensitivity.

The only STEC1 strain that was susceptible to the phage stocks was DEC16A, which is serotype O113:H21. No other O113 strains were among the 28 tested strains. Like the O157:H7 strains, it is possible that the 8 bacteriophage stocks may have activity against many O113 strains.

The majority of STEC2 strains tested, 5 of 6, had the O103 antigen, but only two O103 strains were susceptible to the 8 phage stocks. Both the O antigen and the H antigen varied between susceptible strains within the STEC groupings, indicating that the receptors the 8 bacteriophage use are not likely the O or H antigens. DA-33 is nonmotile, with no detectable flagellar antigen, with the other susceptible strains include the H21, H7, & H2 antigens. O antigens include O157, O113, & O103. Further host range testing with other strains within these serotypes and with other untested serotypes may provide differences in host range not yet detected in the 28 strains tested.

Absorbance Assay

In all four absorbance experiments with EDL933, PS1 had a final absorbance that was higher than the other 7 phage stocks with no overlap of error bars. Additionally, in experiments 1, 3 and 4, the increase in absorbance is fairly linear while the other phage stocks had inflection and/or deflection points. In experiment 2, the final absorbance

readings of the other seven phage stocks are very close with overlapping error bars. However, in experiments 1, 3, and 4 they appear to separate into two different groups. The group with the lowest final absorbance readings contains PS2, PS3 and PS16. These three phage stocks produced similar patterns with their absorbance readings and a significant amount of overlap with error bars at all time points. The second group of phage stocks contains PS4, PS7, PS8 and PS13. Their final absorbance readings all fell below PS1 and shared significant overlap with error bars. However, PS8 and PS13 seem to share a similar shape to their graphs that is different from PS4 and PS7. Both PS8 and PS13 seem to have a larger increase in absorbance over the first four hours than PS7. Between hour 4 and hour 6, PS7 experiences a sharp increase in absorbance readings whereas PS8 and PS13 experience their sharpest increase in absorbance in the first two hours. These pattern differences are the most distinct in experiment 1, shown in Figure 5, but the same pattern can be seen in all four experiments. In experiment 3, PS4 expressed a pattern very similar to PS 7 with significant overlap of error bars at all time points. However, this shared pattern was absent in experiments 1, 2, and 4, with PS4 showing a pattern that more closely resembled PS8 and PS13.

The grouping patterns of the eight phage stocks with EDL933 suggests the possibility that PS2, PS3 and PS16 are the same or very closely related bacteriophage and that PS1 is unique from the other eight phage stocks. The remaining four phage stocks may also be the same or very closely related bacteriophage. However, given the different pattern displayed by PS7 from PS8, PS13 and PS4, this last group may consist of two, possibly three different bacteriophage.

DEC16A demonstrated a similar pattern to EDL933 with phage stock 1. In experiments 1, 2, and 3, PS1 had a final absorbance reading that was around 1.4 times higher than the other phage stocks. This further suggests that PS1 is different from the other eight phage stocks. PS1 is only slightly higher than the other phage stocks in experiment 4. Comparing figure 16 to figures 13, 14 and 15, all of the absorbance readings for the different phage stocks were significantly higher on experiment 4, including PS1. All eight phage stocks increased significantly more in absorbance between hours 4 and 6 during experiment 4 than in the other three experiments. However, comparing figure 12 to figures 9, 10 and 11, the final absorbance reading on the control in experiment 4 is very similar to the final absorbance readings for the controls in the other three experiments. Warm summer weather causing laboratory temperature fluctuations could be the cause of the discrepancy between experiment 4 and the other three experiments.

The only phage stock to demonstrate a different pattern of absorbance readings against DEC16A was PS1. The other 7 phage stocks all had final absorbance readings that were very close with overlapping error bars. The kinetics for each phage stock were not consistent enough between experiments to show any further differences between the phage stocks. Against DEC16A, it would appear that PS2, PS3, PS4, PS7, PS8, PS13 and PS16 could all be the same, or very closely related, bacteriophage.

E. coli strain 8257 also had high absorbance readings for phage stock 1. In experiments 1, 2, and 3, PS1 had a final absorbance reading that was higher than the other phage stocks with no overlapping error bars. Again, suggesting that PS1 is different from the other eight phage stocks. PS1 is only slightly higher than the other phage stocks

in experiment 4. Comparing figure 24 to figures 21, 22 and 23, the absorbance readings for the other phage stocks were very similar in all four experiments. The only discrepancy is PS1, however, despite the final absorbance being lower on experiment 4, the error bars from PS1 do not overlap with the error bars of any other phage stock.

The only phage stock to demonstrate a clearly different pattern of absorbance readings against 8257 was PS1. The other 7 phage stocks all had final absorbance readings that were very close with overlapping error bars. As seen against DEC16A, the kinetics for each phage stock were not consistent enough between experiments to suggest any further differences between the phage stocks. Against 8257, it would appear that PS2, PS3, PS4, PS7, PS8, PS13 and PS16 could all be the same, or very closely related, bacteriophage.

With DA-33, in all four experiments PS1 had a final absorbance that was higher than the other 7 phage stocks with no overlap of error bars. The other 7 phage stocks all had final absorbance readings that were very close with overlapping error bars. Unlike against DEC16A and 8257, there do appear to be consistent differences in kinetics against DA-33. In all four experiments, PS8 and PS13 display a concave curve and follow each other very closely. PS4 has a similar pattern as PS8 and PS13, however, it does not follow quite as closely. In all four experiments, PS2, PS3 and PS16 display a convex curve and follow each other very closely. PS7 has a similar pattern as PS2, PS3 and PS16, however, it does not follow quite as closely. Experiment 4, shown in figure 32, displays these shapes the clearest, however, experiment 3, shown in figure 33, displays the greatest separation from these two groups.

The grouping patterns of the eight phage stocks with DA-33 are very similar to the grouping patterns against EDL933. This further suggests the possibility that PS2, PS3 and PS16 are the same or very closely related bacteriophage and that PS1 is unique from the other eight phage stocks. It is very likely that PS8 and PS13 are also either the same, or very closely related bacteriophage.

EK-33 was the only strain tested that displayed a decrease in absorbance between time points. This was observed in experiments 1, 3 and 4 and was strongly tied to warm summer weather causing laboratory temperature fluctuations. Cooler temperatures caused by air-conditioning vents blowing on the samples appears to increase the effectiveness of PS2, PS3, PS4, PS7, PS8, PS13 and PS16. Only PS1 did not cause a drop in absorbance. These drops occurred between hours 4 and 6 in experiments 1, 3, and 4. Hours 4 and 6 also corresponded to the hottest hours of the day when the air-conditioning vents were running the longest. The kinetics for each phage stock were not consistent enough between experiments to suggest any further differences between the phage stocks. Against EK-33, it would appear that PS2, PS3, PS4, PS7, PS8, PS13 and PS16 could all be the same, or very closely related, bacteriophage, while PS1 is most likely unique.

Cocktail Absorbance Assay

The control experiments for EDL933 when tested with the bacteriophage cocktail all had final average absorbance readings between 0.33 and 0.35 which is lower than the final absorbance readings from the previous absorbance experiments with EDL933. The final average absorbance readings for the three cocktail experiments were between 0.21 and 0.11 which is fairly consistent with the results of PS1 from the previous experiments. Overnight cultures of all five *E. coli* strains did not grow as well as the previous

experiments, resulting in starting absorbance values between 0.030 and 0.038. This likely explains the lower control absorbance readings. However, this should have resulted in lower absorbance readings for the phage stocks as well. It appears that mixing the three phage stocks together did not improve the effectiveness of the bacteriophage against EDL933, and may have even decreased them.

In the cocktail experiments against DEC16A, all three control experiments had final average absorbance readings between 0.36 and 0.37 which is lower than the final absorbance readings from the previous absorbance experiments with DEC16A, consistent with the lower starting absorbance readings. The final average absorbance readings for the three cocktail experiments were all below 0.100 which is lower than the phage stocks from the previous experiments, especially PS1. The lower absorbance readings may be due to the lower starting absorbance or mixing the three phage stocks together improved the effectiveness of the bacteriophage against DEC16A.

For strain 8257, when tested against the cocktail, all three controls had a final average absorbance readings between 0.26 and 0.29. These readings are consistent with the final absorbance readings from the previous absorbance experiments with 8257, despite the lower starting absorbance values. The final average absorbance readings for the three cocktail experiments were all between 0.22 and 0.29 with overlapping error bars with the controls. Previous experiments had shown that individually, all three phage stocks slowed the growth of 8257. However, mixing the three phage stocks seems to have an antagonistic effect eliminating the effectiveness of the bacteriophage against 8257. This antagonistic effect indicates that PS1, PS7 and PS16 are not the same bacteriophage, with at least one of them different from the others.

When DA-33 was mixed with the cocktail, all three controls had final average absorbance reading between 0.30 and 0.33 which is lower than the final absorbance readings from the previous absorbance experiments with DA-33. This was consistent with the lower starting values. The average final absorbance readings for the three cocktail experiments were between 0.14 and 0.17 which is higher than the phage stocks from the previous experiments, but fairly consistent with PS1. Mixing the three phage stocks together did not improve the effectiveness of the bacteriophage against DA-33.

The cocktail experiments with EK33 resulting in the three controls having final average absorbance readings between 0.33 and 0.39. These are slightly lower than the final absorbance readings from the previous absorbance experiments with EK-33 and consistent with the lower starting values. The final average absorbance readings for the three cocktail experiments were between 0.13 and 0.16 which is higher than the phage stocks from the previous experiments, but fairly consistent with PS1. Unlike the previous experiments with EK-33, there was no decrease in absorbance values between hours four and six. It is possible that the laboratory temperatures were more consistent during the week of experimentation. However, mixing the three phage stocks together did not improve the effectiveness of the bacteriophage against EK-33.

Nucleic Acid Isolation

The undigested nucleic acids from all 8 phage stocks produced a band around the upper λ-HindIII band, indicating similar sized genomes for all 8 bacteriophage. When the nucleic acids from all 8 phage stocks were digested with Plasmid-Safe™ ATP-Dependent DNase, no visible bands were observed on the gels, indicating the nucleic acids had been fragmented. Alternatively, when the nucleic acids from all 8 phage stocks were digested

with RNAse A, bands were still visible on the gels, indicating the RNAse was unable to fragment the nucleic acids. These results indicate that the genomes of all 8 bacteriophage stocks are made of DNA rather than RNA. Additionally, the genomes are most likely linear rather than circular. If the genomes were circular, the Plasmid-Safe™ ATP-Dependent DNase would have left the nucleic acids intact.

Conclusion

All of the absorbance experiments were limited by the concentration of the phage stocks and the accuracy of the spectrophotometer. Phage stock 1 had the lowest concentration of the phage stocks, with only $1.17x10^9$ PFU/mL. This limited the concentration of bacteriophage that could be used in the experiments. Additionally, the spectrophotometer used was only accurate with absorbance readings above 0.020. Any sample below 0.020 would have absorbance readings fluctuating rapidly +/- 0.005. This required the starting absorbance to be aimed around 0.050 to accurately detect any drops after the initial reading. At an absorbance of 0.050, the *E. coli* were around $5.0x10^7$ cfu/mL. More dramatic results may have been obtained using more concentrated bacteriophage or lower starting *E. coli* concentrations. Longer running experiments may also allow the phage stocks to differentiate themselves more.

Temperature appeared to be a factor in the absorbance experiment, at least with EK33. It may be worth repeating the experiment in a more controlled temperature setting, to eliminate temperature fluctuations due to heating and cooling cycles. It would also be beneficial to determine the most active temperature for the bacteriophage. Smith *et al.* found that each of their bacteriophage stocks had different ideal temperature ranges, and

that outside of those ideal ranges the bacteriophage virulence dramatically decreased [32].

Given the two different environmental sources for the bacteriophage, phage stocks 13 &16, which came from cow manure from a cattle ranch, are likely different from phage stocks 1, 2, 3, 4, 7, & 8, which came from cow manure from a dairy farm. However, since they are both environmental samples from Idaho, winds could have spread bacteriophage between farms. The different sources alone are not enough to indicate different bacteriophage.

The absorbance experiments with all five strains suggest that PS1 is different from the other seven phage stocks. With both EDL933 and DA-33, the absorbance experiments further divide the remaining seven bacteriophage into three possible groupings, with the first grouping including PS2, PS3 and PS16. The second grouping placed PS4 and PS7 together, while the last grouping placed PS8 and PS13 together. Both PS2 and PS3 had different plaque morphology from PS16 when plated against DEC16A, suggesting PS2 and PS3 may be the same bacteriophage, but PS16 is a different bacteriophage. While it is possible that all eight bacteriophage are unique from each other, these combined experiments suggest five different bacteriophage strains. Further testing is required to conclusively determine the number of unique bacteriophage.

Based on the type of genomes and the isolation procedure, the 8 phage stocks investigated most likely belong to the families *Myoviridae*, *Siphoviridae*, or *Podoviridae*. These families of bacteriophage all fall within the order *Caudovirales*, and are known as the tailed bacteriophages. The only bacteriophage families that possess linear DNA genomes are *Myoviridae, Siphoviridae, Podoviridae* and *Tectiviridae*. However,

Tectiviridae have protein-rich membranes that surround their protein capsids [22]. The chloroform used in the isolation of the bacteriophage would have destroyed any tectivirus membranes. It is unlikely that any of the 8 phage stocks belong to *Tectiviridae*, rather the 8 phage stocks are most likely tailed bacteriophage from the order *Caudovirales*.

The temperature sensitive behavior for 7 of the eight bacteriophage with EK33 indicate that these bacteriophage may be ideally suited for biocontrol in food manufacturing. Slaughterhouses and other food plants often use low temperatures as a measure to control for bacterial growth [2] [6] [17]. Bacteriophage that are especially lytic at these low temperatures may prove to be a useful addition to their sanitation methods.

Table 1. Background plaques from induced prophage and lawn conditions of 72 strains of *E. coli*.

Tube #	Serotype	Isolate	# of plaques	Group	Tube #	Serotype	Isolate	# of plaques	Group
1	O157:H7	EDL933	0	EHEC1	37	O91:H21	B2F1	4	STEC1
2	O157:H7	93-111	1	EHEC1	38	O146:H21	DEC16E	3, Biofilm	STEC1
3	O157:H7	OK-1	0	EHEC1	39	O103:H6	TB154A	0	STEC2
4	O157:H7	86-24	1	EHEC1	40	O15:H27	88-1509	6, Biofilm	
5	O157:H7	2886-75	0	EHEC1	41	0125:NM	8153B-86	12	
6	O157:H-	493/89	5	EHEC1	42	O15:H27	M2133	5	
7	O157:H-	E32511	5	EHEC1	43	O55:N	BCL17	8	
8	O157:H7	G5101	0	EHEC1	44	OX03:NM	90-1787	1	STEC1
9	O55:H7	5905	2	EHEC1	45	O45:NM	DA-21	1	STEC2
10	O55:H7	TB182A	20	EHEC1	46	O45:H2	B8227-C8	3	STEC2
11	O55:H7	DEC5D	2	EHEC1	47	O103:NM	DA-33	2	STEC2
12	O55:H7	3256-97	7	EHEC1	48	O103:NT	89-7321	4	STEC2
13	O103:H2	EK33	1	STEC2	49	O103:H2	RW1372	2	STEC2
14	O111:H8	CL-37	7	EHEC2	50	O121:H19	8-084	20	
15	O111:H8	DEC8B	1	EHEC2	51	O121:H7	403-3	4	
16	O111:NM	3007-85	4, Biofilm	EHEC2	52	O121:H10	8-682	4	
17	O111:H-	TB226A	7	EHEC2	53	O121:NM	DA-69	2	
18	O111:H-	928/91	2	EHEC2	54	O145:H+	314-S	7	
19	O111	412/55	12	EHEC2	55	O145:NT	IH 16	4	
20	O111:NM	DEC8C	1	EHEC2	56	O145	555932	0	
21	O111	C412	14	EHEC2	57	O103:H25	8419	2	STEC2
22	O26:H11	H19	3	EHEC2	58	O91:H7	23/67	0	STEC1
23	O26:H11	DEC10B	5	EHEC2	59	O146:H21	E851/71	6	STEC1
24	O26:H11	DEC10C	0	EHEC2	60	O142:H6	C771	9, thin lawn	
25	O26:NM	DEC9F	1, Biofilm	EHEC2	61	O111:NM	8361	2	EHEC2
26	O26:H-	TB285C	3	EHEC2	62	O157:H7	8257	1	EHEC1
27	O26	VP30	12	EHEC2	63	O146:H21	7606	3, Biofilm	STEC1
28	O157:NM	RDEC-1	8	EHEC2	64	O157:H7	7004	6	EHEC1
29	N:NM	BCL19	2	EHEC2	65	O145:NM	6940	7	
30	O70:H11	DEC10J	17	EHEC2	66	O145:NM	6986	7, Biofilm	
31	O111:H-	ED-31	10	EHEC2	67	unknown:NM	6650	5	
32	O45:NM	4309-65	3	EHEC2	68	O157:H7	6581	7	EHEC1
33	O45:N	2566-58	3	EHEC2	69	O157:H7	5533	3	EHEC1
34	O113:H21	CL-3	7	STEC1	70	O121:H19	5518	25, Biofilm	
35	O113:H21	DEC16A	3	STEC1	71	O26:H11	5365	3	EHEC2
36	O104:H21	G5506	0	STEC1	72	K-12		3	

Table 2. Host range of eight phage stocks tested against 28 different strains of *E. coli* separated based off EHEC groupings from the STEC Center. All eight phage stocks diluted to ~10^4 PFU/ml. Highlighted strains selected for absorbance testing.

ISOLATE	Serotype	PHAGE STOCK								
EHEC1		PS1	PS2	PS3	PS4	PS7	PS8	PS13	P16	Control
86-24	O157:H7	~300	~500	~500	~500	~500	~500	~500	>1000	0
EDL933	O157:H7	~500	~200	~500	~300	~500	~300	~600	>1000	0
93-111	O157:H7	~300	~400	~500	~300	~300	~300	~500	>1000	0
OK-1	O157:H7	~300	~700	~700	~500	~500	~500	~500	>1000	0
2886-75	O157:H7	>1000	~700	~700	~500	~500	~500	~500	>1000	0
5905	O55:H7	1	0	0	0	0	0	0	1	0
DEC5D	O55:H7	6	0	0	0	0	0	0	0	0
8257	O157:H7	~500	>1000	~300	~700	>1000	>1000	>1000	~700	0
G5101	O157:H7	0	0	0	0	1	0	0	0	0
EHEC2										
928/91	O111:H-	0	0	0	0	0	0	0	1	0
DEC8C	O111:NM	5	0	0	0	0	0	0	3	0
BCL19	N:NM	2	0	0	0	0	0	0	0	0
DEC10C	O26:H11	2	0	0	18	0	0	5	0	0
DEC8B	O111:H8	2	0	0	0	0	0	0	1	0
8361	O111:NM	0	0	0	0	0	0	0	0	0
STEC1										
G5506	O104:H21	1	0	0	0	0	3	0	0	0
90-1787	OX03:NM	0	0	0	0	0	0	0	0	0
23/67	O91:H7	0	0	0	0	0	0	0	14	0
DEC16A	O113:H21	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	0
STEC2										
EK33	O103:H2	>1000	~150	~200	~200	~200	~200	~300	~300	0
TB154A	O103:H6	0	0	0	0	0	0	0	0	0
DA-33	O103:NM	~300	~320	~300	~100	~100	~200	~200	>1000	0
RW1372	O103:H2	0	0	26	0	0	0	0	0	0
8419	O103:H25	0	0	0	0	0	0	0	1	0
DA-21	O45:NM	3	0	20	0	0	0	0	0	0
UNKNOWN										
DA-69	O121:NM	0	0	0	0	0	0	2	1	0
555932	O145	4	0	0	0	0	0	0	0	1
K-12		0	0	0	0	0	0	0	1	0

Table 3. Recorded absorbance values for *E. coli* EDL933 mixed with 8 different phage stocks, measured at 600nm every 2 hours for 6 hour. Experiments 1, 2, 3, & 4 all performed on different days.

		Experiment 1			Experiment 2			Experiment 3			Experiment 4		
Sample	Time	Rep1	Rep2	Rep3	Rep4	Rep5	Rep6	Rep7	Rep8	Rep9	Rep10	Rep11	Rep12
EDL933 Control	0 hr	0.060	0.058	0.054	0.059	0.054	0.053	0.040	0.038	0.039	0.050	0.050	0.055
	2 hr	0.129	0.143	0.119	0.158	0.155	0.154	0.117	0.120	0.118	0.135	0.139	0.138
	4 hr	0.224	0.231	0.213	0.286	0.283	0.288	0.264	0.263	0.253	0.286	0.282	0.287
	6 hr	0.388	0.382	0.383	0.512	0.498	0.502	0.475	0.469	0.461	0.468	0.463	0.463
EDL933 + PS1	0 hr	0.068	0.057	0.062	0.062	0.066	0.062	0.051	0.052	0.047	0.060	0.060	0.065
	2 hr	0.077	0.071	0.073	0.083	0.087	0.084	0.073	0.077	0.071	0.083	0.083	0.089
	4 hr	0.099	0.090	0.092	0.113	0.114	0.115	0.094	0.097	0.090	0.110	0.111	0.119
	6 hr	0.131	0.119	0.117	0.179	0.184	0.189	0.127	0.133	0.123	0.145	0.143	0.158
EDL933 + PS2	0 hr	0.057	0.058	0.050	0.065	0.063	0.063	0.053	0.054	0.052	0.060	0.067	0.058
	2 hr	0.062	0.066	0.058	0.079	0.074	0.076	0.061	0.058	0.057	0.073	0.070	0.075
	4 hr	0.062	0.059	0.056	0.090	0.084	0.088	0.071	0.066	0.062	0.080	0.083	0.080
	6 hr	0.076	0.074	0.068	0.144	0.156	0.147	0.087	0.086	0.081	0.117	0.113	0.115
EDL933 + PS3	0 hr	0.056	0.050	0.052	0.064	0.060	0.063	0.050	0.050	0.049	0.064	0.072	0.066
	2 hr	0.061	0.057	0.058	0.078	0.075	0.077	0.060	0.057	0.060	0.071	0.078	0.079
	4 hr	0.061	0.059	0.059	0.089	0.084	0.090	0.067	0.065	0.064	0.075	0.080	0.078
	6 hr	0.074	0.074	0.071	0.140	0.135	0.141	0.089	0.092	0.091	0.116	0.113	0.106
EDL933 + PS4	0 hr	0.064	0.053	0.051	0.059	0.056	0.061	0.052	0.053	0.054	0.066	0.073	0.064
	2 hr	0.070	0.075	0.071	0.097	0.091	0.095	0.067	0.067	0.070	0.080	0.090	0.081
	4 hr	0.088	0.079	0.078	0.111	0.104	0.110	0.085	0.087	0.079	0.108	0.110	0.107
	6 hr	0.107	0.099	0.092	0.142	0.140	0.149	0.111	0.122	0.109	0.134	0.127	0.116
EDL933 + PS7	0 hr	0.064	0.059	0.061	0.062	0.064	0.058	0.054	0.054	0.055	0.070	0.060	0.065
	2 hr	0.065	0.067	0.071	0.085	0.082	0.086	0.069	0.068	0.065	0.081	0.083	0.081
	4 hr	0.073	0.069	0.070	0.103	0.092	0.093	0.078	0.081	0.082	0.102	0.101	0.096
	6 hr	0.095	0.088	0.086	0.145	0.136	0.143	0.106	0.109	0.108	0.134	0.127	0.119
EDL933 + PS8	0 hr	0.055	0.054	0.059	0.058	0.056	0.058	0.051	0.055	0.054	0.064	0.057	0.064
	2 hr	0.078	0.074	0.079	0.096	0.092	0.098	0.069	0.075	0.071	0.095	0.089	0.093
	4 hr	0.084	0.078	0.082	0.110	0.104	0.108	0.093	0.097	0.092	0.116	0.117	0.112
	6 hr	0.093	0.089	0.095	0.144	0.133	0.140	0.114	0.118	0.113	0.127	0.125	0.124
EDL933 + PS13	0 hr	0.059	0.056	0.054	0.058	0.059	0.056	0.050	0.051	0.052	0.065	0.059	0.060
	2 hr	0.089	0.083	0.080	0.099	0.101	0.102	0.070	0.076	0.075	0.107	0.095	0.109
	4 hr	0.091	0.084	0.085	0.108	0.110	0.112	0.091	0.097	0.095	0.116	0.112	0.116
	6 hr	0.095	0.089	0.087	0.129	0.132	0.137	0.112	0.117	0.112	0.127	0.124	0.122
EDL933 + PS16	0 hr	0.056	0.051	0.051	0.055	0.056	0.062	0.051	0.050	0.050	0.061	0.064	0.061
	2 hr	0.067	0.065	0.067	0.072	0.071	0.076	0.056	0.058	0.059	0.066	0.068	0.069
	4 hr	0.062	0.059	0.057	0.087	0.083	0.086	0.060	0.063	0.064	0.080	0.079	0.076
	6 hr	0.075	0.070	0.070	0.127	0.126	0.130	0.081	0.083	0.079	0.109	0.107	0.109

Table 4. Recorded absorbance values for *E. coli* DEC16A mixed with 8 different phage stocks, measured at 600nm every 2 hours for 6 hour. Experiments 1, 2, 3, & 4 all performed on different days.

		Experiment 1			Experiment 2			Experiment 3			Experiment 4		
Sample	Time	Rep1	Rep2	Rep3	Rep4	Rep5	Rep6	Rep7	Rep8	Rep9	Rep10	Rep11	Rep12
DEC16A Control	0 hr	0.063	0.059	0.061	0.064	0.060	0.066	0.045	0.047	0.047	0.069	0.066	0.061
	2 hr	0.135	0.124	0.136	0.136	0.144	0.143	0.12	0.118	0.12	0.133	0.146	0.144
	4 hr	0.231	0.218	0.221	0.293	0.315	0.310	0.269	0.266	0.27	0.263	0.280	0.281
	6 hr	0.380	0.369	0.379	0.431	0.453	0.439	0.441	0.435	0.431	0.444	0.455	0.453
DEC16A + PS1	0 hr	0.068	0.066	0.069	0.064	0.066	0.062	0.06	0.059	0.058	0.073	0.073	0.074
	2 hr	0.085	0.085	0.088	0.092	0.096	0.095	0.084	0.086	0.085	0.104	0.106	0.105
	4 hr	0.109	0.111	0.113	0.134	0.146	0.135	0.121	0.123	0.119	0.144	0.147	0.146
	6 hr	0.141	0.143	0.144	0.181	0.191	0.182	0.169	0.168	0.162	0.251	0.260	0.256
DEC16A + PS2	0 hr	0.059	0.058	0.059	0.070	0.065	0.064	0.058	0.058	0.056	0.074	0.076	0.075
	2 hr	0.070	0.067	0.072	0.087	0.081	0.078	0.07	0.07	0.073	0.109	0.106	0.105
	4 hr	0.079	0.077	0.083	0.105	0.100	0.098	0.084	0.086	0.085	0.123	0.121	0.127
	6 hr	0.092	0.095	0.105	0.131	0.137	0.135	0.118	0.116	0.104	0.221	0.231	0.238
DEC16A + PS3	0 hr	0.062	0.057	0.062	0.069	0.073	0.065	0.057	0.057	0.056	0.073	0.074	0.075
	2 hr	0.074	0.067	0.073	0.085	0.083	0.090	0.072	0.07	0.073	0.101	0.104	0.104
	4 hr	0.086	0.075	0.081	0.110	0.099	0.109	0.088	0.089	0.09	0.114	0.118	0.123
	6 hr	0.104	0.093	0.103	0.135	0.137	0.139	0.117	0.116	0.112	0.189	0.233	0.242
DEC16A + PS4	0 hr	0.052	0.056	0.052	0.066	0.069	0.062	0.057	0.056	0.055	0.070	0.069	0.070
	2 hr	0.081	0.080	0.084	0.094	0.086	0.086	0.074	0.076	0.072	0.115	0.112	0.111
	4 hr	0.094	0.090	0.098	0.114	0.102	0.104	0.095	0.102	0.101	0.145	0.144	0.142
	6 hr	0.092	0.092	0.101	0.126	0.121	0.128	0.113	0.124	0.117	0.232	0.230	0.232
DEC16A + PS7	0 hr	0.050	0.045	0.045	0.074	0.070	0.076	0.056	0.057	0.058	0.069	0.068	0.070
	2 hr	0.075	0.072	0.071	0.096	0.094	0.091	0.068	0.066	0.065	0.110	0.104	0.109
	4 hr	0.085	0.075	0.086	0.116	0.102	0.109	0.095	0.092	0.093	0.133	0.134	0.136
	6 hr	0.088	0.092	0.089	0.135	0.129	0.133	0.111	0.107	0.129	0.223	0.227	0.229
DEC16A + PS8	0 hr	0.069	0.071	0.063	0.069	0.063	0.070	0.056	0.056	0.06	0.072	0.067	0.070
	2 hr	0.086	0.083	0.083	0.096	0.088	0.087	0.08	0.081	0.082	0.121	0.115	0.123
	4 hr	0.094	0.083	0.095	0.115	0.108	0.106	0.098	0.099	0.104	0.150	0.145	0.156
	6 hr	0.107	0.095	0.088	0.126	0.128	0.131	0.104	0.105	0.135	0.222	0.230	0.241
DEC16A + PS13	0 hr	0.054	0.065	0.063	0.072	0.069	0.072	0.057	0.056	0.062	0.068	0.068	0.068
	2 hr	0.086	0.086	0.084	0.101	0.098	0.099	0.085	0.085	0.088	0.118	0.117	0.123
	4 hr	0.090	0.093	0.091	0.116	0.109	0.104	0.104	0.093	0.095	0.148	0.149	0.155
	6 hr	0.097	0.100	0.085	0.132	0.125	0.121	0.105	0.102	0.115	0.221	0.228	0.229
DEC16A + PS16	0 hr	0.067	0.070	0.071	0.064	0.062	0.069	0.059	0.058	0.054	0.067	0.069	0.075
	2 hr	0.066	0.064	0.064	0.076	0.078	0.079	0.065	0.064	0.067	0.101	0.104	0.114
	4 hr	0.079	0.074	0.073	0.082	0.085	0.086	0.083	0.078	0.082	0.119	0.115	0.128
	6 hr	0.098	0.091	0.093	0.124	0.120	0.117	0.11	0.108	0.111	0.218	0.225	0.240

Table 5. Recorded absorbance values for *E. coli* 8257 mixed with 8 different phage stocks, measured at 600nm every 2 hours for 6 hour. Experiments 1, 2, 3, & 4 all performed on different days.

		Experiment 1			Experiment 2			Experiment 3			Experiment 4		
Sample	Time	Rep1	Rep2	Rep3	Rep4	Rep5	Rep6	Rep7	Rep8	Rep9	Rep10	Rep11	Rep12
8257 Control	0 hr	0.054	0.053	0.055	0.054	0.050	0.053	0.049	0.049	0.047	0.056	0.056	0.054
	2 hr	0.104	0.103	0.106	0.111	0.105	0.107	0.095	0.095	0.09	0.092	0.088	0.087
	4 hr	0.182	0.182	0.184	0.179	0.168	0.172	0.158	0.156	0.153	0.149	0.144	0.141
	6 hr	0.293	0.292	0.305	0.281	0.267	0.271	0.289	0.286	0.278	0.249	0.239	0.236
8257 + PS1	0 hr	0.063	0.065	0.064	0.055	0.053	0.053	0.055	0.055	0.052	0.058	0.055	0.061
	2 hr	0.084	0.083	0.085	0.078	0.073	0.077	0.072	0.069	0.066	0.071	0.067	0.071
	4 hr	0.121	0.119	0.134	0.118	0.110	0.112	0.108	0.16	0.1	0.094	0.087	0.091
	6 hr	0.168	0.170	0.181	0.183	0.162	0.164	0.173	0.172	0.163	0.139	0.134	0.140
8257 + PS2	0 hr	0.067	0.062	0.063	0.057	0.055	0.059	0.05	0.051	0.053	0.058	0.057	0.056
	2 hr	0.082	0.078	0.079	0.077	0.075	0.077	0.066	0.065	0.067	0.069	0.068	0.069
	4 hr	0.098	0.092	0.101	0.093	0.091	0.095	0.084	0.084	0.083	0.079	0.077	0.083
	6 hr	0.141	0.140	0.147	0.139	0.134	0.138	0.137	0.136	0.135	0.115	0.111	0.116
8257 + PS3	0 hr	0.059	0.063	0.064	0.057	0.056	0.058	0.051	0.05	0.049	0.058	0.057	0.060
	2 hr	0.077	0.079	0.081	0.074	0.072	0.073	0.066	0.064	0.064	0.068	0.069	0.072
	4 hr	0.099	0.098	0.106	0.096	0.097	0.095	0.09	0.086	0.088	0.091	0.087	0.094
	6 hr	0.144	0.145	0.155	0.136	0.135	0.147	0.144	0.134	0.14	0.134	0.128	0.132
8257 + PS4	0 hr	0.063	0.060	0.063	0.054	0.057	0.055	0.049	0.047	0.05	0.053	0.054	0.060
	2 hr	0.092	0.090	0.091	0.078	0.082	0.081	0.069	0.069	0.074	0.073	0.071	0.076
	4 hr	0.112	0.109	0.112	0.095	0.099	0.101	0.087	0.086	0.098	0.085	0.086	0.093
	6 hr	0.143	0.145	0.150	0.127	0.131	0.137	0.125	0.124	0.134	0.110	0.107	0.113
8257 + PS7	0 hr	0.063	0.064	0.060	0.061	0.058	0.061	0.051	0.049	0.048	0.060	0.063	0.064
	2 hr	0.085	0.087	0.082	0.082	0.081	0.083	0.067	0.064	0.067	0.073	0.076	0.076
	4 hr	0.111	0.109	0.108	0.101	0.097	0.099	0.082	0.079	0.083	0.087	0.090	0.089
	6 hr	0.152	0.155	0.160	0.136	0.134	0.137	0.131	0.124	0.13	0.122	0.123	0.121
8257 + PS8	0 hr	0.063	0.061	0.060	0.062	0.055	0.059	0.053	0.05	0.05	0.058	0.068	0.067
	2 hr	0.095	0.092	0.088	0.088	0.081	0.088	0.076	0.073	0.071	0.068	0.081	0.080
	4 hr	0.117	0.109	0.108	0.106	0.098	0.108	0.097	0.091	0.09	0.085	0.098	0.101
	6 hr	0.153	0.146	0.148	0.128	0.126	0.128	0.13	0.123	0.122	0.118	0.124	0.123
8257 + PS13	0 hr	0.064	0.062	0.060	0.058	0.057	0.056	0.052	0.054	0.052	0.068	0.062	0.065
	2 hr	0.101	0.093	0.093	0.092	0.090	0.088	0.078	0.073	0.074	0.085	0.079	0.081
	4 hr	0.116	0.108	0.108	0.111	0.109	0.106	0.096	0.092	0.089	0.108	0.103	0.105
	6 hr	0.150	0.146	0.143	0.127	0.134	0.123	0.118	0.114	0.112	0.122	0.119	0.118
8257 + PS16	0 hr	0.065	0.059	0.062	0.057	0.060	0.059	0.055	0.052	0.05	0.063	0.062	0.065
	2 hr	0.081	0.074	0.078	0.078	0.074	0.077	0.067	0.065	0.066	0.071	0.069	0.073
	4 hr	0.095	0.084	0.089	0.097	0.092	0.098	0.079	0.078	0.077	0.089	0.091	0.091
	6 hr	0.146	0.137	0.141	0.133	0.134	0.134	0.133	0.125	0.122	0.129	0.128	0.131

Table 6. Recorded absorbance values for *E. coli* DA-33 mixed with 8 different phage stocks, measured at 600nm every 2 hours for 6 hour. Experiments 1, 2, 3, & 4 all performed on different days.

		Experiment 1			Experiment 2			Experiment 3			Experiment 4		
Sample	Time	Rep1	Rep2	Rep3	Rep4	Rep5	Rep6	Rep7	Rep8	Rep9	Rep10	Rep11	Rep12
DA-33 Control	0 hr	0.067	0.065	0.066	0.059	0.057	0.055	0.055	0.056	0.055	0.061	0.062	0.060
	2 hr	0.114	0.111	0.117	0.137	0.140	0.134	0.117	0.129	0.124	0.123	0.126	0.122
	4 hr	0.231	0.227	0.237	0.240	0.247	0.236	0.216	0.226	0.222	0.224	0.227	0.224
	6 hr	0.408	0.405	0.420	0.392	0.407	0.397	0.393	0.415	0.419	0.388	0.394	0.396
DA-33 + PS1	0 hr	0.079	0.070	0.073	0.059	0.061	0.059	0.059	0.061	0.061	0.061	0.062	0.059
	2 hr	0.086	0.082	0.086	0.076	0.077	0.078	0.074	0.074	0.075	0.069	0.073	0.070
	4 hr	0.112	0.109	0.113	0.101	0.106	0.104	0.089	0.09	0.091	0.096	0.098	0.094
	6 hr	0.144	0.159	0.158	0.129	0.143	0.135	0.128	0.129	0.131	0.128	0.135	0.127
DA-33 + PS2	0 hr	0.068	0.068	0.068	0.059	0.059	0.063	0.057	0.06	0.057	0.060	0.060	0.062
	2 hr	0.086	0.086	0.086	0.075	0.075	0.080	0.073	0.075	0.07	0.072	0.071	0.074
	4 hr	0.102	0.101	0.101	0.092	0.094	0.097	0.074	0.077	0.073	0.082	0.084	0.086
	6 hr	0.127	0.131	0.136	0.119	0.125	0.121	0.11	0.115	0.108	0.114	0.115	0.116
DA-33 + PS3	0 hr	0.072	0.070	0.073	0.058	0.062	0.059	0.059	0.058	0.057	0.060	0.062	0.063
	2 hr	0.083	0.084	0.087	0.072	0.077	0.073	0.073	0.072	0.073	0.069	0.073	0.072
	4 hr	0.103	0.104	0.108	0.088	0.096	0.098	0.077	0.076	0.077	0.083	0.087	0.086
	6 hr	0.136	0.136	0.139	0.120	0.128	0.119	0.11	0.108	0.109	0.121	0.122	0.119
DA-33 + PS4	0 hr	0.072	0.075	0.070	0.056	0.059	0.060	0.055	0.056	0.055	0.056	0.058	0.061
	2 hr	0.098	0.102	0.096	0.084	0.084	0.087	0.082	0.085	0.086	0.078	0.081	0.083
	4 hr	0.118	0.119	0.110	0.096	0.098	0.099	0.096	0.098	0.097	0.091	0.093	0.096
	6 hr	0.127	0.129	0.128	0.116	0.113	0.114	0.114	0.115	0.114	0.108	0.111	0.112
DA-33 + PS7	0 hr	0.074	0.072	0.072	0.063	0.063	0.062	0.059	0.061	0.06	0.060	0.061	0.064
	2 hr	0.097	0.092	0.092	0.082	0.079	0.080	0.079	0.086	0.081	0.076	0.076	0.078
	4 hr	0.111	0.104	0.105	0.097	0.093	0.095	0.086	0.094	0.086	0.086	0.089	0.090
	6 hr	0.128	0.121	0.124	0.127	0.114	0.117	0.112	0.119	0.114	0.111	0.114	0.115
DA-33 + PS8	0 hr	0.074	0.070	0.068	0.065	0.066	0.067	0.063	0.059	0.061	0.065	0.066	0.067
	2 hr	0.099	0.096	0.095	0.094	0.091	0.088	0.088	0.086	0.09	0.084	0.086	0.086
	4 hr	0.118	0.111	0.111	0.109	0.104	0.101	0.105	0.1	0.103	0.101	0.104	0.102
	6 hr	0.121	0.118	0.124	0.125	0.119	0.116	0.116	0.112	0.114	0.112	0.114	0.115
DA-33 + PS13	0 hr	0.071	0.068	0.069	0.058	0.058	0.056	0.061	0.059	0.061	0.064	0.067	0.062
	2 hr	0.102	0.098	0.098	0.089	0.093	0.092	0.095	0.091	0.092	0.086	0.091	0.083
	4 hr	0.119	0.112	0.114	0.105	0.106	0.104	0.111	0.105	0.105	0.105	0.106	0.100
	6 hr	0.121	0.122	0.129	0.119	0.115	0.117	0.117	0.115	0.112	0.108	0.112	0.107
DA-33 + PS16	0 hr	0.069	0.068	0.070	0.061	0.059	0.062	0.062	0.061	0.061	0.067	0.064	0.061
	2 hr	0.086	0.082	0.083	0.081	0.078	0.077	0.075	0.076	0.077	0.077	0.072	0.069
	4 hr	0.100	0.098	0.098	0.093	0.091	0.091	0.076	0.079	0.079	0.090	0.086	0.083
	6 hr	0.123	0.121	0.124	0.125	0.117	0.121	0.11	0.111	0.112	0.121	0.116	0.113

Table 7. Recorded absorbance values for *E. coli* EK33 mixed with 8 different phage stocks, measured at 600nm every 2 hours for 6 hour. Experiments 1, 2, 3, & 4 all performed on different days.

		Experiment 1			Experiment 2			Experiment 3			Experiment 4		
Sample	Time	Rep1	Rep2	Rep3	Rep4	Rep5	Rep6	Rep7	Rep8	Rep9	Rep10	Rep11	Rep12
EK33 Control	0 hr	0.062	0.064	0.062	0.048	0.045	0.047	0.051	0.046	0.049	0.045	0.047	0.046
	2 hr	0.108	0.110	0.105	0.097	0.095	0.097	0.092	0.088	0.09	0.078	0.079	0.079
	4 hr	0.270	0.276	0.259	0.228	0.223	0.226	0.221	0.215	0.218	0.214	0.217	0.210
	6 hr	0.464	0.477	0.445	0.441	0.445	0.443	0.451	0.445	0.446	0.386	0.394	0.387
EK33 + PS1	0 hr	0.083	0.079	0.080	0.048	0.044	0.044	0.05	0.052	0.05	0.049	0.048	0.051
	2 hr	0.104	0.103	0.105	0.060	0.059	0.060	0.07	0.072	0.069	0.061	0.059	0.062
	4 hr	0.143	0.143	0.146	0.089	0.089	0.090	0.104	0.106	0.108	0.094	0.095	0.100
	6 hr	0.211	0.208	0.217	0.149	0.164	0.160	0.138	0.143	0.149	0.110	0.115	0.123
EK33 + PS2	0 hr	0.080	0.079	0.080	0.045	0.046	0.044	0.049	0.047	0.051	0.047	0.048	0.048
	2 hr	0.101	0.100	0.103	0.061	0.062	0.062	0.068	0.064	0.069	0.061	0.059	0.060
	4 hr	0.102	0.106	0.107	0.088	0.089	0.085	0.083	0.102	0.088	0.086	0.083	0.087
	6 hr	0.104	0.107	0.107	0.144	0.155	0.148	0.079	0.084	0.082	0.074	0.071	0.076
EK33 + PS3	0 hr	0.080	0.081	0.080	0.043	0.042	0.043	0.047	0.047	0.048	0.048	0.049	0.050
	2 hr	0.099	0.100	0.098	0.058	0.056	0.058	0.065	0.064	0.068	0.061	0.058	0.061
	4 hr	0.103	0.106	0.102	0.085	0.081	0.082	0.104	0.103	0.102	0.114	0.100	0.087
	6 hr	0.107	0.108	0.107	0.150	0.154	0.149	0.09	0.088	0.091	0.126	0.093	0.076
EK33 + PS4	0 hr	0.078	0.078	0.080	0.044	0.045	0.045	0.046	0.045	0.043	0.048	0.045	0.044
	2 hr	0.103	0.101	0.107	0.063	0.064	0.064	0.068	0.067	0.065	0.064	0.061	0.061
	4 hr	0.106	0.105	0.109	0.102	0.099	0.099	0.104	0.087	0.094	0.107	0.104	0.075
	6 hr	0.099	0.102	0.100	0.153	0.150	0.161	0.094	0.062	0.067	0.082	0.083	0.062
EK33 + PS7	0 hr	0.079	0.079	0.080	0.044	0.046	0.044	0.045	0.047	0.047	0.048	0.046	0.048
	2 hr	0.103	0.100	0.102	0.062	0.063	0.064	0.064	0.066	0.068	0.062	0.059	0.061
	4 hr	0.102	0.101	0.106	0.091	0.093	0.089	0.113	0.091	0.098	0.104	0.101	0.079
	6 hr	0.094	0.098	0.101	0.151	0.149	0.149	0.105	0.07	0.073	0.093	0.099	0.064
EK33 + PS8	0 hr	0.079	0.077	0.080	0.046	0.048	0.050	0.047	0.05	0.05	0.048	0.052	0.050
	2 hr	0.108	0.101	0.106	0.067	0.069	0.070	0.067	0.071	0.072	0.063	0.066	0.065
	4 hr	0.109	0.105	0.114	0.101	0.097	0.101	0.107	0.093	0.093	0.104	0.095	0.081
	6 hr	0.097	0.092	0.098	0.148	0.126	0.151	0.098	0.064	0.066	0.094	0.073	0.062
EK33 + PS13	0 hr	0.080	0.079	0.081	0.047	0.046	0.044	0.046	0.047	0.045	0.046	0.052	0.046
	2 hr	0.112	0.106	0.111	0.069	0.070	0.068	0.071	0.069	0.068	0.062	0.066	0.062
	4 hr	0.115	0.112	0.119	0.091	0.092	0.093	0.085	0.087	0.085	0.103	0.079	0.082
	6 hr	0.095	0.095	0.098	0.115	0.106	0.114	0.057	0.058	0.056	0.087	0.058	0.059
EK33 + PS16	0 hr	0.079	0.079	0.080	0.047	0.047	0.047	0.047	0.05	0.048	0.044	0.048	0.048
	2 hr	0.098	0.097	0.101	0.060	0.060	0.062	0.063	0.065	0.064	0.056	0.059	0.057
	4 hr	0.137	0.102	0.112	0.076	0.080	0.079	0.089	0.089	0.088	0.095	0.090	0.084
	6 hr	0.143	0.101	0.104	0.142	0.136	0.135	0.071	0.074	0.073	0.118	0.073	0.075

Table 8. Recorded absorbance values for *E. coli* strains mixed with cocktail of 3 different phage stocks (PS1, PS7 PS16), measured at 600nm every 2 hours for 6 hour. Experiments 1, 2, & 3 all performed on different days**.**

		Experiment 1			Experiment 2			Experiment 3		
Sample	Time	Rep1	Rep2	Rep3	Rep4	Rep5	Rep6	Rep7	Rep8	Rep9
EDL 933 Control	0 hr	0.033	0.034	0.030	0.032	0.031	0.034	0.033	0.035	0.034
	2 hr	0.068	0.069	0.064	0.066	0.065	0.068	0.066	0.066	0.065
	4 hr	0.175	0.173	0.168	0.171	0.168	0.173	0.164	0.165	0.166
	6 hr	0.334	0.336	0.328	0.346	0.345	0.354	0.326	0.332	0.330
EDL 933 'Cocktail'	0 hr	0.031	0.031	0.030	0.035	0.037	0.036	0.031	0.032	0.034
	2 hr	0.044	0.045	0.045	0.048	0.049	0.047	0.053	0.051	0.054
	4 hr	0.067	0.068	0.066	0.078	0.080	0.078	0.111	0.108	0.109
	6 hr	0.110	0.114	0.112	0.128	0.128	0.127	0.208	0.206	0.210
DEC16A Control	0 hr	0.033	0.031	0.033	0.034	0.033	0.032	0.035	0.033	0.033
	2 hr	0.072	0.070	0.071	0.070	0.067	0.068	0.067	0.064	0.068
	4 hr	0.202	0.194	0.198	0.199	0.198	0.199	0.190	0.187	0.195
	6 hr	0.370	0.362	0.368	0.371	0.372	0.374	0.360	0.356	0.362
DEC16A 'Cocktail'	0 hr	0.033	0.032	0.033	0.034	0.033	0.032	0.031	0.032	0.030
	2 hr	0.044	0.045	0.043	0.047	0.046	0.046	0.047	0.048	0.046
	4 hr	0.053	0.053	0.051	0.058	0.057	0.058	0.068	0.071	0.069
	6 hr	0.078	0.078	0.076	0.092	0.087	0.086	0.101	0.099	0.096
DA-33 Control	0 hr	0.033	0.031	0.029	0.032	0.031	0.033	0.031	0.032	0.033
	2 hr	0.058	0.056	0.054	0.059	0.058	0.059	0.055	0.056	0.058
	4 hr	0.157	0.149	0.152	0.167	0.167	0.171	0.155	0.155	0.163
	6 hr	0.305	0.298	0.307	0.326	0.330	0.341	0.308	0.312	0.318
DA-33 'Cocktail'	0 hr	0.032	0.032	0.031	0.033	0.033	0.034	0.030	0.031	0.033
	2 hr	0.045	0.047	0.047	0.046	0.046	0.047	0.045	0.046	0.048
	4 hr	0.083	0.084	0.084	0.079	0.080	0.081	0.096	0.096	0.098
	6 hr	0.148	0.143	0.151	0.142	0.143	0.145	0.166	0.167	0.170
8257 Control	0 hr	0.030	0.032	0.032	0.034	0.033	0.035	0.035	0.034	0.033
	2 hr	0.061	0.062	0.061	0.063	0.061	0.064	0.062	0.059	0.058
	4 hr	0.147	0.142	0.155	0.170	0.161	0.173	0.166	0.170	0.159
	6 hr	0.257	0.254	0.269	0.291	0.284	0.299	0.285	0.280	0.275
8257 'Cocktail'	0 hr	0.033	0.036	0.034	0.036	0.034	0.034	0.032	0.033	0.037
	2 hr	0.055	0.060	0.056	0.063	0.062	0.062	0.058	0.059	0.062
	4 hr	0.130	0.112	0.121	0.160	0.147	0.168	0.164	0.162	0.162
	6 hr	0.225	0.220	0.212	0.287	0.276	0.295	0.289	0.288	0.290
EKs33 Control	0 hr	0.033	0.032	0.033	0.038	0.036	0.034	0.034	0.035	0.035
	2 hr	0.060	0.060	0.062	0.064	0.065	0.065	0.065	0.063	0.064
	4 hr	0.154	0.152	0.156	0.190	0.187	0.198	0.189	0.184	0.190
	6 hr	0.334	0.328	0.336	0.386	0.385	0.402	0.379	0.372	0.390
EK33 'Cocktail'	0 hr	0.033	0.036	0.031	0.035	0.034	0.033	0.033	0.031	0.032
	2 hr	0.046	0.048	0.044	0.049	0.048	0.050	0.052	0.050	0.050
	4 hr	0.068	0.069	0.067	0.081	0.080	0.082	0.096	0.085	0.094
	6 hr	0.145	0.128	0.120	0.138	0.139	0.143	0.174	0.137	0.163

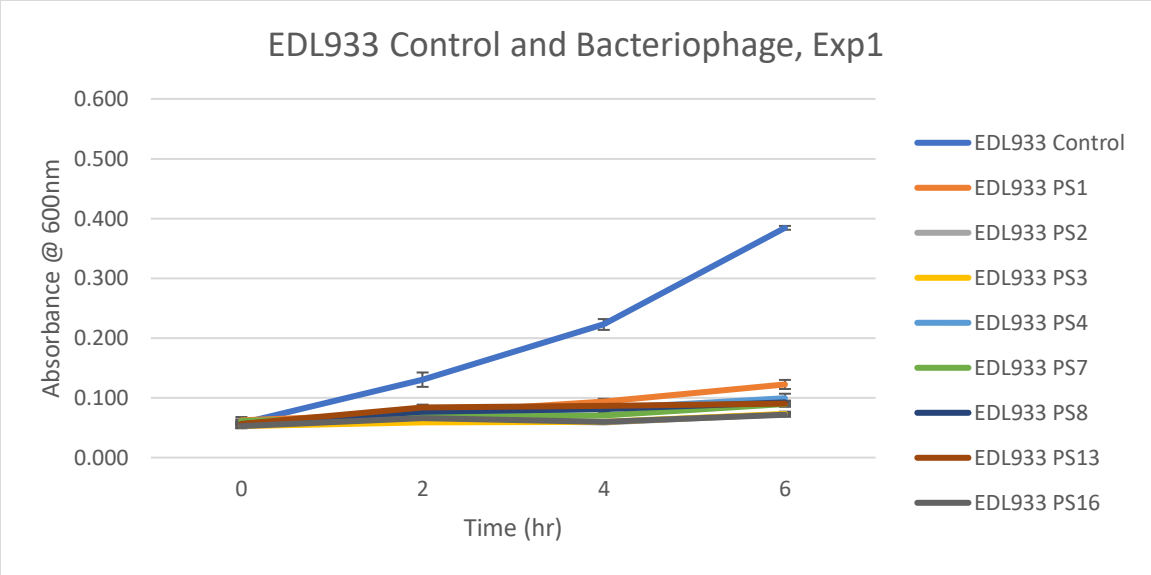

Figure 1. Average absorbance of *E. coli* EDL933 at 600nm mixed with phage stocks, measured over six hours from Experiment 1. Standard deviation for each time point shown with error bars.

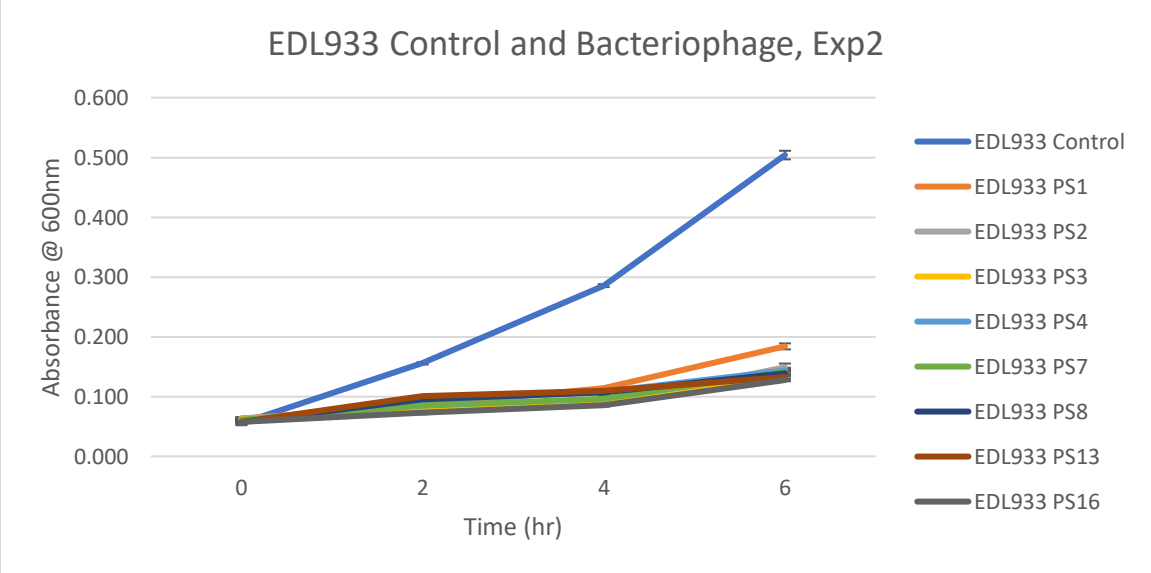

Figure 2. Average absorbance of *E. coli* EDL933 at 600nm mixed with phage stocks, measured over six hours from Experiment 2. Standard deviation for each time point shown with error bars.

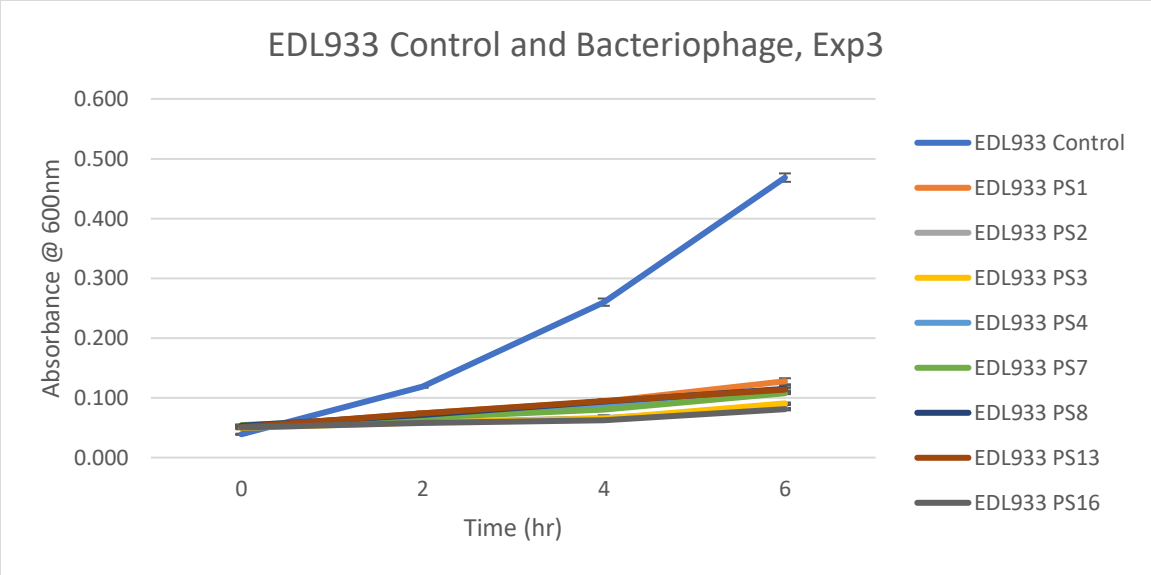

Figure 3. Average absorbance of *E. coli* EDL933 at 600nm mixed with phage stocks, measured over six hours from Experiment 3. Standard deviation for each time point shown with error bars.

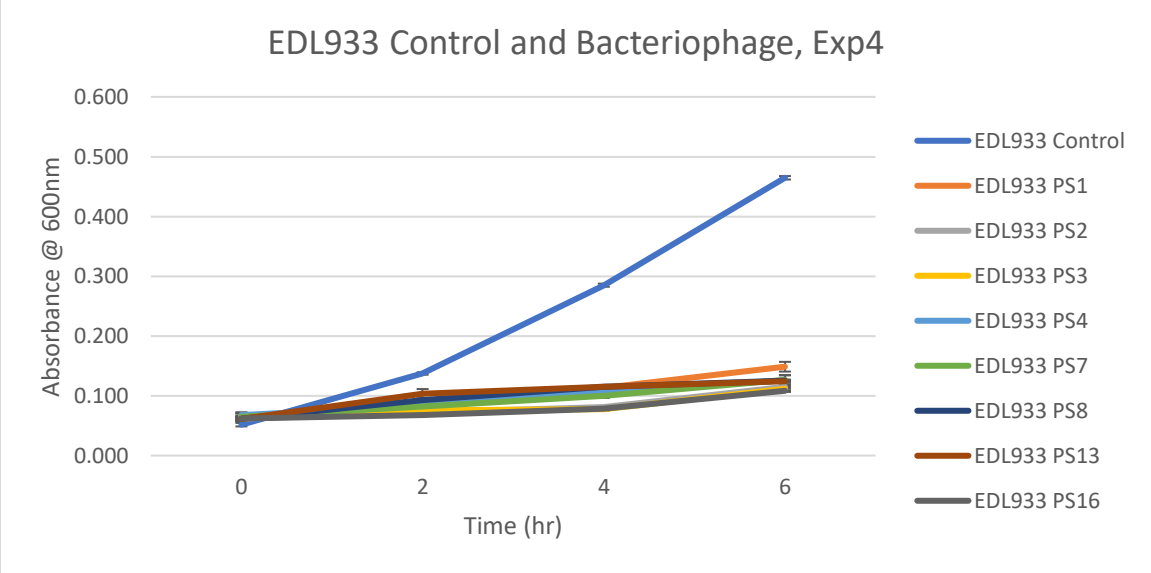

Figure 4. Average absorbance of *E. coli* EDL933 at 600nm mixed with phage stocks, measured over six hours from Experiment 4. Standard deviation for each time point shown with error bars.

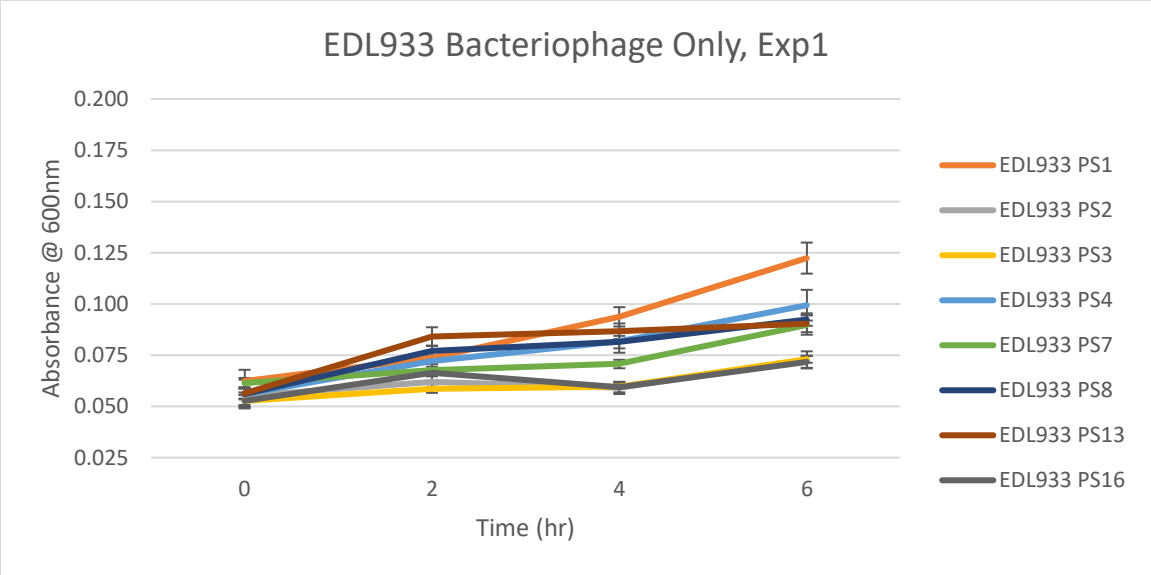

Figure 5. Average absorbance of *E. coli* EDL933 at 600nm mixed with phage stocks, measured over six hours from Experiment 1, control data removed. Standard deviation for each time point shown with error bars.

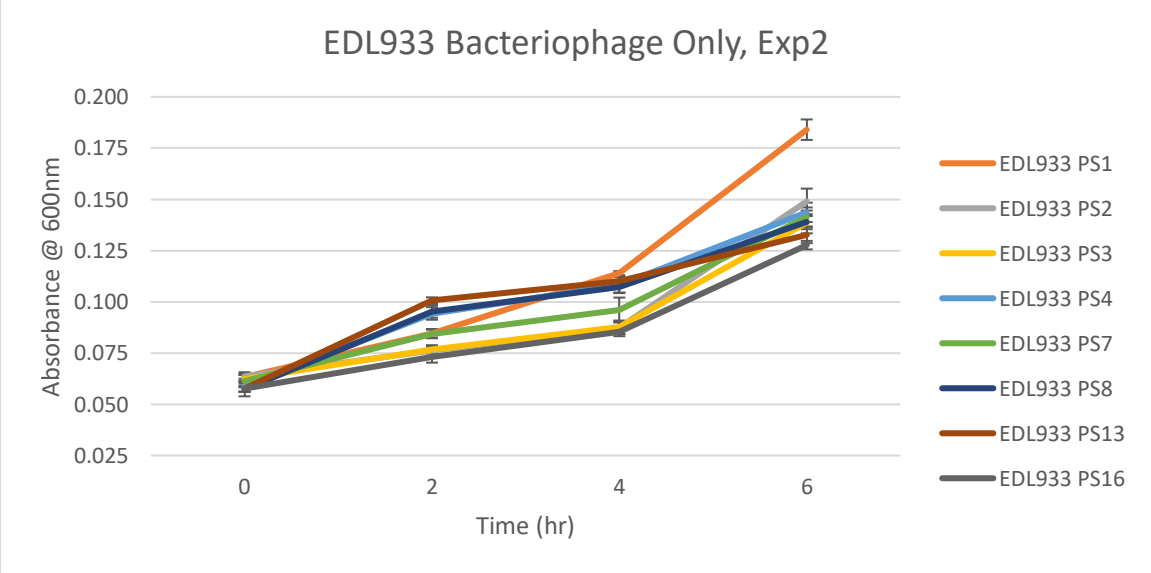

Figure 6. Average absorbance of *E. coli* EDL933 at 600nm mixed with phage stocks, measured over six hours from Experiment 2, control data removed. Standard deviation for each time point shown with error bars.

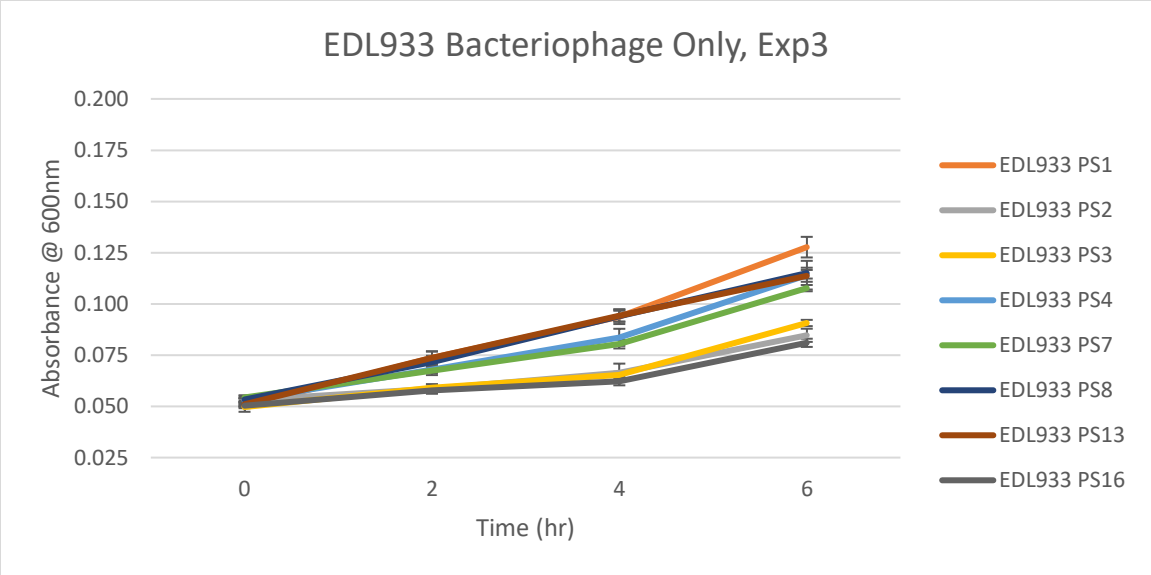

Figure 7. Average absorbance of *E. coli* EDL933 at 600nm mixed with phage stocks, measured over six hours from Experiment 3, control data removed. Standard deviation for each time point shown with error bars.

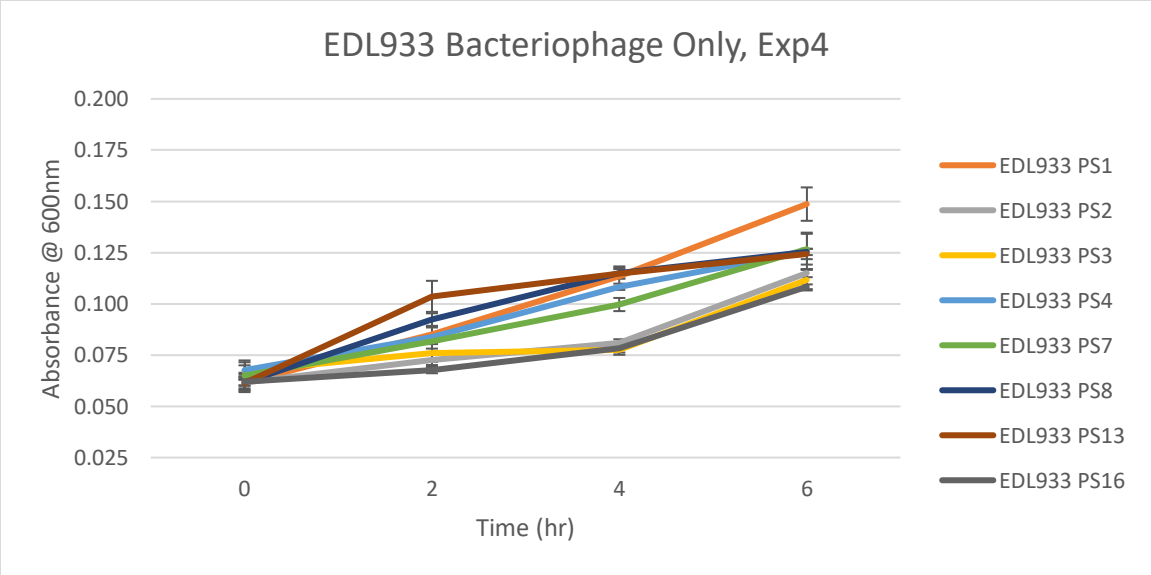

Figure 8. Average absorbance of *E. coli* EDL933 at 600nm mixed with phage stocks, measured over six hours from Experiment 4, control data removed. Standard deviation for each time point shown with error bars.

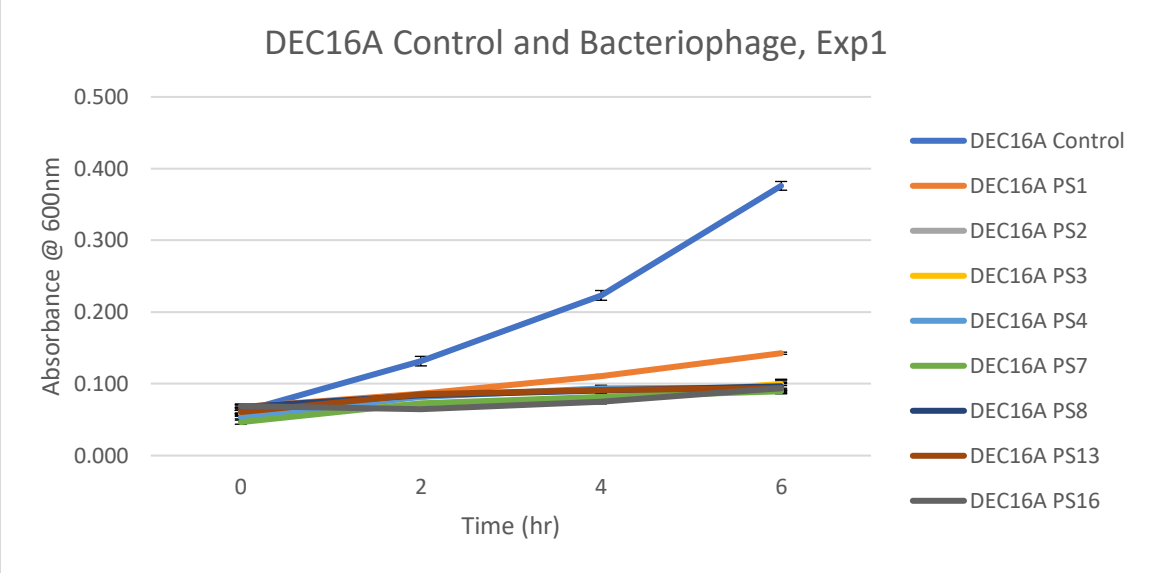

Figure 9. Average absorbance of *E. coli* DEC16A at 600nm mixed with phage stocks, measured over six hours from Experiment 1. Standard deviation for each time point shown with error bars.

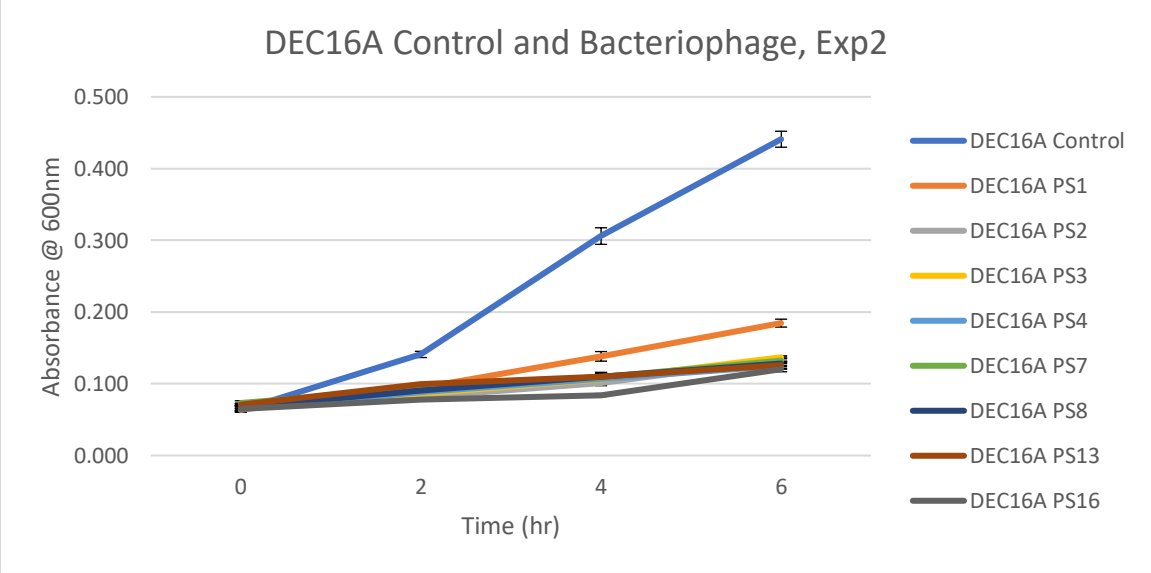

Figure 10. Average absorbance of *E. coli* DEC16A at 600nm mixed with phage stocks, measured over six hours from Experiment 2. Standard deviation for each time point shown with error bars.

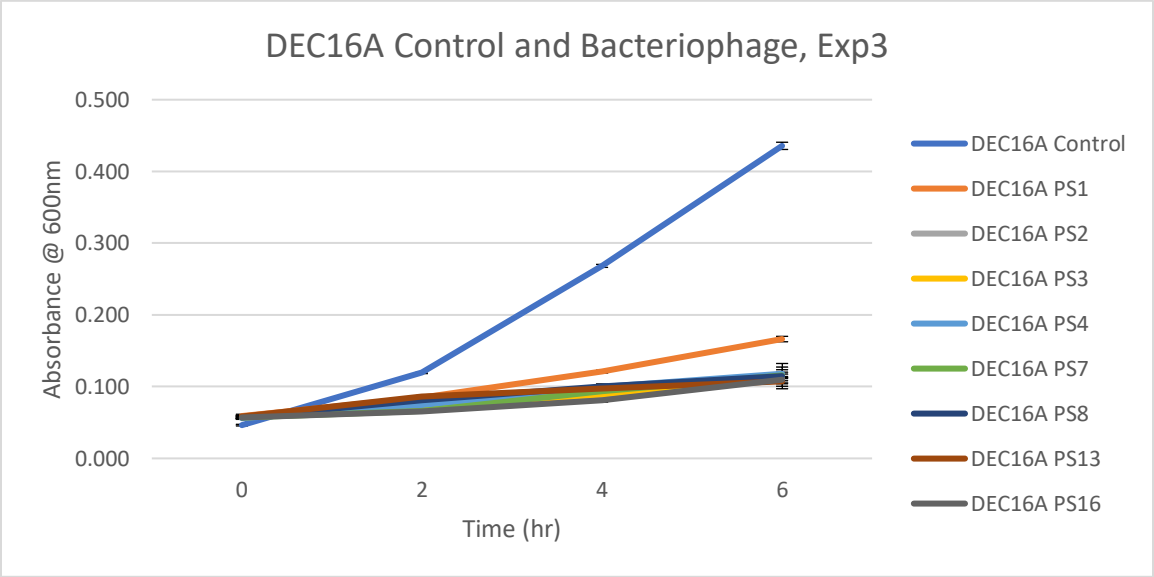

Figure 11. Average absorbance of *E. coli* at 600nm mixed with phage stocks, measured over six hours from Experiment 3. Standard deviation for each time point shown with error bars.

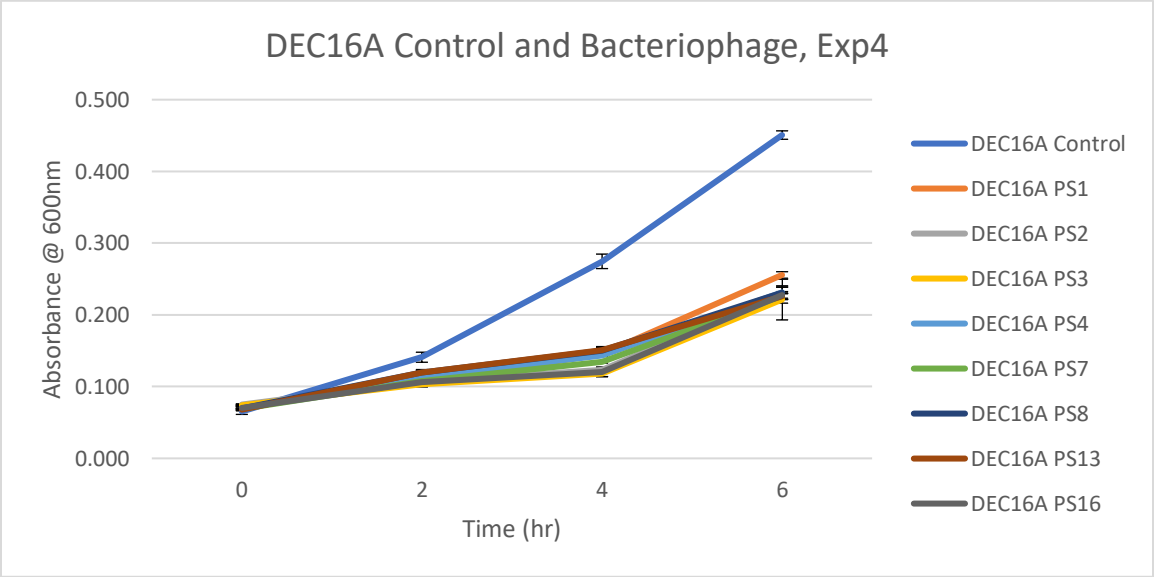

Figure 12. Average absorbance of *E. coli* DEC16A at 600nm mixed with phage stocks, measured over six hours from Experiment 4. Standard deviation for each time point shown with error bars.

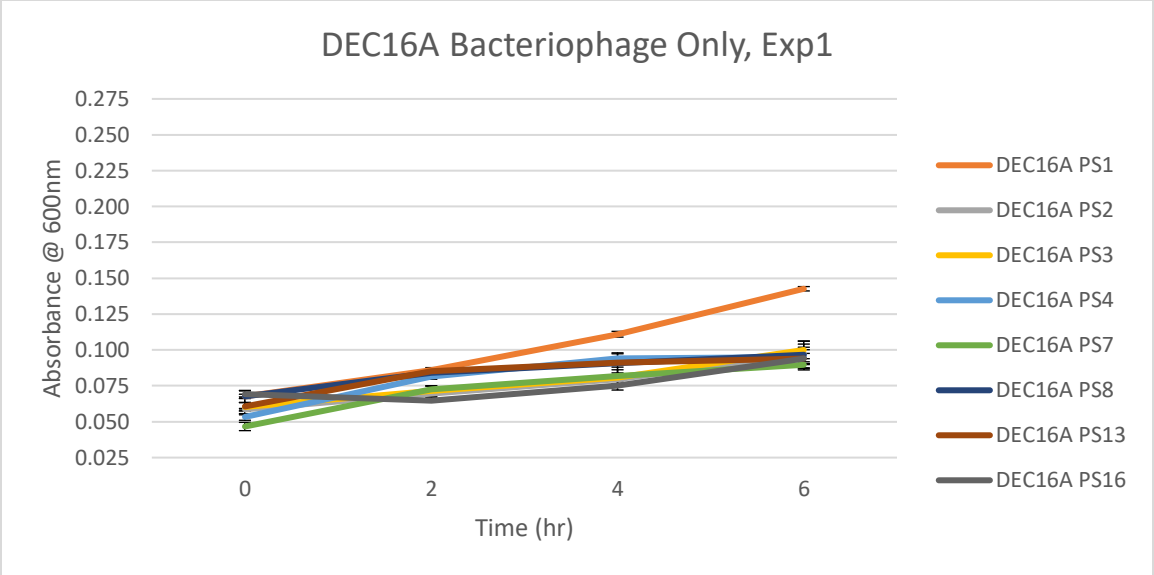

Figure 13. Average absorbance of *E. coli* DEC16A at 600nm mixed with phage stocks, measured over six hours from Experiment 1, control data removed. Standard deviation for each time point shown with error bars.

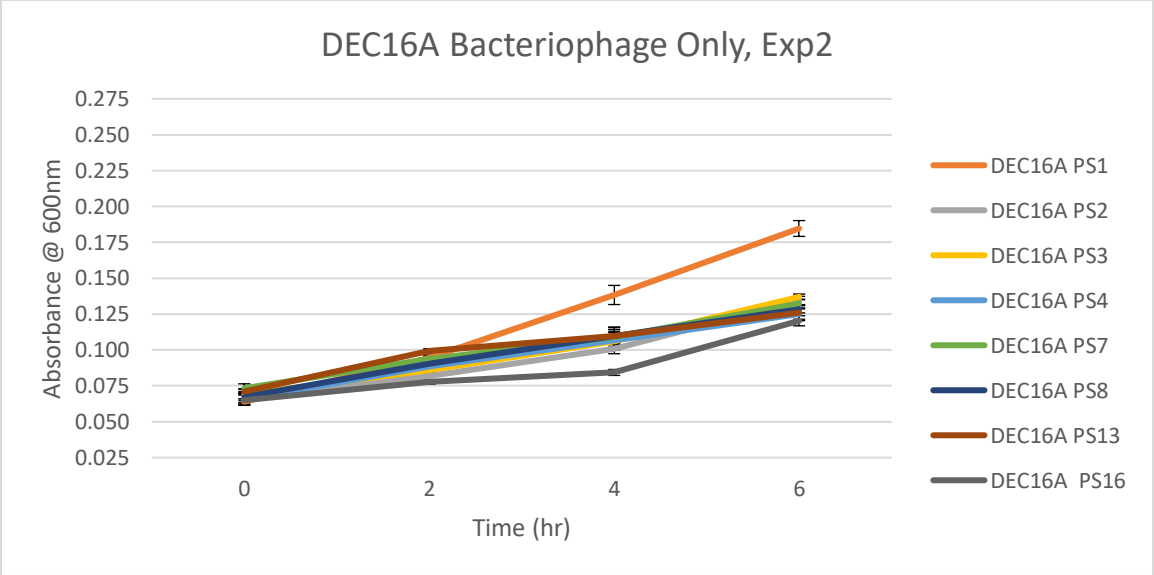

Figure 14. Average absorbance of *E. coli* DEC16A at 600nm mixed with phage stocks, measured over six hours from Experiment 2, control data removed. Standard deviation for each time point shown with error bars.

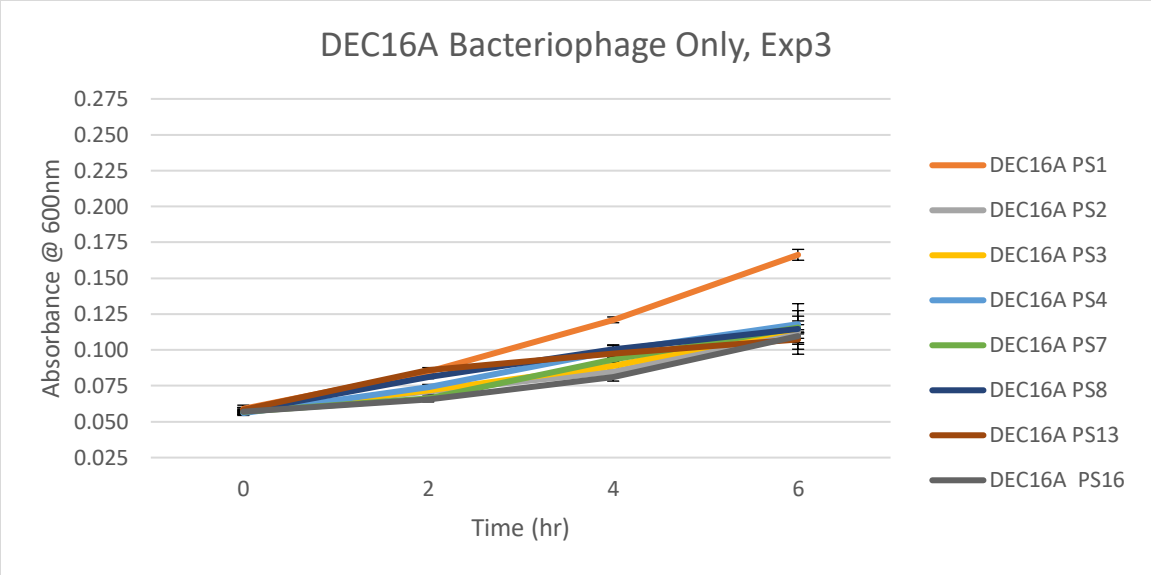

Figure 15. Average absorbance of *E. coli* DEC16A at 600nm mixed with phage stocks, measured over six hours from Experiment 3, control data removed. Standard deviation for each time point shown with error bars.

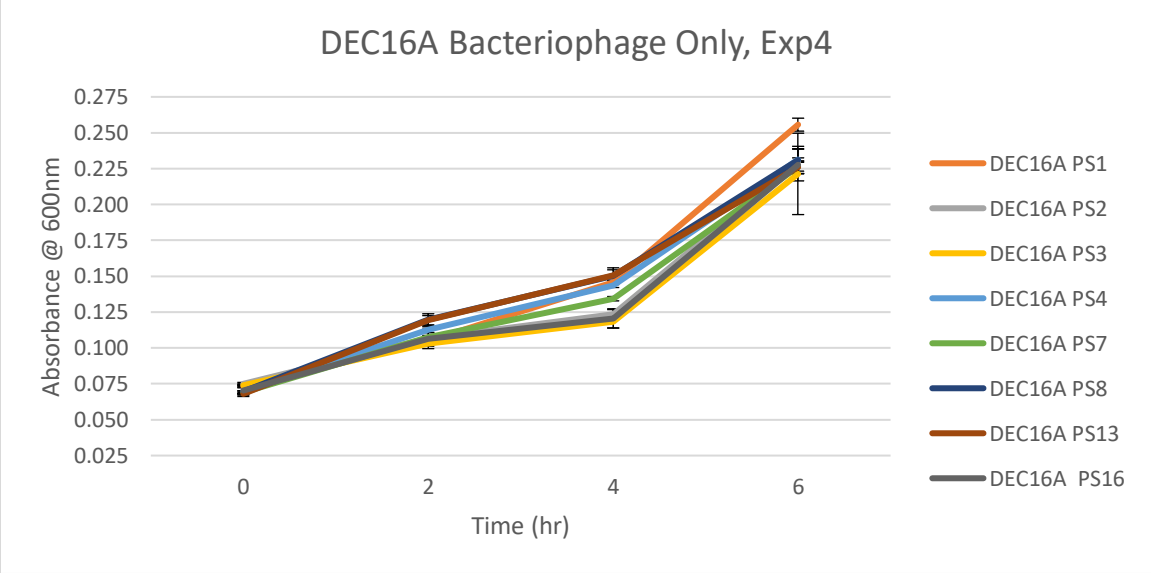

Figure 16. Average absorbance of *E. coli* DEC16A at 600nm mixed with phage stocks, measured over six hours from Experiment 4, control data removed. Standard deviation for each time point shown with error bars.

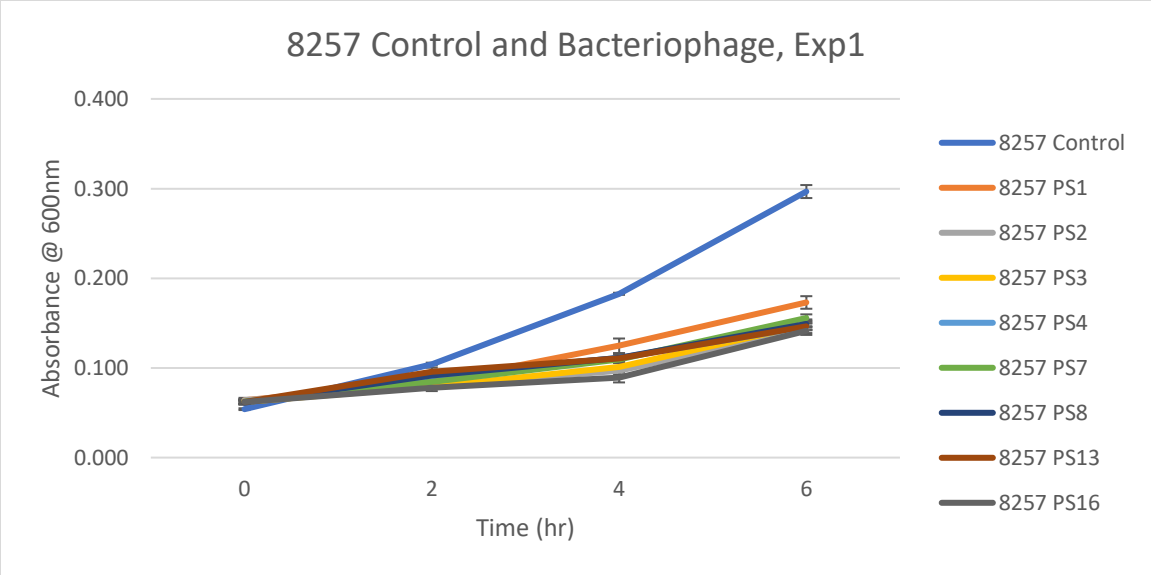

Figure 17. Average absorbance of *E. coli* 8257 at 600nm mixed with phage stocks, measured over six hours from Experiment 1. Standard deviation for each time point shown with error bars.

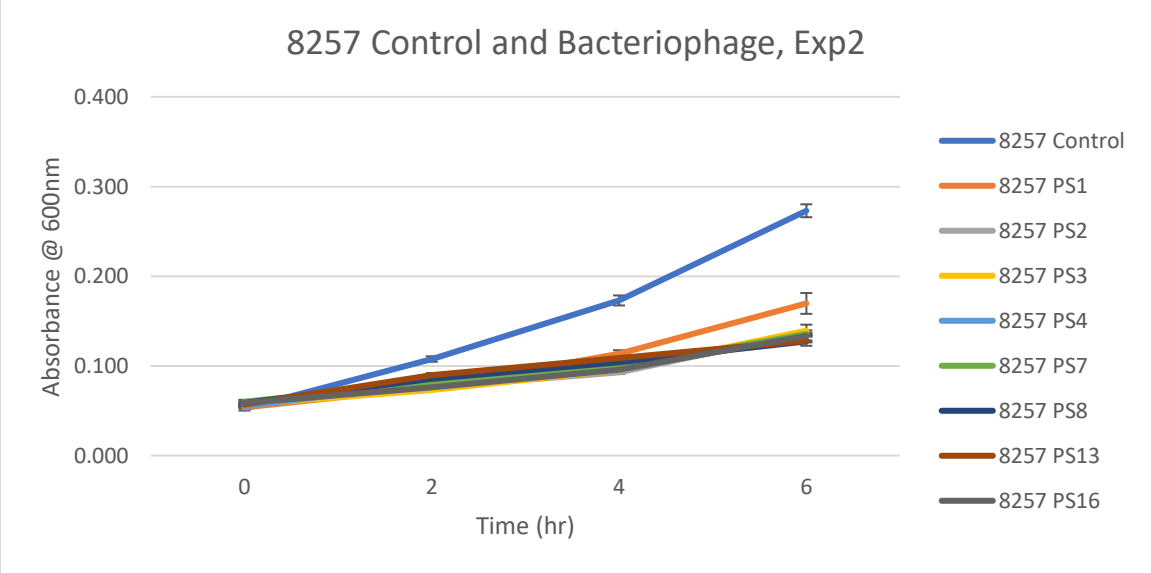

Figure 18. Average absorbance of *E. coli* 8257 at 600nm mixed with phage stocks, measured over six hours from Experiment 2. Standard deviation for each time point shown with error bars.

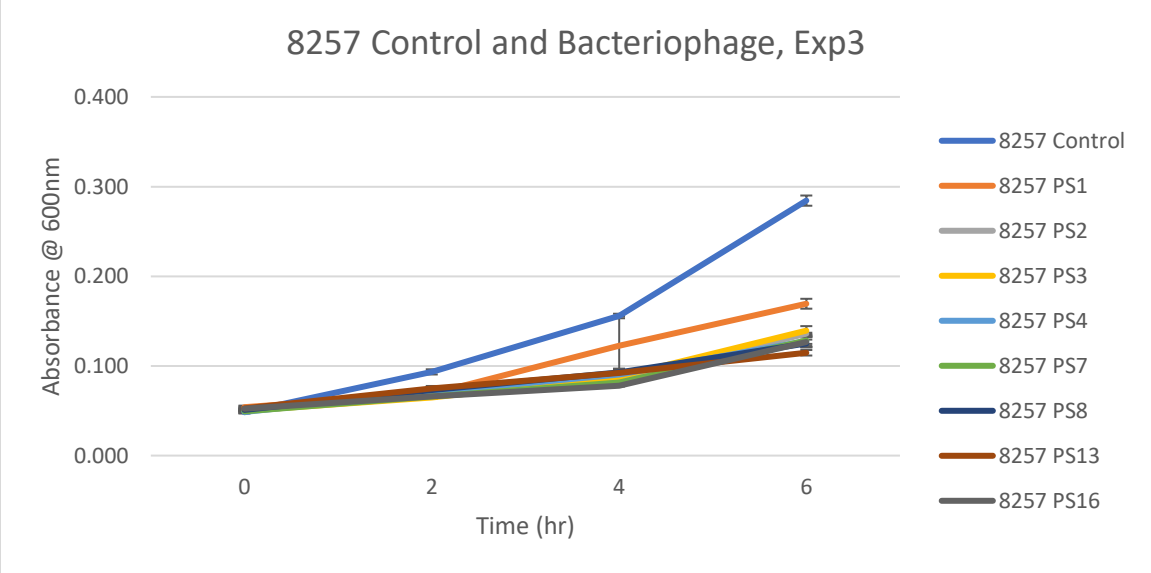

Figure 19. Average absorbance of *E. coli* 8257 at 600nm mixed with phage stocks, measured over six hours from Experiment 3. Standard deviation for each time point shown with error bars.

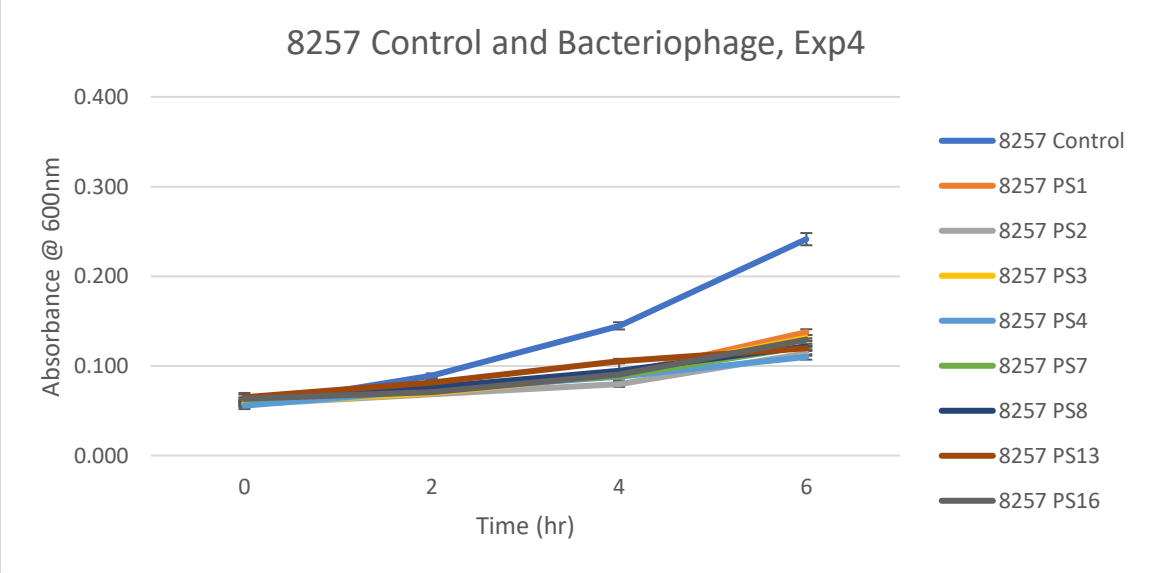

Figure 20. Average absorbance of *E. coli* 8257 at 600nm mixed with phage stocks, measured over six hours from Experiment 4. Standard deviation for each time point shown with error bars.

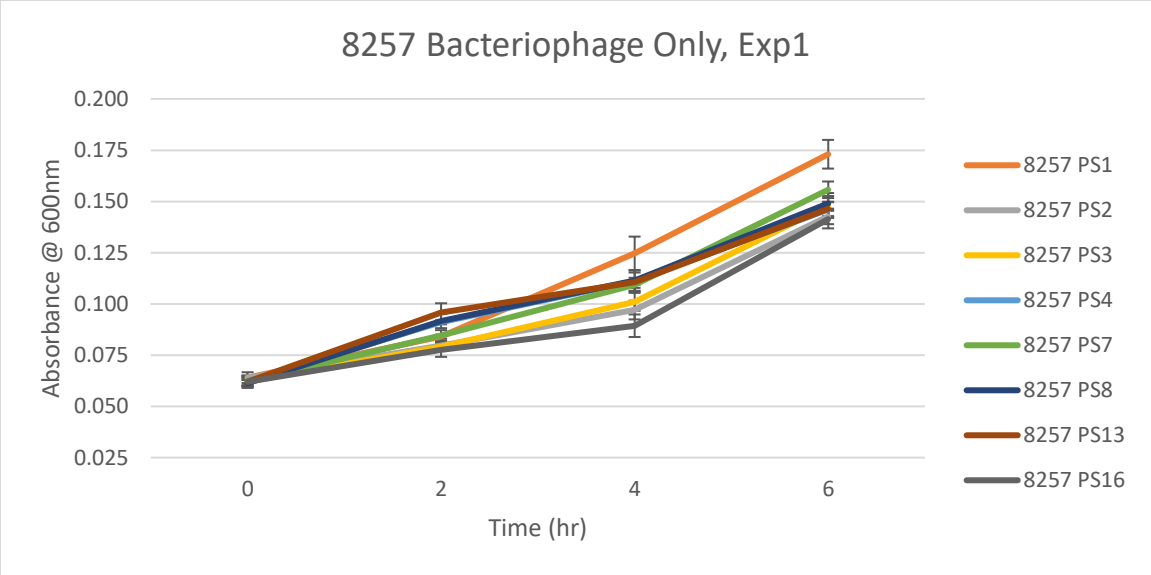

Figure 21. Average absorbance of *E. coli* 8257 at 600nm mixed with phage stocks, measured over six hours from Experiment 1, control data removed. Standard deviation for each time point shown with error bars.

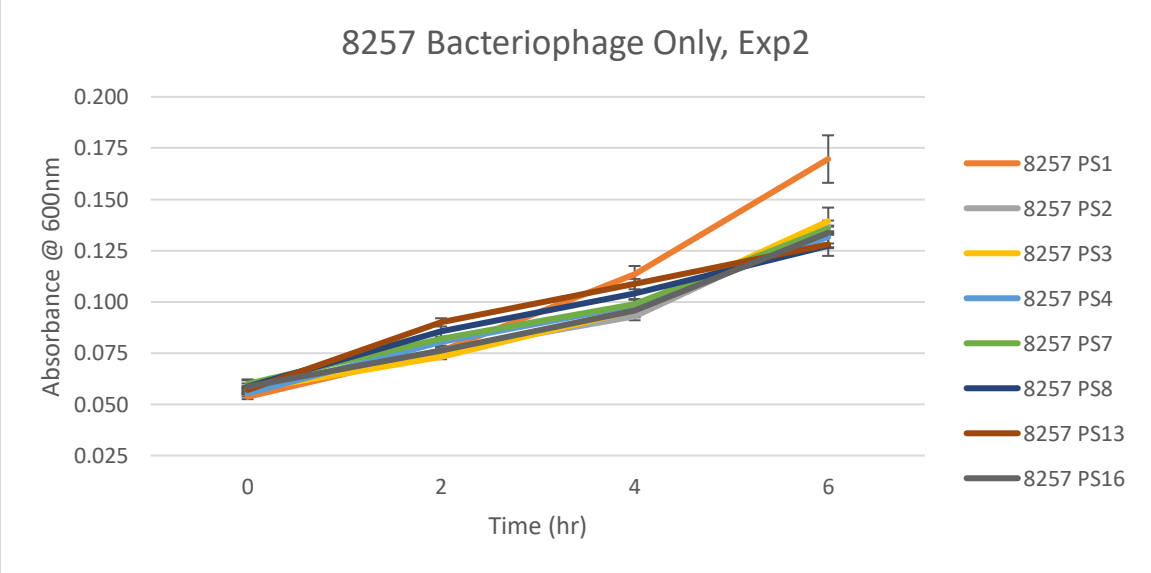

Figure 22. Average absorbance of *E. coli* 8257 at 600nm mixed with phage stocks, measured over six hours from Experiment 2, control data removed. Standard deviation for each time point shown with error bars.

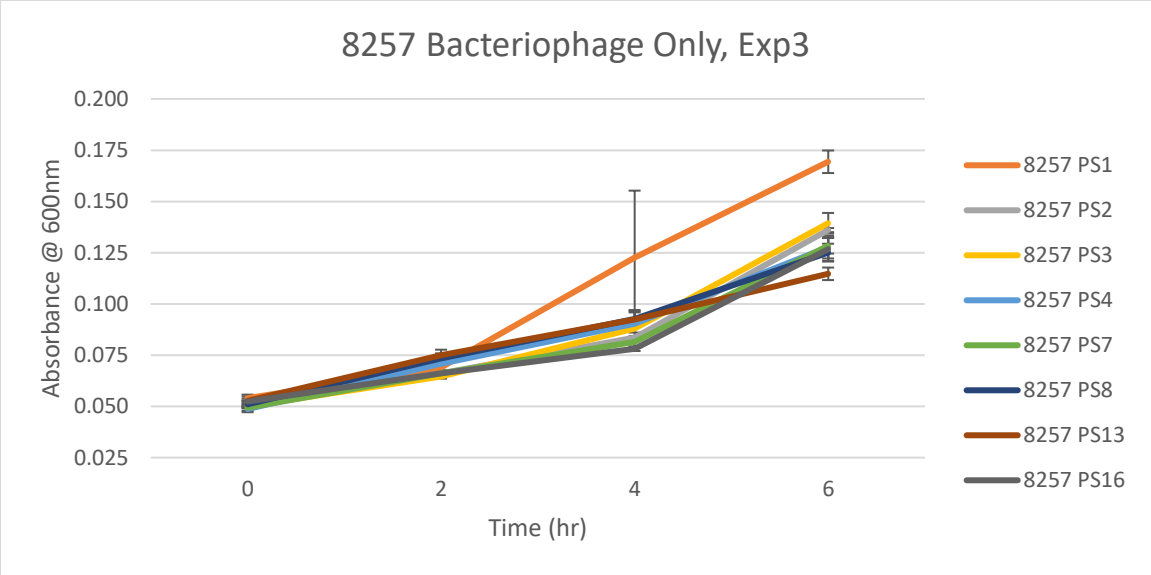

Figure 23. Average absorbance of *E. coli* 8257 at 600nm mixed with phage stocks, measured over six hours from Experiment 3, control data removed. Standard deviation for each time point shown with error bars.

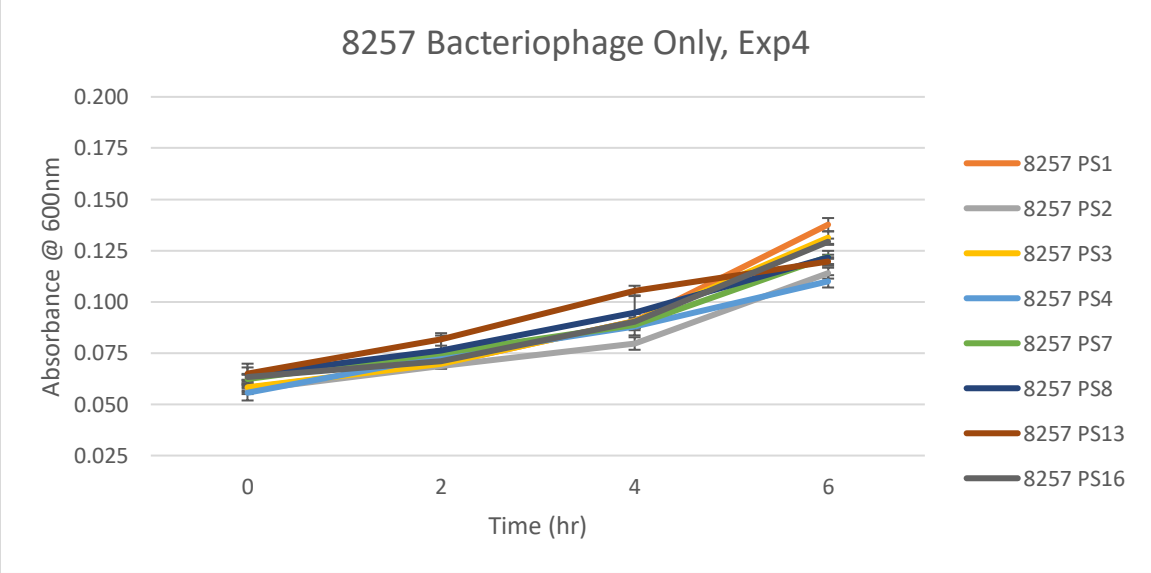

Figure 24. Average absorbance of *E. coli* 8257 at 600nm mixed with phage stocks, measured over six hours from Experiment 4, control data removed. Standard deviation for each time point shown with error bars.

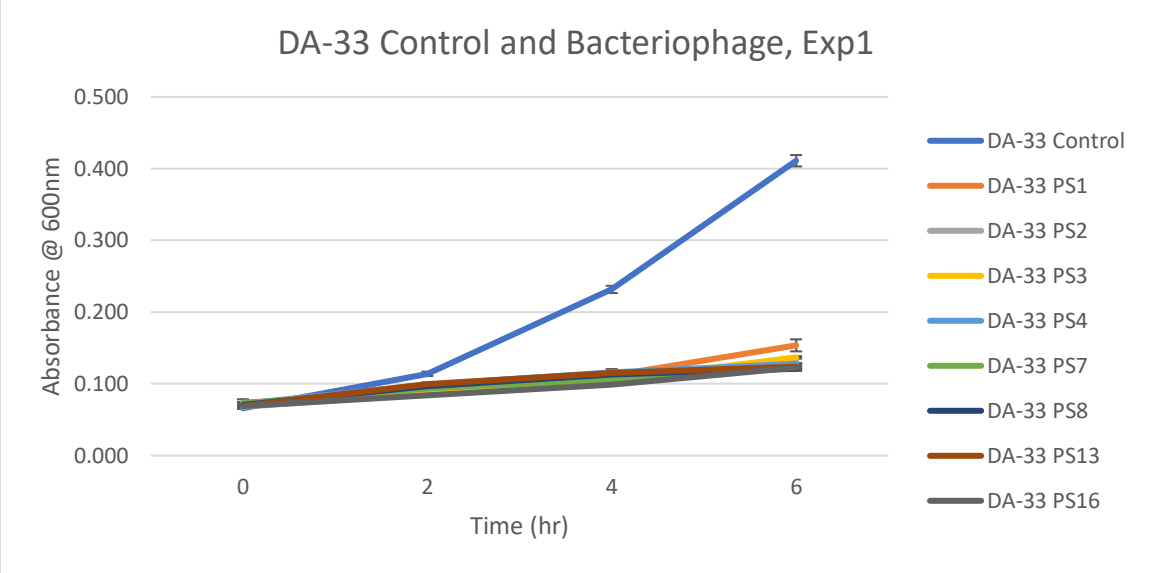

Figure 25. Average absorbance of *E. coli* DA-33 at 600nm mixed with phage stocks, measured over six hours from Experiment 1. Standard deviation for each time point shown with error bars.

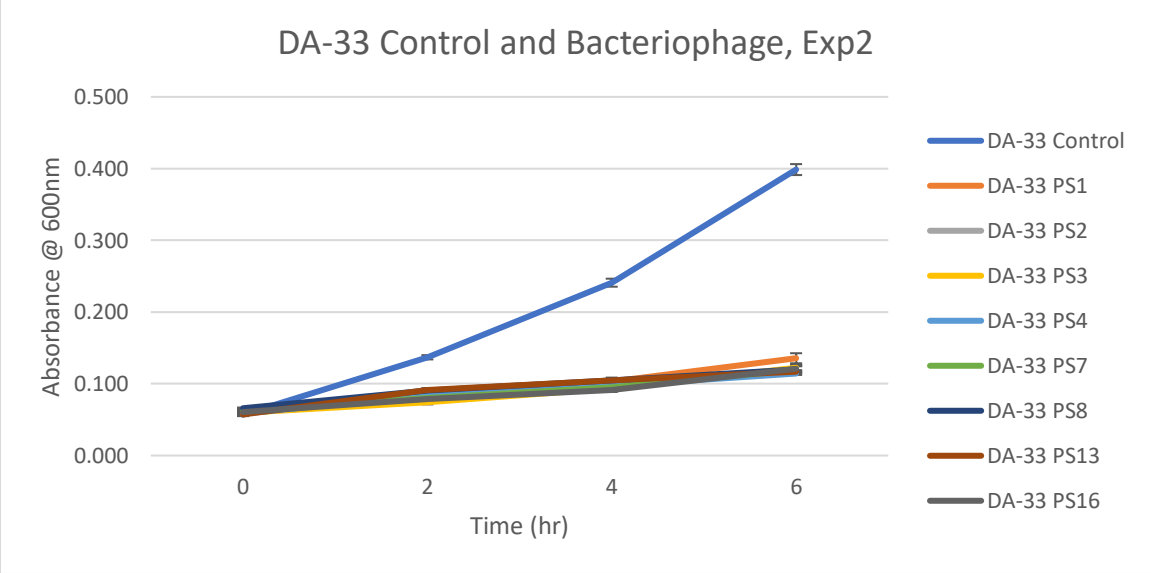

Figure 26. Average absorbance of *E. coli* DA-33 at 600nm mixed with phage stocks, measured over six hours from Experiment 2. Standard deviation for each time point shown with error bars.

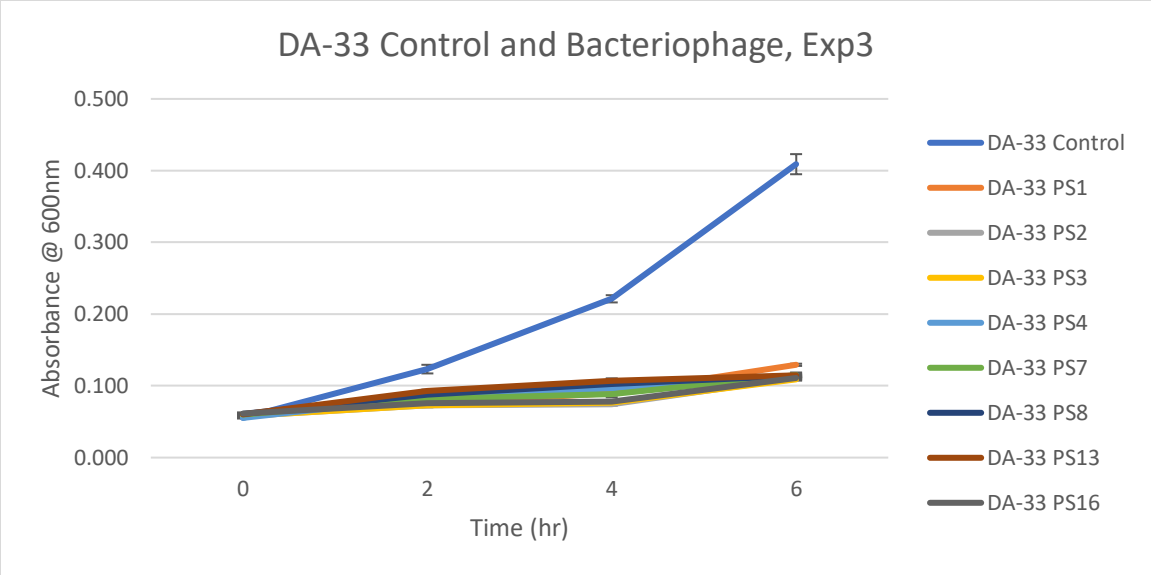

Figure 27. Average absorbance of *E. coli* DA-33 at 600nm mixed with phage stocks, measured over six hours from Experiment 3. Standard deviation for each time point shown with error bars.

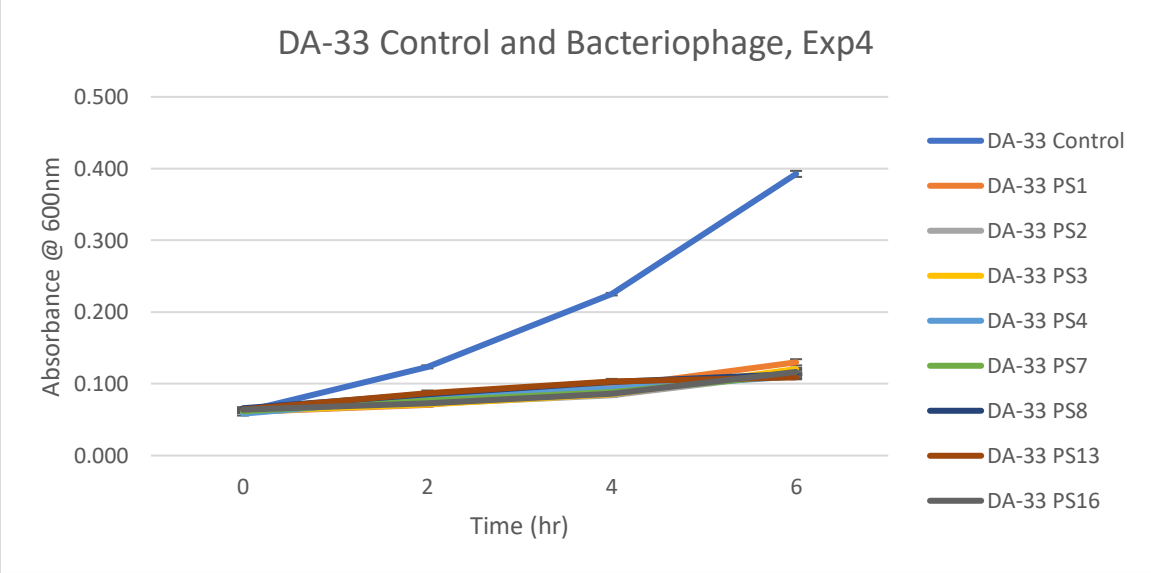

Figure 28. Average absorbance of *E. coli* DA-33 at 600nm mixed with phage stocks, measured over six hours from Experiment 4. Standard deviation for each time point shown with error bars.

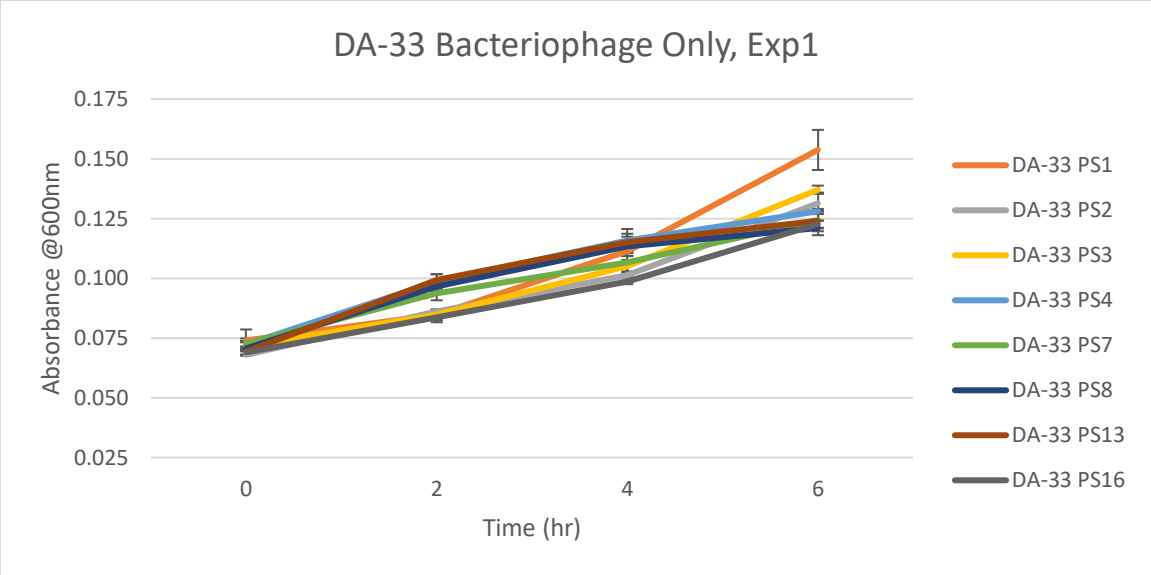

Figure29. Average absorbance of *E. coli* DA-33 at 600nm mixed with phage stocks, measured over six hours from Experiment 1, control data removed. Standard deviation for each time point shown with error bars.

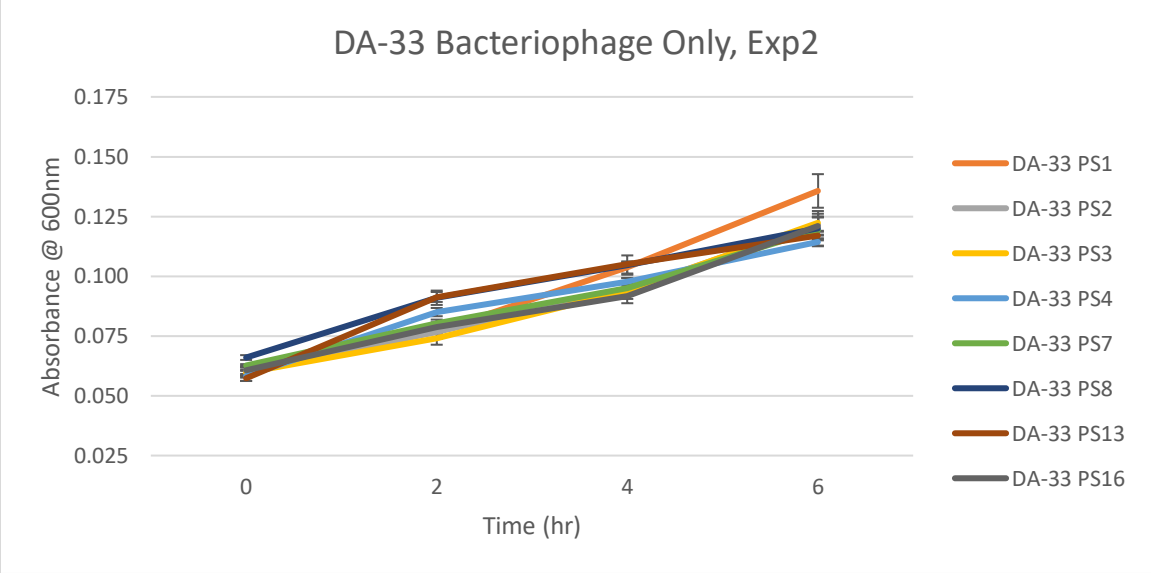

Figure 30. Average absorbance of *E. coli* DA-33 at 600nm mixed with phage stocks, measured over six hours from Experiment 2, control data removed. Standard deviation for each time point shown with error bars.

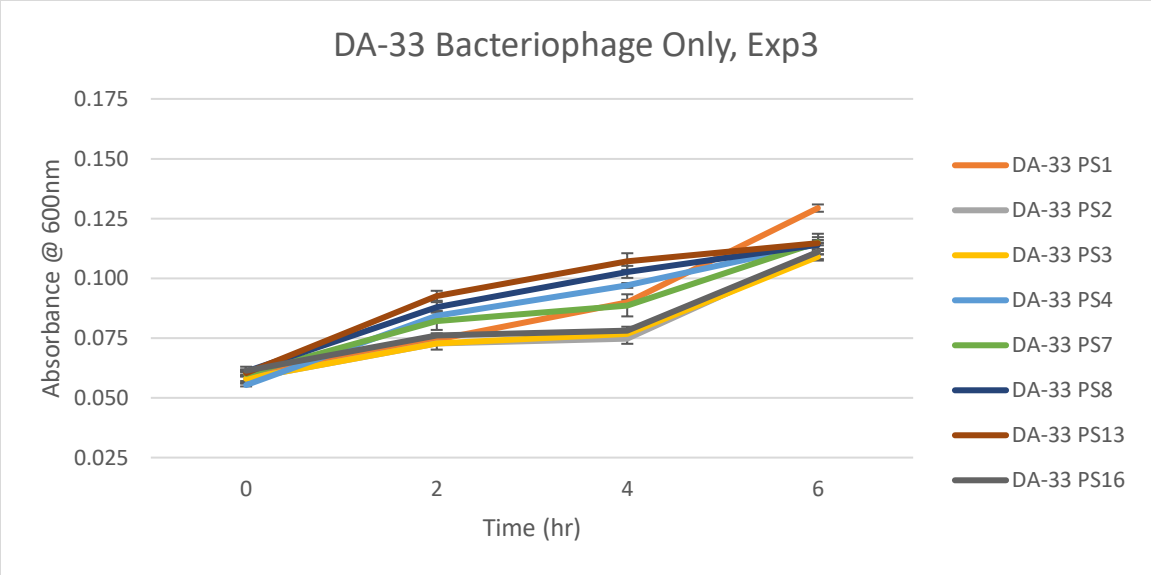

Figure 31. Average absorbance of *E. coli* DA-33 at 600nm mixed with phage stocks, measured over six hours from Experiment 3, control data removed. Standard deviation for each time point shown with error bars.

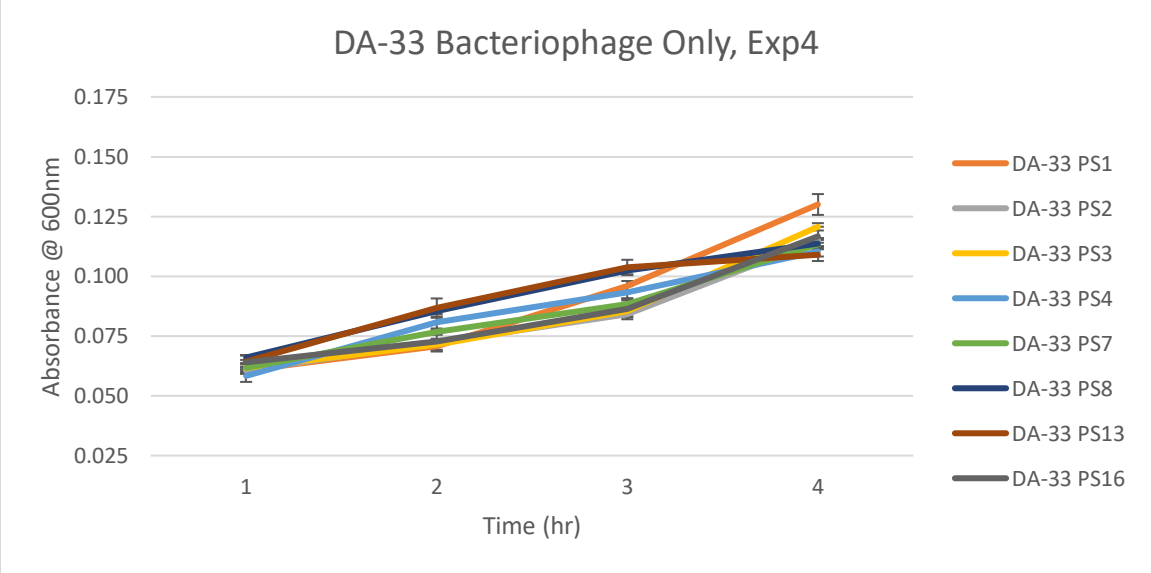

Figure 32. Average absorbance of *E. coli* DA-33 at 600nm mixed with phage stocks, measured over six hours from Experiment 4, control data removed. Standard deviation for each time point shown with error bars.

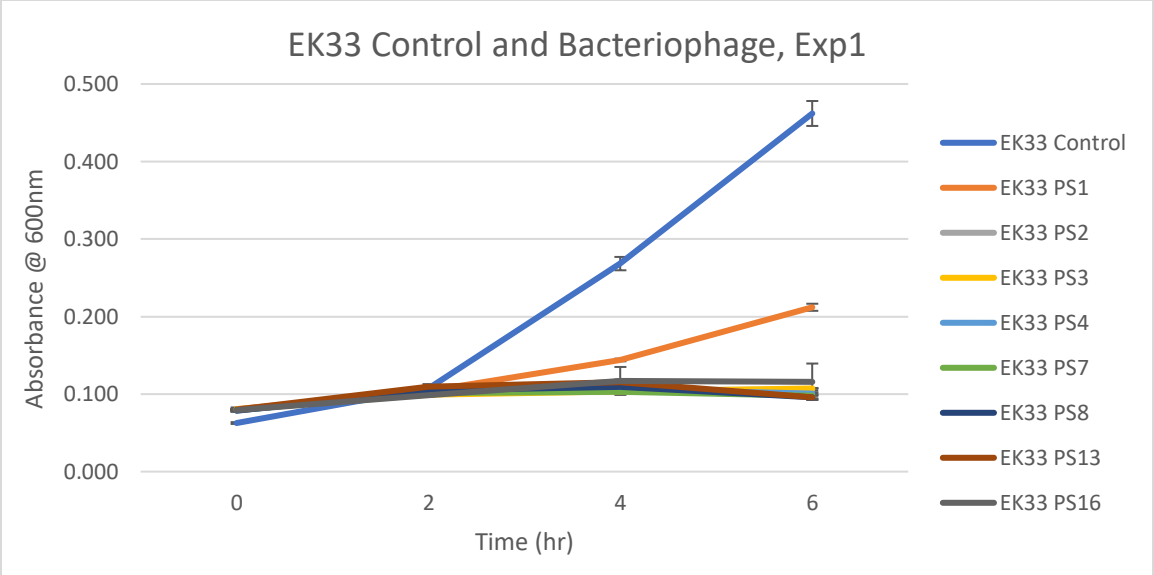

Figure 33. Average absorbance of *E. coli* EK33 at 600nm mixed with phage stocks, measured over six hours from Experiment 1. Standard deviation for each time point shown with error bars.

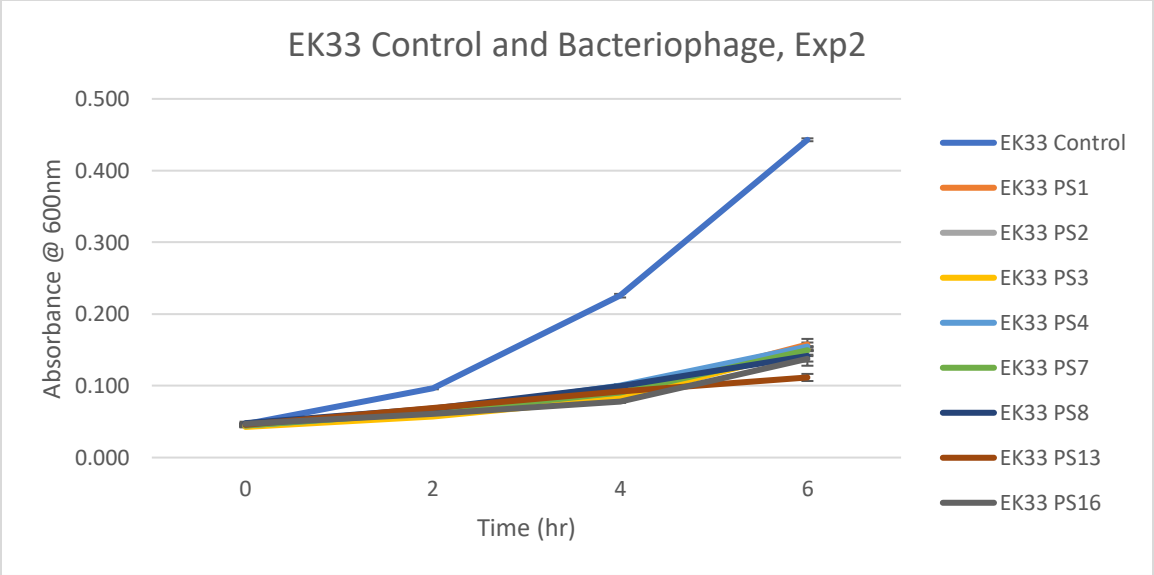

Figure 34. Average absorbance of *E. coli* EK33 at 600nm mixed with phage stocks, measured over six hours from Experiment 2. Standard deviation for each time point shown with error bars.

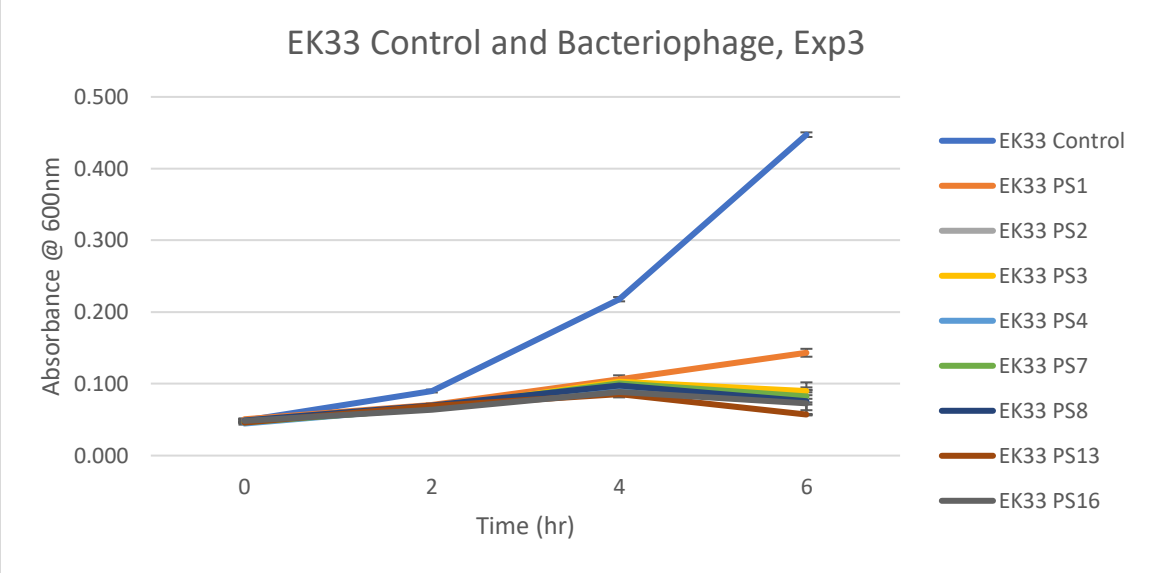

Figure 35. Average absorbance of *E. coli* EK33 at 600nm mixed with phage stocks, measured over six hours from Experiment 3. Standard deviation for each time point shown with error bars.

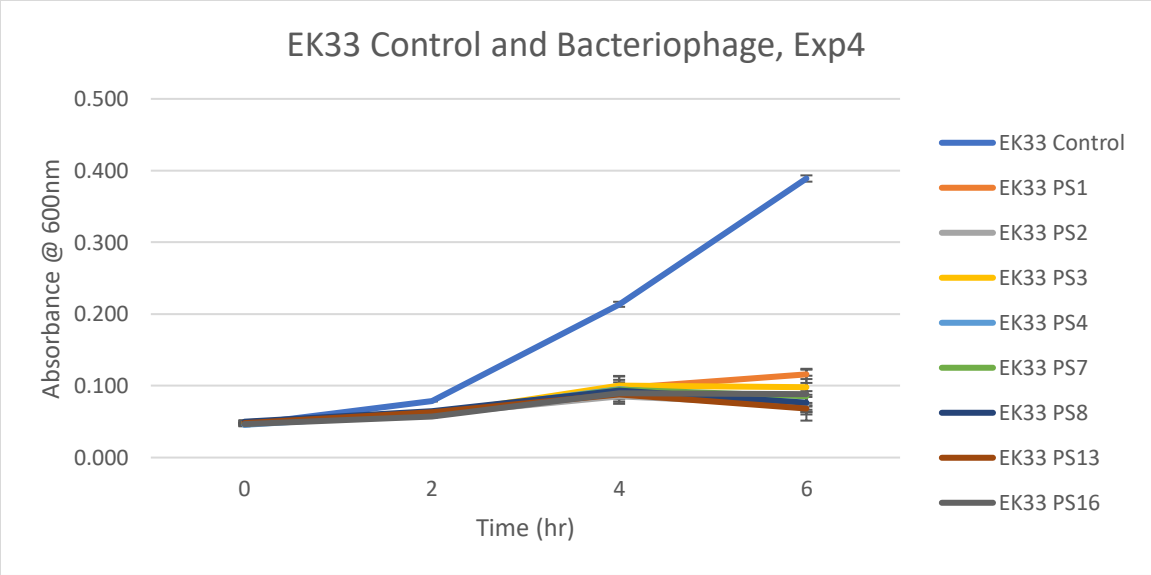

Figure 36. Average absorbance of *E. coli* EK33 at 600nm mixed with phage stocks, measured over six hours from Experiment 4. Standard deviation for each time point shown with error bars.

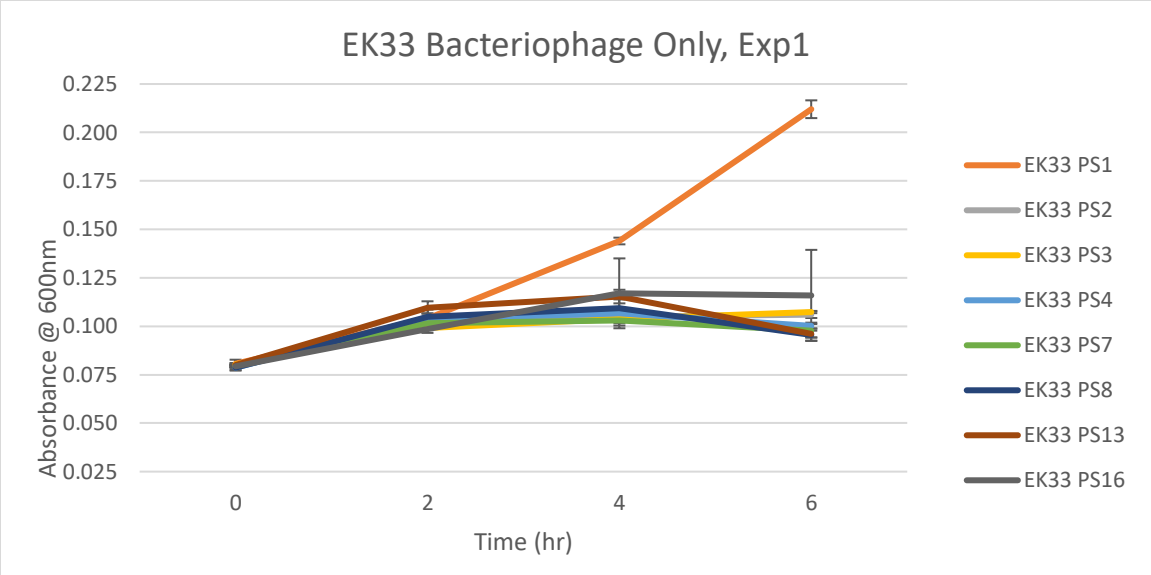

Figure 37. Average absorbance of *E. coli* EK33 at 600nm mixed with phage stocks, measured over six hours from Experiment 1, control data removed. Standard deviation for each time point shown with error bars.

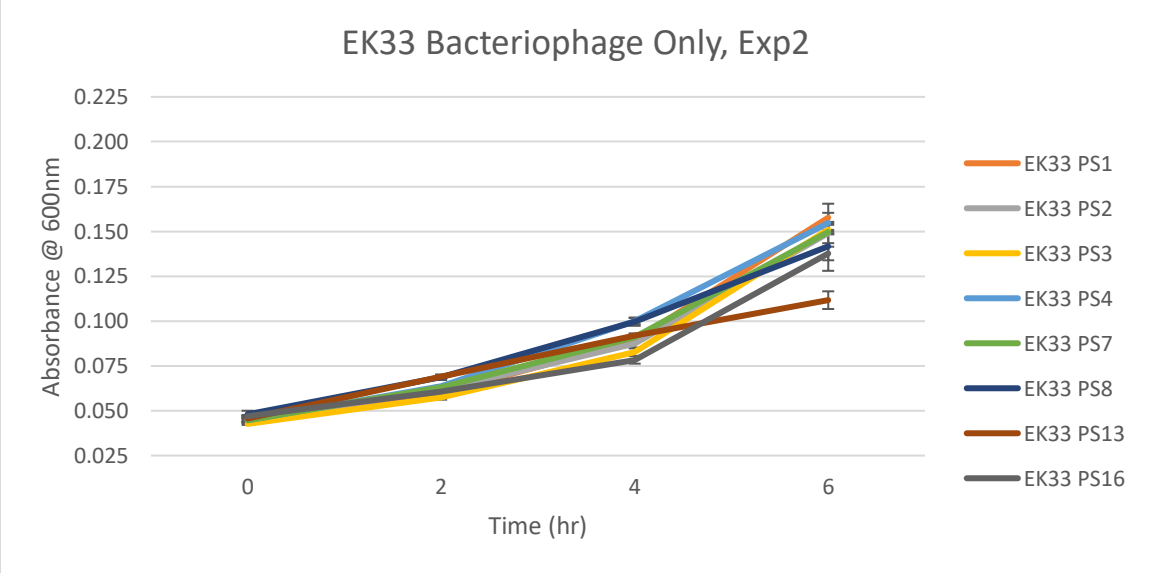

Figure 38. Average absorbance of *E. coli* EK33 at 600nm mixed with phage stocks, measured over six hours from Experiment 2, control data removed. Standard deviation for each time point shown with error bars.

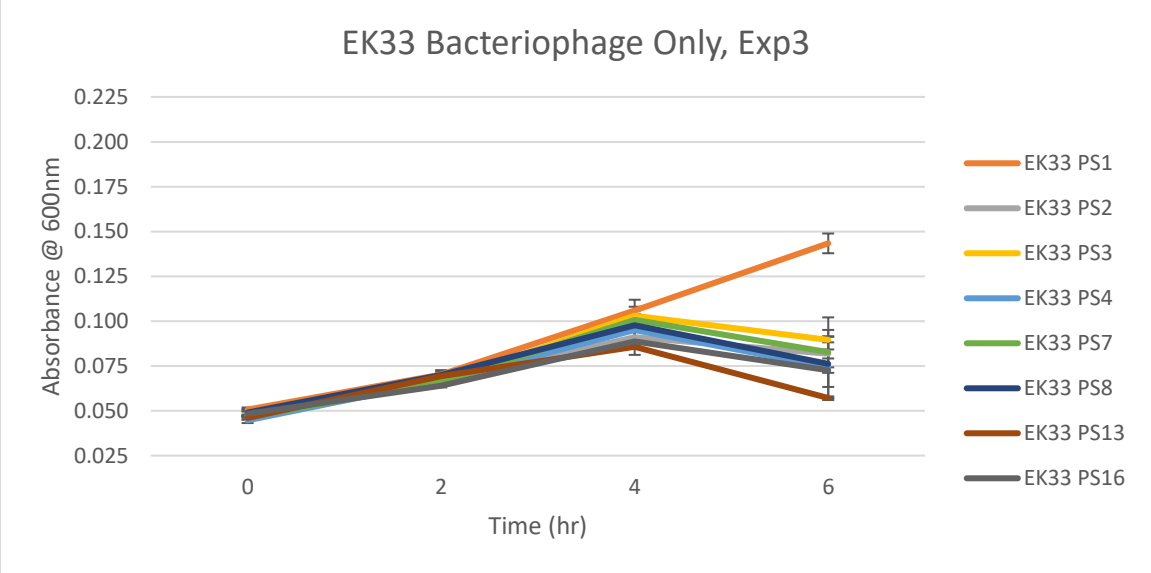

Figure 39. Average absorbance of *E. coli* EK33 at 600nm mixed with phage stocks, measured over six hours from Experiment 3, control data removed. Standard deviation for each time point shown with error bars.

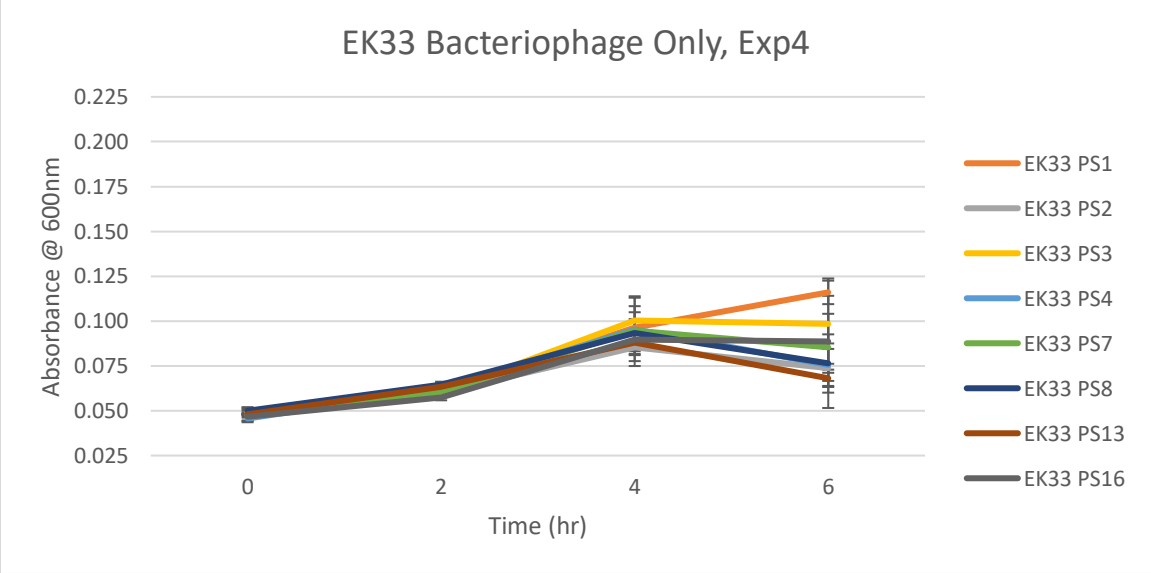

Figure 40. Average absorbance of *E. coli* EK33 at 600nm mixed with phage stocks, measured over six hours from Experiment 4, control data removed. Standard deviation for each time point shown with error bars.

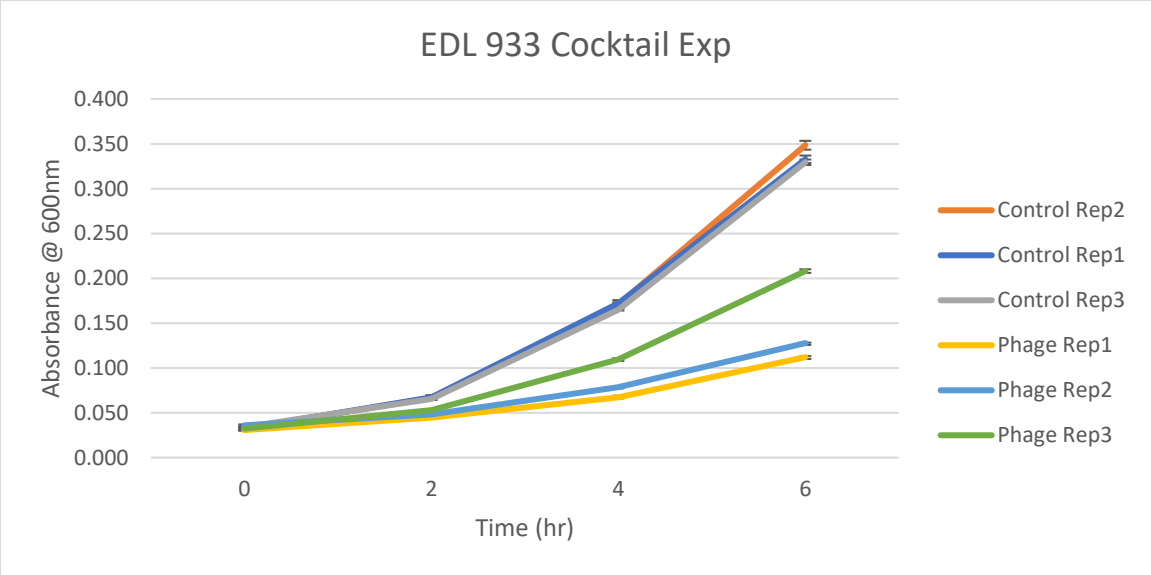

Figure 41. Average absorbance of *E. coli* EDL933 33 at 600nm mixed with phage stock cocktail (PS1, PS7, & PS16), measured over six hours. Standard deviation for each time point shown with error bars.

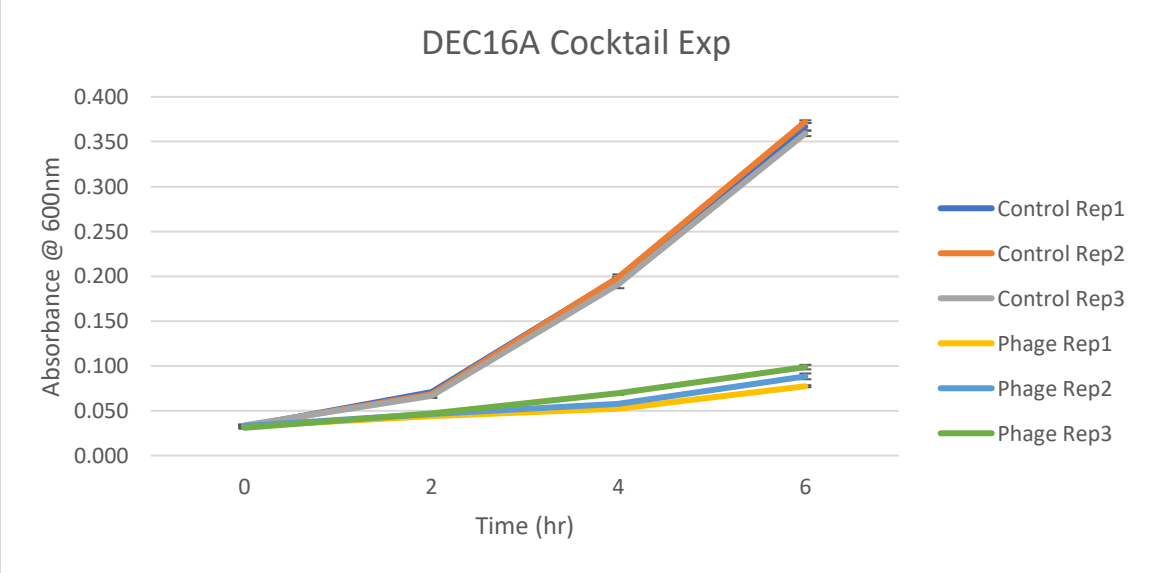

Figure 42. Average absorbance of *E. coli* DEC16A 33 at 600nm mixed with phage stock cocktail (PS1, PS7, & PS16), measured over six hours. Standard deviation for each time point shown with error bars.

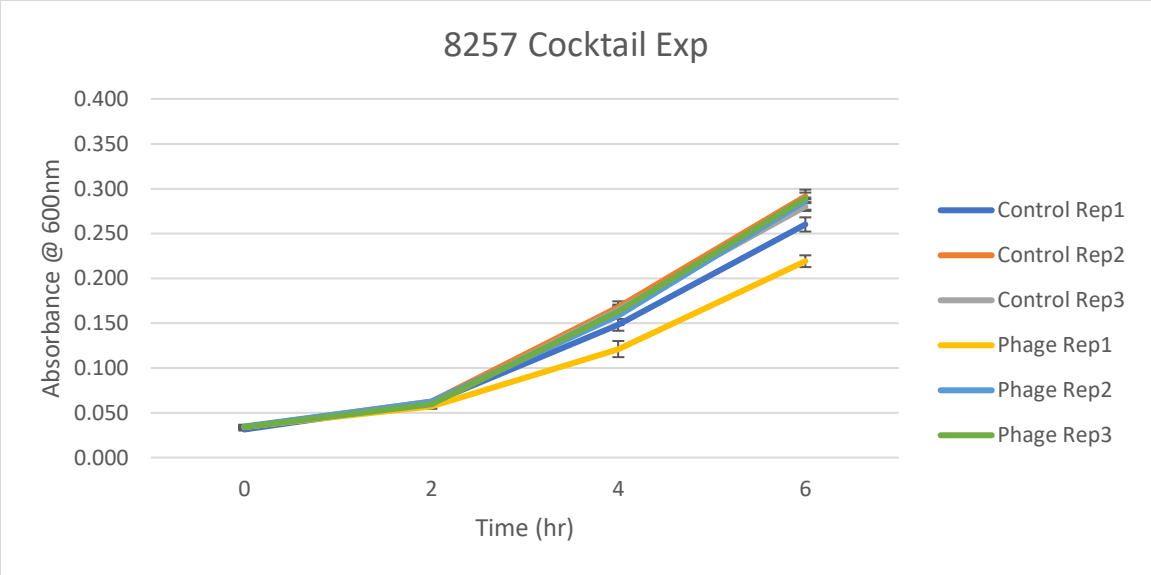

Figure 43. Average absorbance of *E. coli* 8257 33 at 600nm mixed with phage stock cocktail (PS1, PS7, & PS16), measured over six hours. Standard deviation for each time point shown with error bars.

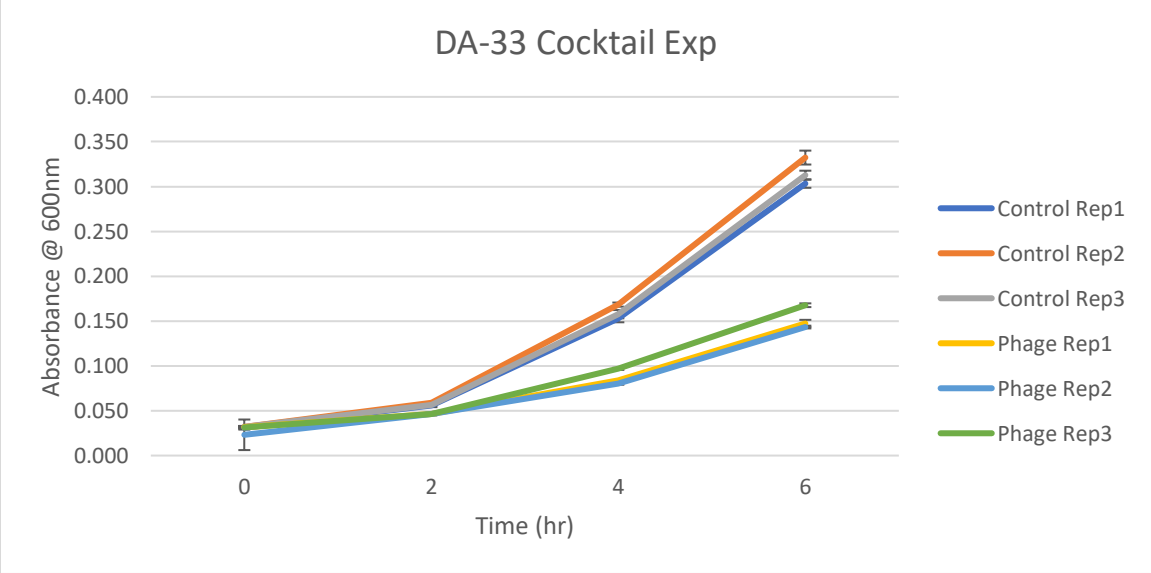

Figure 44. Average absorbance of *E. coli* DA-33 33 at 600nm mixed with phage stock cocktail (PS1, PS7, & PS16), measured over six hours. Standard deviation for each time point shown with error bars.

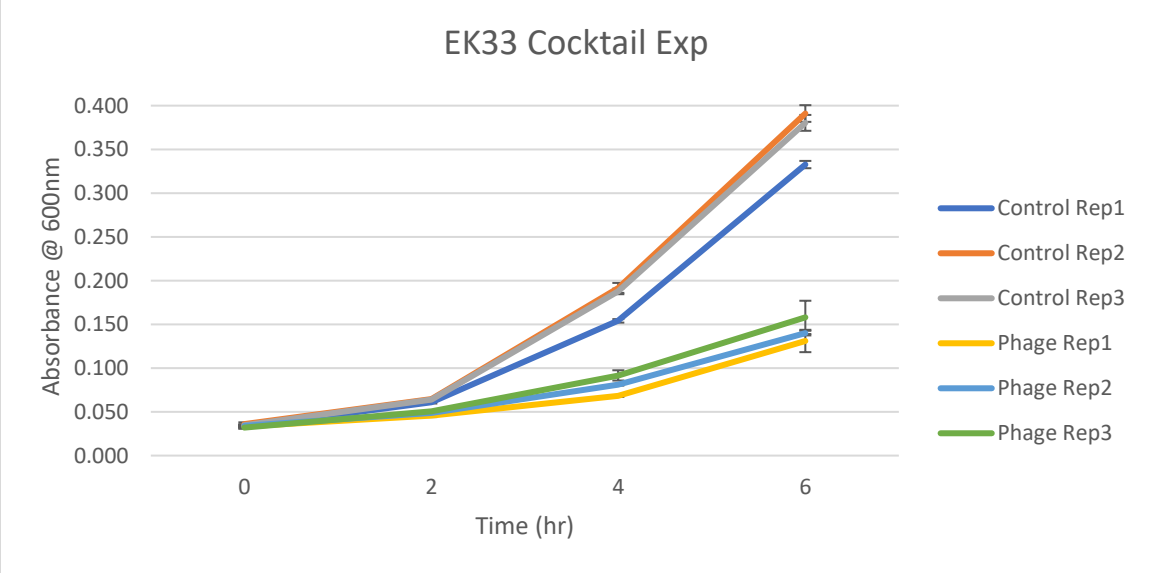

Figure 45. Average absorbance of *E. coli* EK33 33 at 600nm mixed with phage stock cocktail (PS1, PS7, & PS16), measured over six hours. Standard deviation for each time point shown with error bars.

Figure 46. Agarose gel of nucleic acids from Phage Stocks 1, 2, & 3. Lanes 1 and 12 contain λ DNA cut with Hind III. Lanes 2, 3, and 4 contain PS1 nucleic acid treatments; undigested in lane 2, digested with DNAse in lane 3, and digested with RNAse in lane 4. Lanes 5, 6 and 7 contain PS2 nucleic acid treatments; undigested in lane 5, digested with DNAse in lane 6, and digested with RNAse in lane 7. Lanes 8, 9 and 10 contain PS3 nucleic acid treatments; undigested in lane 8, digested with DNAse in lane 9, and digested with RNAse in lane 10. Lane 11 was intentionally left empty.

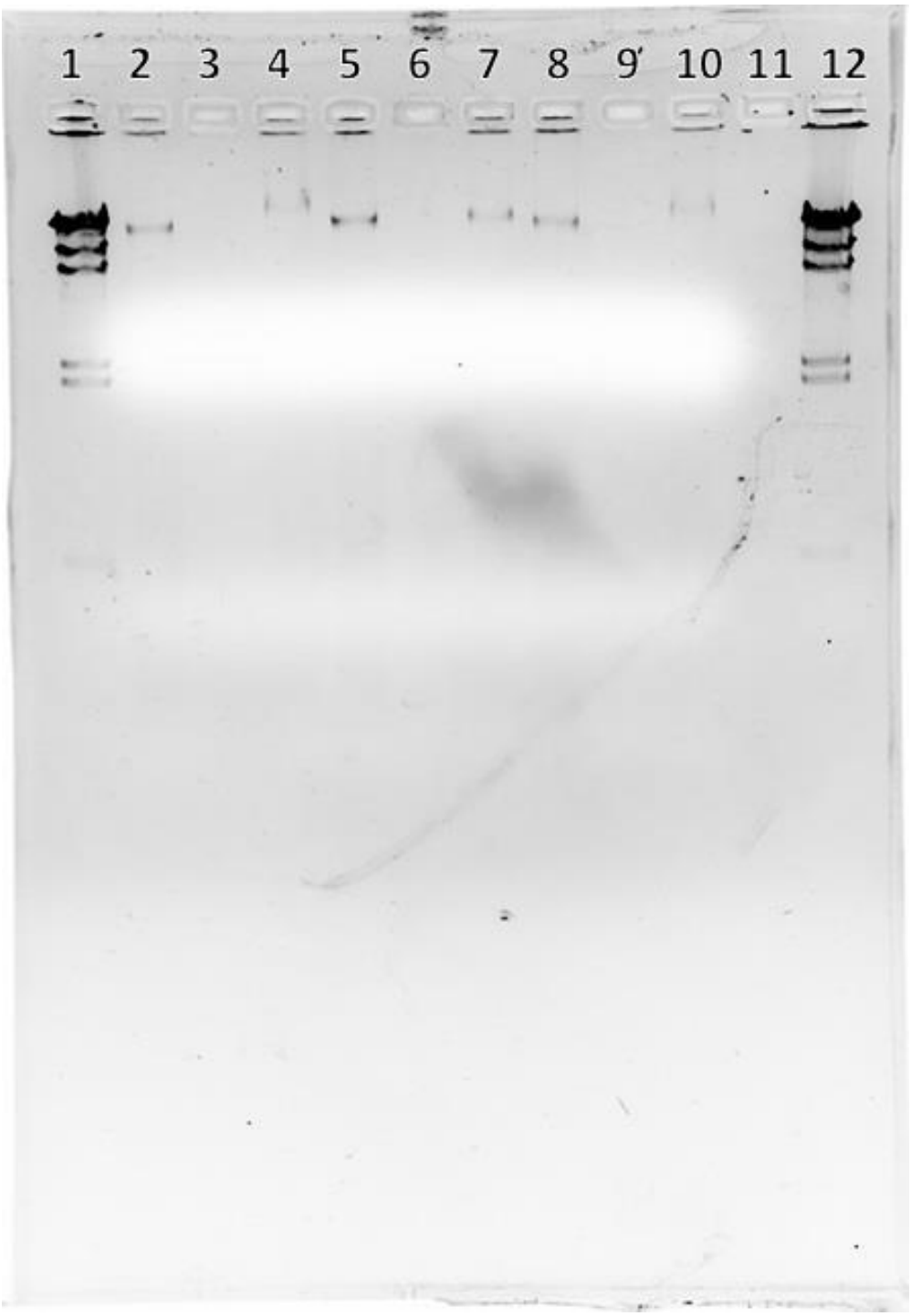

Figure 47. Agarose gel of nucleic acids from Phage Stocks 4,7 & 8. Lanes 1 and 12 contain λ DNA cut with Hind III. Lanes 2, 3, and 4 contain PS4 nucleic acid treatments; undigested in lane 2, digested with DNAse in lane 3, and digested with RNAse in lane 4. Lanes 5, 6 and 7 contain PS7 nucleic acid treatments; undigested in lane 5, digested with DNAse in lane 6, and digested with RNAse in lane 7. Lanes 8, 9 and 10 contain PS8 nucleic acid treatments; undigested in lane 8, digested with DNAse in lane 9, and digested with RNAse in lane 10. Lane 11 was intentionally left empty.

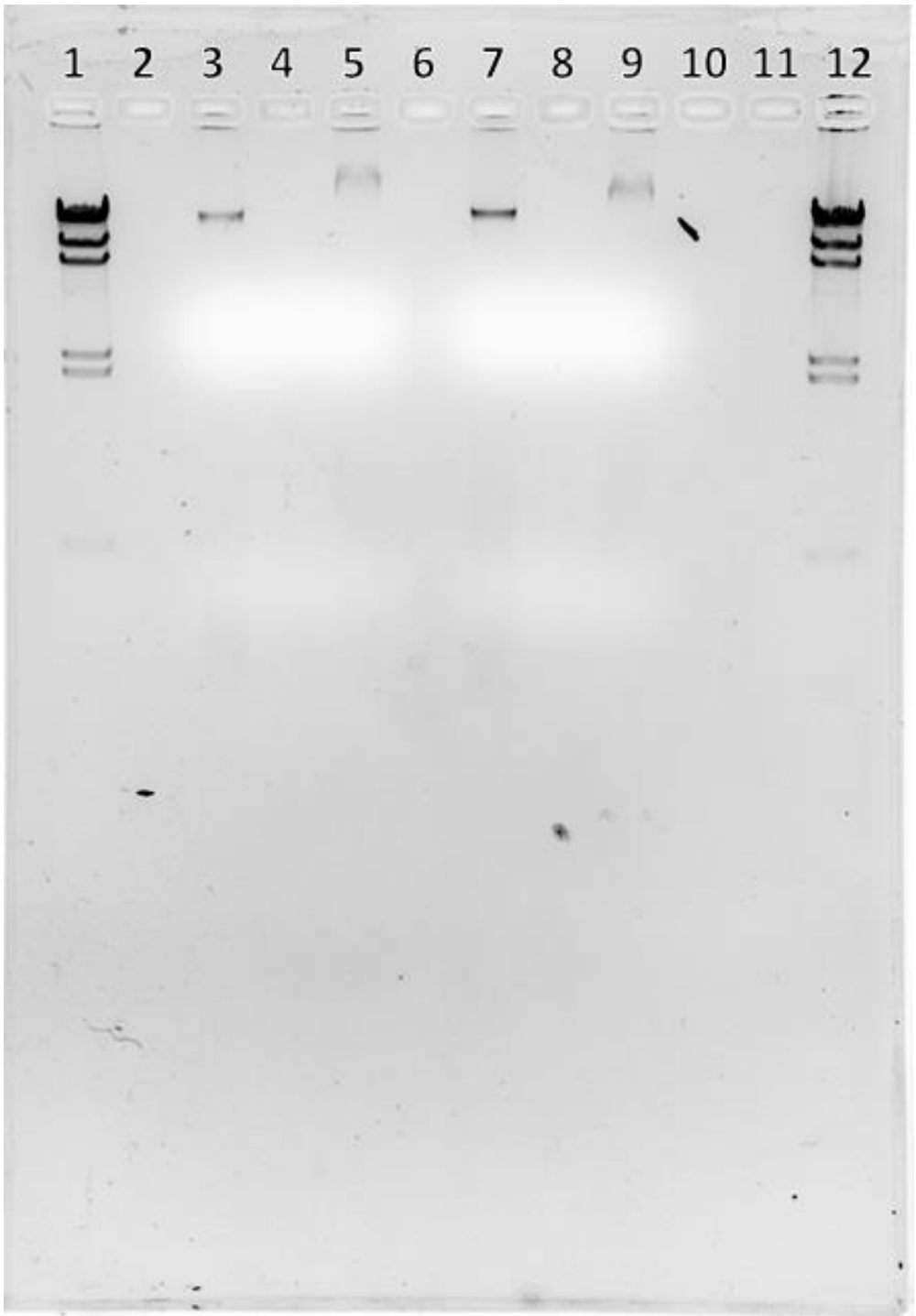

Figure 48. Agarose gel of nucleic acids from Phage Stocks 13 & 16. Lanes 1 and 12 contain λ DNA cut with Hind III. Lanes 3, 4, and 5 contain PS13 nucleic acid treatments; undigested in lane 2, digested with DNAse in lane 4, and digested with RNAse in lane 5. Lanes 7, 8 and 9 contain PS16 nucleic acid treatments; undigested in lane 7, digested with DNAse in lane 8, and digested with RNAse in lane 9. Lanes 2, 6, 10 and 11 were intentionally left empty.

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CHAPTER III

Future Directions

The next step in studying these bacteriophage is to examine them under a scanning electron microscope (SEM). The use of chloroform in isolating the bacteriophage should have selected for bacteriophage with low lipid counts, likely *Caudovirales*[48]. Determining what type of bacteriophage was selected would be beneficial in knowing whether or not any of these 8 bacteriophage could be used in bacteriophage therapy, which requires obligately lytic bacteriophage. The morphology of the 8 bacteriophage not only tells us what family of bacteriophage they fall under, but can also give insight into possible life cycles and may confirm suspected differences between the eight bacteriophage. However, genetic sequencing is key to determine if they are all different, how much they differ from each other, and whether or not they have any genes for lysogeny. Genome analysis will also determine if these bacteriophage are potential candidates for bacteriophage therapy by looking for resistance genes and toxins.

In addition to genome analysis, greater host range specificity needs to be carried out. We only tested 28 out of the 72 STEC strains within our lab. These strains included EHEC 1, EHEC 2, STEC 1 and STEC 2 strains in addition to several other strains that do not fall into one of the STEC groupings. Viazis *et al.* tested 123 different *E. coli* O157:H7 strains when developing their bacteriophage mixture[143].

In addition to determining greater host range, temperature ranges for each bacteriophage need to be established to determine greatest activity and when activity is lost. Smith *et al.* found that bacteriophage virulence is largely impacted by temperature, and that outside of the optimal range bacteriophage virulence dramatically drops. The

experiments with EK33 indicate that for 7 of the eight bacteriophage, their optimal temperature range may be below room temperature. The optimal temperature ranges of the bacteriophage would also determine their suitability for bacteriophage therapy. Patient therapy requires highly virulent bacteriophage around 35^{o}C while surface biocontrol requires bacteriophage virulence around 20^{o}C.

The present study was limited by the concentration of phage stock 1, which was only $1.2x10^{9}$ pfu/mL. Due to the volumes required for experimentation, all phage stocks were diluted to $1.4x10^{8}$ pfu/mL. The spectrophotometer was only accurate with absorbance readings above 0.020, which required bacterial concentrations of $5.0x10^{7}$ cfu/mL. Increasing the concentrations of all phage stocks would allow the absorbance assays to be repeated with higher bacteriophage titers. Methods to increase the titers include PEG precipitation or membrane capture followed by dilution in media of choice.

Once temperature ranges are determined, and genomic analysis determines the number of unique bacteriophage and which one(s) are obligately lytic, surface testing can be completed. Testing on spinach leaves or other fresh produce with known concentrations of sensitive strains at both commonly encountered contamination levels and increased contamination levels should be done. In addition to testing produce, testing solid surfaces such as stainless steel, wood and high density polyethylene should be performed. These surfaces are commonly used in food preparation and once contaminated, can spread bacteria onto clean food[143]. Similar to produce, testing should be done with known concentrations of sensitive strains at both commonly encountered contamination levels and increased contamination levels. Testing both produce and surface materials would indicate what concentrations of bacteriophage are

effective, to what extent can *E. coli* concentrations be reduced, and whether these bacteriophage can be practical in the food industry.

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