Escherichia coli O157:H7 bacteriophage Φ241 isolated from an industrial cucumber fermentation at high acidity and salinity

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A novel phage, Φ241, specific for Escherichia coli O157:H7 was isolated from an industrial cucumber fermentation where both acidity (pH ≤ 3.7) and salinity (>5% NaCl) were high. The phage belongs to the Myoviridae family. Its latent period was 15 min and average burst size was 53 phage particles per infected cell. The phage was able to lyse 48 E. coli O157:H7 strains, but none of the 18 non-O157 strains (including E. coli O104:H7 or the 2 O antigen-negative mutants of O157:H7 strain, 43895Δper (also lacking H7 antigen) and F12 (still expressing H7 antigen). However, the phage was able to lyse a per-complemented strain (43895ΔperComp) which expresses O157 antigen. These results indicated that phage Φ241 is specific for O157 antigen, and E. coli strains lacking O157 antigen were resistant to the phage infection, regardless of the presence or absence of H7 antigen. SDS-PAGE profile revealed at least 13 structural proteins of the phage. The phage DNA was resistant to many commonly used restriction endonucleases, suggesting the presence of modified nucleotides in the phage genome. At the multiplicity of infection of 10, 3, or 0.3, the phage caused a rapid cell lysis within 1 or 2 h, resulting in 3.5- 4.5-log-unit reduction in cell concentration. The high lytic activity, specificity and tolerance to low pH and high salinity make phage Φ241 a potentially ideal biocontrol agent of E. coli O157:H7 in various foods. To our knowledge, this is the first report on E. coli O157:H7 phage isolated from high acidity and salinity environment.

Keywords: bacteriophage, phage Φ241, Escherichia coli O157:H7, biocontrol agent, cucumber fermentation, high acidity and salinity, food safety

INTRODUCTION

Escherichia coli O157:H7 has emerged as one of the major foodborne pathogens. Each year, it causes more than 73,000 illnesses, 2,100 hospitalizations, and 60 deaths in the U.S. (Mead and Griffin, 1998; Mead et al., 1999; Rangel et al., 2005). A variety of foods have been associated with these outbreaks such as undercooked ground beef (Griffin and Tauxe, 1991; Anonymous, 1993, 2014; Bell et al., 1994), raw milk (Riley et al., 1983), cheese (Anonymous, 1989), water (Swerdlow et al., 1992; Bopp et al., 2003), unpasteurized apple juice (Anonymous, 1996), sprouts, lettuce, spinach, and other vegetables (Como-Sebetti et al., 1997; Jinneman et al., 2003; Anonymous, 2006, 2012a,b, 2013). Healthy cattle are the primary reservoir of E. coli O157:H7. Human infection by E. coli O157:H7 can frequently be traced to the food or water contaminated with cattle manure (Gyles, 2007). The infection by this pathogen can result in severe hemorrhagic colitis and life-threatening hemolytic uremic syndrome (Remis et al., 1984; Cleary, 1988; Tarr, 1995; Nataro and Kaper, 1998). E. coli O157:H7 has a very low infectious dose (as low as 10 cells; Griffin and Tauxe, 1991; Griffin et al., 1994; Tuttle et al., 1999) partly due to its very efficient mechanisms of stress resistance (Price et al., 2004). Acid resistance is one of the characteristics of E. coli O157:H7. The bacterium has evolved multiple mechanisms to survive in low-pH environments (Lin et al., 1996; Castanie-Cornet et al., 1999; Jordan et al., 1999; Price et al., 2000, 2004; Large et al., 2005) such as gastrointestinal tracts and various acidic foods (Weagant et al., 1994; Diez-Gonzalez and Russell, 1999; Price et al., 2004). Acid resistance is especially crucial for food-borne pathogens that must survive the hostile acidic condition in the stomach before entering and colonizing the small intestines or colon (Berk et al., 2005; Chen and Jiang, 2014).

Acid adaptation can further enhance the survival of E. coli O157:H7 in fermented or acidified foods, and induce the cross-protection against heat, salt, and acids (Farber and Pagotto, 1992; Leyer and Johnson, 1993; Leyer et al., 1995; Cheville et al., 1996). A variety of acidic foods have been involved in the outbreaks caused by E. coli O157:H7. These include apple cider (Besser et al., 1993; Hilborn et al., 2000), unpasteurized apple juice (Cody et al., 1999), salami (Anonymous, 1995), and fermented sausage (Glass et al., 1992). E. coli O157:H7 can also tolerate high concentration of NaCl (Glass et al., 1992).

Many physical, chemical, and biological methods (such as pasteurization, radiation, addition of preservatives, or addition of lactic acid bacteria) have been used to control E. coli O157:H7 in foods. However, these control methods are not very effective for certain foods or they can alter the color, flavor, or texture of the foods. Safe and effective alternative methods are needed to control E. coli O157:H7 in foods. Recent studies have showed that the use...
of phages to control pathogenic bacteria in foods is a promising novel strategy.

The use of phages as antibacterial agents has several advantages over traditional antibacterial methods. First of all, phages are highly host specific. They only infect specific bacterial hosts and cause rapid bacterial lysis. They do not infect humans and other eukaryotes. Phages specific for pathogenic bacteria do not disrupt normal microflora in humans (Kudva et al., 1999) or in animals. Secondly, phages are not toxic to humans. Although certain cell lysis may release endotoxins, phages themselves do not generate any toxic products during their multiplication (Hagens and Loessner, 2010). Thirdly, phages do not alter food quality because they do not produce any substances that can change the taste, composition, aroma, or color of foods. In addition, phages are stable (Coffey et al., 2010), but also self-limiting in foods. They do not replicate unless their bacterial hosts are present (Hagens and Loessner, 2010). Moreover, phages are the most abundant biological entities and naturally present in the environment and a wide variety of foods (Guenther et al., 2009). It is relatively easy to isolate phages from the environment and propagate them in laboratories. All these features make phages promising novel biocontrol agents of bacterial pathogens in foods.

Recent studies have shown high efficacy of using phages against several major food-borne pathogens including E. coli O157:H7, Listeria monocytogenes, and Salmonella enterica in food products or on food contact surfaces. Use of phages specific for E. coli O157:H7 resulted in significant, log-unit reductions in E. coli O157:H7 counts in a variety of foods such as tomato, spinach, broccoli, and ground beef (Abuladze et al., 2008), beef (Carter et al., 2012), cantaloupe (Sharma et al., 2009), lettuce (Sharma et al., 2009; Ferguson et al., 2013), and other leafy green vegetables (Viazis et al., 2011). Such reductions could substantially decrease a risk of food-borne infections by the pathogen.

Significant progress in phage research for food safety has been made toward phage applications in foods. Several phage-based food additives have been recently approved or cleared by the U.S. Food and Drug Administration (FDA). These approvals have increased the impetus of phage research to uncover phage-mediated applications against other food-borne pathogens (Mahony et al., 2011). It is likely that more phage products will be developed and gradually gain market acceptance by the food industry and the consumers as a means of a safe, natural, and effective prevention of food-borne diseases (O’Flaherty et al., 2009; Sharma, 2013).

Phages specific for E. coli O157 have previously been isolated from human fecal materials or animal manures from bovine, ovine, swine, and chicken (Kudva et al., 1999; Morita et al., 2002; O’Flynn et al., 2004; Tomat et al., 2013), lake or pond water (Shahrbabak et al., 2013), and sewage (Sheng et al., 2006; Shahrbaban et al., 2013). No E. coli O157-specific phages were isolated from the environment where both acidity and salinity are high. The objectives of this study were to isolate an E. coli O157:H7-specific phage from a cucumber fermentation with low pH (3.7) and high salt concentration (5% NaCl), to characterize the phage, and to evaluate the potential of the phage as an effective biocontrol agent against E. coli O157:H7 in various foods.

**MATERIALS AND METHODS**

**BACTERIAL STRAINS AND CULTURE CONDITIONS**

The E. coli strains used in this study are listed in Tables 1 and 2. A total of 46 E. coli O157:H7 strains, and 18 E. coli non-O157:H7 strains from various sources were obtained from the culture collection of USDA Agricultural Research Service located at North Carolina State University. The non-O157 strains included a variety of E. coli strains that express a variety of H antigens including H7 antigen. Two previously described O antigen-negative mutants (43895Δper and F12), one per-complemented mutant (43895ΔperComp), and two E. coli O157:H7 parent strains (ATCC 43895 and 8624) were kindly provided by Pina Fratamico (Table 3). All strains were stored in tryptic soy broth (TSB; Difco) supplemented with 16% (v/v) glycerol at −80°C until use. Fresh overnight culture of each E. coli strain was prepared by inoculating 10 ml of TSB with an isolated colony from a tryptic soy agar (TSA) plate and incubating statically for 12 h at 37°C. For phage lyase preparation, TSB broth was supplemented with 10 mM CaCl2 (Sigma–Aldrich, St. Louis, MO, USA) unless otherwise stated. Soft TSA agar used in plaque assay was prepared with TSB broth supplemented with 0.6% agar.

**BRINE SAMPLE COLLECTION AND TREATMENT**

To isolate E. coli O157:H7-specific phages, brine samples (40 ml each) were taken from seven industrial cucumber fermentation tanks (capacity: 32,000 l) from a commercial processing plant. The tanks contained approximately 55% pickling cucumbers in 5 to 8% recycled NaCl brine, prepared essentially as described by Breidt et al. (2013). These samples were taken during the fermentation (3–5 days after the tanks were packed and brined). Samples were transported to the laboratory at ambient temperature (~23°C), stored at 4°C, and processed within 24 h. The pH of each brine sample was measured and adjusted to around 6.4 with 5 M NaOH. The pH-adjusted brine samples were then centrifuged (5,000 × g for 10 min). The supernatants were filtered through syringe filters (0.45 μm pore size) to remove cellular materials and solid particles. The filtrates were stored at 4°C until used as potential phage source for phage isolation.

**PHAGE ISOLATION**

Ten E. coli O157:H7 strains (shown in bold text, Table 1) were used as potential hosts for phage isolation. Overnight cultures of these O157 strains (~10⁹ CFU/ml) were prepared in TSB. A 96-well microplate was used to enrich phages potentially present in the filtered brines. Each well of the microplate contained 200 μl of TSB, 5 μl of one of the 10 E. coli O157:H7 strains and 45 μl of one of the eight filtered brines, so the eight wells in the same column received the same O157:H7 strain. The first 10 wells in the same row received the same filtered brine. After incubation at 37°C for 20 h, the microplate was centrifuged (SH-3000 rotor, RC-5B centrifuge, Sorvall, Newtown, CT, USA) at 4,000 rpm, 4°C for 20 min. The supernatant (lysate) in each well was collected and used in spot tests to detect the presence of phages. Each spot test was performed by adding 10 μl of a phage lysate onto a lawn of E. coli O157:H7 in a soft agar overlay on a TSA plate. After
Table 1 | *Escherichia coli* O157:H7 strains that are sensitive to phage Φ241.

| ID   | Serotype       | Source                          |
|------|----------------|--------------------------------|
| B0201b | O157:H7        | Apple cider outbreak            |
| B0349 | O157:H7        | Spinach outbreak                |
| B0264 | O157:H7        | Apple juice outbreak, 1996      |
| B0204 | O157:H7        | Pork                            |
| B0202 | O157:H7        | Salami outbreak                 |
| B0203 | O157:H7        | Ground beef                     |
| B0348 | O157:H7        | Salami                          |
| B0350 | O157:H7        | Sakai                           |
| B0243 | O157:H7        | Bovine carcass                  |
| B0242 | O157:H7        | Bovine carcass                  |
| B0240 | O157:H7        | Bovine carcass                  |
| B0239 | O157:H7        | Bovine carcass                  |
| B0238 | O157:H7        | Bovine carcass                  |
| B0241 | O157:H7        | Bovine carcass                  |
| B0258 | O157:H7        | Bovine feces                    |
| B0259 | O157:H7        | Bovine feces                    |
| B0301 | O157:H7        | Water                           |
| B0307 | O157:H7        | Water                           |
| B0306 | O157:H7        | Water                           |
| B0309 | O157:H7        | Water                           |
| B0302 | O157:H7        | Water                           |
| B0297 | O157:H7        | Water                           |
| B0299 | O157:H7        | Water                           |
| B0285 | O157:H7        | Water                           |
| B0275 | O157:H7        | Water                           |
| B0305 | O157:H7        | Water                           |
| B0281 | O157:H7        | Water                           |
| B0289 | O157:H7        | Water                           |
| B0280 | O157:H7        | Water                           |
| B0287 | O157:H7        | Water                           |
| B0283 | O157:H7        | Water                           |
| B0269 | O157:H7        | Human, outbreak, 2000, waterborne |
| B0273 | O157:H7        | Human, outbreak, 2002, leafy vegetable |
| B0247 | O157:H7        | Human, outbreak                 |
| B0296 | O157:H7        | Human, outbreak, 2005, leafy vegetable |
| B0311 | O157:H7        | Human, outbreak, 2006, leafy vegetable |
| B0246 | O157:H7        | Human, outbreak                 |
| B0271 | O157:H7        | Human, outbreak, 2003, leafy vegetable |
| B0250 | O157:H7        | Human, outbreak                 |
| B0263 | O157:H7        | Human, sporadic, 1997           |
| B0251 | O157:H7        | Human, outbreak                 |
| B0249 | O157:H7        | Human, outbreak                 |
| B0266 | O157:H7        | Human, outbreak, 1999, taco meat |
| B0245 | O157:H7        | Human, outbreak                 |
| B0265 | O157:H7        | Human, outbreak, 1999, lettuce  |
| B0244 | O157:H7        | Human, outbreak                 |

*a* ID, identification number in the culture collection of USDA-ARS Food Fermentation Laboratory.  
bThe strains with ID bolded were used for initial phage isolation.

Table 2 | Non-O157 strains of *E. coli* that are resistant to phage Φ241.

| ID   | Serotype       | Source                          |
|------|----------------|--------------------------------|
| B0445 | O26:H11        | Human                           |
| B0449 | O26:H11        | Human                           |
| B0463 | O103:H6        | Human diarrhea                  |
| B0460 | O103:H25       | Human                           |
| B0469 | O104:H4        | Human                           |
| B0467 | O104:H21       | Human, milk outbreak            |
| B0475 | O111:NMb       | Human                           |
| B0478 | O111:H8        | Human                           |
| B0479 | O121:NM        | Human diarrhea                  |
| B0485 | O145:NM        | Human                           |
| B0457 | O45:H2         | Cow (cafl)                      |
| B0468 | O104:H7        | Ground beef                     |
| B0235 | Non-O157c      | Bovine feces                    |
| B0237 | Non-O157       | Bovine feces                    |
| B0234 | Non-O157       | Bovine feces                    |
| B0236 | Non-O157       | Bovine feces                    |
| B0233 | Non-O157       | Bovine feces                    |
| 25922 | O6:H1          | ATCCd                           |

*a* ID, identification number.  
bNM, non-motile.  
cThe strains were not completely serotyped. But the data showed that they did not respond to the serum antibody against O157 strains.  
dATCC, American Type Culture Collection.

overnight incubation at 37°C, the plates were checked for a zone of bacterial lysis.

PHAGE PURIFICATION AND CONCENTRATION

Phage from a positive spot-test plate was purified and concentrated using the methods described by Lu et al. (2003) with minor modification. Briefly, an isolated single plaque was picked and propagated against its natural host in TSB at 37°C. After two runs of plaque purification, the phage lysate was prepared and then centrifuged at 5,000 × g for 10 min. The supernatant was filtered through bottle-top filter (0.45 μm pore size). The filtered high titer phage stock (typically ca. 1010 PFU/ml) was stored at 4°C. To further purify and concentrate the phage, a portion of the phage stock were treated with DNase I and RNase A, and then concentrated by PEG precipitation. The concentrated phage was further purified by CsCl step density gradient ultracentrifugation at 600,000 × g for 6 h at 4°C followed by dialysis as described by Lu et al. (2003). The ultracentrifuge-purified phage was used for electron microscopy analysis, SDS-PAGE, and DNA extraction.

ELECTRON MICROSCOPY

Phage samples were negatively stained with 2% (w/v) aqueous uranyl acetate (pH 4) on carbon-coated grids and examined by transmission electron microscopy (JEM 1200EX TEM, JEOL) at an accelerating voltage of 80 kV. Electron micrographs were taken at a magnification of 50,000 × (Center for Electron Microscopy, North Carolina State University, Raleigh, NC, USA).
Table 3 | Phage susceptibility of E. coli O157:H7 strains and their O antigen-negative mutants.

| E. coli strain   | Description                                                                 | Plaque formation\(^a\) | Source or reference   |
|------------------|------------------------------------------------------------------------------|-------------------------|----------------------|
| ATCC 43895       | Wild-type E. coli O157:H7, clinical isolate, stx\(^+\)/stx\(^+\)             | +                       | ATCC\(^b\)           |
| 43895\(\Delta\)per| O antigen-negative mutant of ATCC 43895 with perosamine synthetase deleted | –                       | Sheng et al. (2008)  |
| 43895\(\Delta\)perComp | 43895\(\Delta\)per transformed with pCRII:per                          | +                       | Sheng et al. (2008)  |
| 8624             | Wild-type E. coli O157:H7, clinical isolate, stx\(^+\)/stx\(^+\)            | +                       | Bilge et al. (1996)  |
| F12              | O antigen-negative mutant of strain 8624                                   | –                       | Bilge et al. (1996)  |

\(^a\)\(^+\), susceptible to \(\Phi241\); –, not susceptible to \(\Phi241\).

\(^b\)ATCC, American Type Culture Collection.

ONE-STEP GROWTH KINETICS

One step growth experiments were carried out based on the method described by Leuschner et al. (1993) and Foschino et al. (1995) with some modifications. Briefly, the experiment started at a multiplicity of infection (MOI) of 0.01 in a 15-ml tube containing the phage (approximately 1 \(\times\) \(10^6\) PFU/ml) and its natural host O157:H7 strain B0241 in 10 ml TSB. After incubation in a water bath at 37\(^\circ\)C for 10 min (to allow phage adsorption), the tube was centrifuged at 13,000 \(\times\) \(g\) for 30 s. The supernatant was removed and subjected to plaque assay to determine the size of the un-absorbed phage. The pellet containing (partially) infected cells was immediately re-suspended in 10 ml of pre-warmed TSB. After taking the first sample, the tube was returned to the water bath (37\(^\circ\)C). A sample (100 \(\mu\)l) was collected every 5 min (up to 60 min). Each sample was immediately diluted and subjected to plaque assay. All assays were carried out in triplicate. The experiment was repeated three times. Latent period was defined as the time interval between the end of the adsorption and the beginning of the first burst, as indicated by the initial rise in phage titer (Ellis and Delbruck, 1939; Adams, 1959). Burst size was calculated as the ratio of the final number of liberated phage particles to the initial number of infected bacterial cells during the latent period (Adams, 1959).

HOST RANGE

Phage \(\Phi241\) was the only phage isolated from one of the seven samples. The host range of \(\Phi241\) was determined by spot tests against 46 E. coli O157:H7 strains (Table 1) and 18 non-O157 strains (Table 2) on TSA. In each test, 10 \(\mu\)l of high titer phage stock (10\(^{10}\) PFU/ml) was used to spot a bacterial lawn of a strain on a plate. Each test was done in duplicate. The O antigen-negative mutants of E. coli O157:H7 and their parent strains (Table 3) were also tested using the agar overlay method.

PHAGE STRUCTURAL PROTEINS

The phage structural proteins were analyzed using the method previously described by Lu et al. (2003) with some modifications. Briefly, the ultracentrifuge-purified phage particles were mixed with SDS-PAGE sample buffer and then heated in a boiling water bath for 10 min. The boiled sample was loaded onto a NuPAGE precast gradient minigel (4–12% Bis-Tris, Invitrogen Corporation, Carlsbad, CA, USA). Electrophoresis was carried out at 75 V for 2 h. Pre-stained protein standard (Invitrogen) was used to estimate the molecular weights of the proteins. The gel was stained with SimplyBlue SafeStain (Invitrogen).

PHAGE DNA EXTRACTION AND RESTRICTION

Phage DNA was prepared from the concentrated lysate using the phenol–chloroform extraction method as described by Lu et al. (2003), and digested with restriction endonucleases (AluI, BamHI, Clal, EcoRI, EcoRV, HindIII, MspI, SwaI, and XbaI; New England BioLabs, Beverly, MA, USA) according to the manufacturer’s instructions. The resulting DNA fragments were separated on the 1% agarose gel containing 0.001% SYBR Safe DNA gel stain (Invitrogen) by gel electrophoresis in Tris-borate-EDTA buffer at 70 V for 2 h. The 1 kb DNA ladder (Promega, Madison, WI, USA) was used to estimate the size of the digested phage DNA.

PHAGE INFECTION

The lytic activity of phage \(\Phi241\) against host E. coli O157:H7 B0241 was investigated in TSB medium at three different MOIs. A bacterial overnight culture was diluted with TSB to a concentration of 9 \(\times\) \(10^6\) CFU/ml. Ten milliliter of the diluted bacterial culture was then transferred into each of the four 15-ml tubes. One of these tubes served as a control. To each of other three tubes, a high titer phage stock (2.8 \(\times\) \(10^9\) PFU/ml) was added to achieve an initial MOI of 10, 3, or 0.3, respectively. The four tubes were incubated statically at 37\(^\circ\)C. Samples were taken from each tube at 60-min intervals for a 12-h period. After serial dilution, each sample was plated onto TSA plates using a spiral autoplater (Model 4000, Spiral Biotech, Bethesda, MD, USA). The plates were incubated at 37\(^\circ\)C overnight. The colonies on each plate were enumerated using Q-Count system (Model 510, Spiral Biotech, Norwood, MA, USA). The experiment was repeated two more times.

STATISTICAL ANALYSIS

Differences in bacterial cell concentration between various grouping of MOIs were analyzed by using one-way analysis of variance (ANOVA) and Tukey’s multiple comparison.

RESULTS AND DISCUSSION

ISOLATION OF PHAGE \(\Phi241\)

Seven brine samples from 32,000-l cucumber fermentation tanks (all from the same commercial plant) were enriched for phage isolation. One sample was found to contain a phage that infects E. coli O157:H7. The phage-containing sample was taken from a tank...
3 days after the tank was packed with size 2A cucumbers (∼27–32 mm in diameter). The pH and the salt (NaCl) concentration of the sample were 3.7 and 5%, respectively. In contrast, the pH and salt concentration of the samples from other six tanks were in the range of 3.42–3.92, and 6 to 8%, respectively. The higher salinity in these six tanks may greatly inhibit phages, which may explain why no O157:H7 phages were isolated from them. The isolated O157:H7 phage was designated /Phi1 241. The presence of phage /Phi1 241 specifically active against E. coli O157:H7 in an early stage of the commercial cucumber fermentation indicates that the host strain(s) may be present as well. The most likely source for E. coli O157:H7 in the commercial fermentation would be the fresh cucumbers. Application of animal waste as fertilizer and irrigation of crops with waste water have been recognized as important routes through which E. coli O157:H7 can contaminate fresh vegetables during primary production (Ongeng et al., 2013). However, we are unaware of any reports of disease outbreaks caused by vegetative pathogens from fermented vegetables. Previous research has shown that E. coli O157:H7 will be killed during fermentation of cucumbers in a pH and time dependent manner (Breidt and Caldwell, 2011).

The isolated phage /Phi1 241 forms small (ca. 1 mm in diameter) plaques on the lawn of its natural host, E. coli O157:H7 strain B0241 which contains stx2 gene and was originally isolated from bovine carcass (Table 1). The concentration of high-titer phage stock (ca. 1010 PFU/ml) remained unchanged during 2 years of storage at a refrigeration temperature, indicating that the phage is very stable.

MORPHOLOGY

The electron micrograph (Figure 1) showed that phage /Phi1 241 has an icosahedral head (about 80 nm in diameter) and a contractile tail (ca. 33 nm long in the contracted state) with a base plate and several tail fibers. The overall morphology of /Phi1 241 indicates that it is a T4-like phage, belonging to the Myoviridae family of the Caudovirales order. Interestingly, several phage particles appeared to cluster together through the tail fibers (Figure 1). The base plate and tail fibers are usually involved in the host cell recognition and receptor-binding by many tailed phages (Riede, 1987; Leiman et al., 2004; Bartual et al., 2010; Garcia-Doval and van Raaij, 2012).

ONE-STEP GROWTH KINETICS

Figure 2 shows the one-step growth of phage /Phi1 241. The latent period was only 15 min (excluding 10 min for adsorption), which is shorter than the typical latent periods (21–120 min) for most Myoviridae phages. A short latent period allows phage /Phi1 241 to replicate faster than most Myoviridae phages. The average burst size of /Phi1 241 was about 53 phage particles per infected cell, which is in the range of 50–100 PFU/cell for many Myoviridae phages (Foschino et al., 1995; Chang et al., 2005; Raya et al., 2006; Bao et al., 2011; Park et al., 2012). A few Myoviridae phages have very large burst sizes. The burst size of phage PhaxI (another O157:H7 phage) is 420 PFU per cell (Shahrbabak et al., 2013). A phage with both a short latent period (15 min or less) and a large burst size (>50 PFU/cell) may have a selective advantage over competing phages, resulting in very high lytic activity (Park et al., 2012).

HOST RANGE

A total of 69 E. coli strains from various sources (Tables 1–3) were tested to determine the host range of phage /Phi1 241. The phage was able to lyse all 46 O157:H7 strains (Table 1), but none of the 18 non-O157 strains (Table 2) including O104:H7 strain which has the same H antigen as that of O157:H7. E. coli O104:H7 was originally isolated from ground beef (Bosilevac and Koohmaraie, 2011). It is also Shiga toxin-producing strain containing two uncommon Shiga toxin gene variants, stx1c and stx3c (Bosilevac and Koohmaraie, 2011). The data suggested that the phage is O157 antigen specific, and H7 antigen may not be involved in the host recognition and binding. Phage infection requires specific receptors on bacterial cells. The common receptors on E. coli include O antigen of lipopolysaccharide (LPS), outer membrane proteins, pili, fimbriae, and flagella (H) antigen (Topley and Wilson, 1990; Bokete et al., 1997). Many cell wall receptors can be shared by different bacterial strains and serotypes (Topley and Wilson, 1990). To confirm that O157 antigen (not H7 antigen) serves as the receptor during /Phi1 241 adsorption, two previously described O antigen-negative mutants (43895Δper and F12), one per-complemented mutant (43895ΔperComp), and two E. coli O157:H7 parent strains (ATCC 43895 and 8624) were...
tested for their susceptibility to Φ241 infection (Table 3). The mutant 43895Δper was generated by deletion of a putative per- osamine synthetase gene (per) in the rfb gene cluster (Sheng et al., 2008). The mutant F12 was created by transposon insertion of TnphoA in the per gene (Bilge et al., 1996). Deletion of per gene or insertion in per gene resulted in a mutant lacking the O antigen. The Δper mutant (43895Δper) also lacked H7 antigen, but the transposon insertion mutant (F12) still expressed the H7 antigen. The per-complemented mutant (43895ΔperComp) was constructed by cloning per in the E. coli vector pCRRII and transforming pCRRII:per into the mutant to restore O157 antigenicity (Sheng et al., 2008). Table 3 showed that phage Φ241 lysed the two O157:H7 parent strains (ATCC 43895 and 8624) which had the full-length O157 antigen, and the per-complemented strain (43895ΔperComp) which was able to express O157 antigen. But the phage did not lyse the two O157 antigen-negative mutants, 43895Δper (also lacking H7 antigen) and F12 (still having H7 antigen). These results supported our hypothesis that O157 antigen is required for the infection by phage Φ241, and strains lacking O157 antigen were resistant to the phage infection, regardless of the presence or absence of H7 antigen in the strains. Similar observations have been reported for other O157-specific phages. Kudva et al. (1999) studied three O157-specific phages isolated from bovine and ovine fecal samples. They found that the three phages lysed all of the eight tested E. coli O157 strains including the strain 8624 and did not lyse non-O157 E. coli strains, or O157-negative mutants including F12. In addition, the three phages did not lyse the complement of the O157-deficient mutant, F12(pF12), which produces a truncated O157 LPS (Kudva et al., 1999). They found that phage infection and plaque formation were influenced by the structure of the host cell O157 LPS. Strains that did not express the O157 antigen or expressed a truncated LPS were not susceptible to plaque formation or lysis by phage. Interestingly, strains that expressed abundant mid-range-molecular-weight LPS were lysed in broth media but did not support plaque formation. They explained that in broth media, the excess mid-range-molecular-weight LPS can diffuse from cells into the broth. But on soft agar, those molecules may accumulate around cells, thereby preventing phage attachment (Kudva et al., 1999). An appropriate length of the O side chains and an optimal LPS concentration may be necessary to make the receptor available for phage interaction and/or to allow irreversible phage binding (Calendar, 1988). The high specificity of phage Φ241 for O157 antigen makes it an ideal biocontrol agent of E. coli O157:H7 without disrupting the beneficial bacteria such as probiotics in foods, normal flora in humans, or other microflora in cattle.

**STRUCTURAL PROTEINS**

SDS-PAGE gel revealed at least 13 protein bands from Φ241 (Figure 3), indicating that the phage contains many types of structural proteins. Four of the protein bands are in the molecular weight (MW) range of 26 to 50 kDa. These include three weak bands and one strong band (band 7 in Figure 3, MW≈44 kDa). In fact, this strong band is the strongest one among all bands, indicating that the protein in this band is the most abundant protein. In many tailed phages, the most abundant proteins are usually identified as the major head proteins (Santos et al., 2011). The MWs of major head proteins generally fall within the range of 26–50 kDa. For example, the sequence-predicted MWs of the major capsid protein in Lactobacillus plantarum phage ΦIL-1 (Lu et al., 2005), O157:H7 phage PhaxI (Shahrbabak et al., 2013), Pseudomonas aeruginosa phages LKA1 and LKD16 (Ceyssens et al., 2006), Salmonella enterica phage PVP-SE1 (Santos et al., 2011) are 30.4, 48.0, 36.7, 37.7, and 38.5 kDa, respectively. Since SDS-PAGE analysis can only reveal a very limited number of structural proteins, genomic studies are needed in order to better understand phage structural proteins and their functions.

**DNA RESTRICTION**

The Φ241 genome could be digested by rare-cutters, AluI, MspI, and SwaI (Figure 4). Restriction by AluI or MspI generated more...
than 15 bands on agarose gel while restriction by SmaI only generated a single band with a high MW. The phage genome could not be digested by many commonly used restriction endonucleases such as BamHI, ClaI, EcoRI, EcoRV, HindIII, and XbaI. Similar phenomenon was also observed for other O157:H7-specific phages. Shahrbabak et al. (2013) reported that the genome of phage PhaxI was resistant to eight tested restriction endonucleases including BamHI, EcoRI, EcoRV, HindIII, and a few others (Shahrbabak et al., 2013). The resistance suggested the presence of modifications such as methylation and glycosylation in the phage DNA, allowing the phage to evade the restriction by the host enzymes (Bickle and Kruger, 1993; Nechaev and Severinov, 2008; Vasu and Nagaraja, 2013). Sequence analysis may provide insight into the anti-restriction modification system in phage genome.

**PHAGE INFECTION**

The lytic activity of phage Φ241 against its natural host *E. coli* O157:H7 B0241 was investigated at three different MOIs. Figure 5 shows the growth curves of phage-free and phage-infected cultures in TSB medium at 37°C. The phage-free culture (the control culture) grew steadily during the first 4 h of incubation. After 4 h, the control culture entered the stationary phase and remained unchanged (Figure 5). In contrast, the phage infection at the MOI of 3 or 10 caused a rapid cell lysis within 1 h, resulting in 3- or 4.5-log decrease in the cell concentration. Such a high lytic activity within 1 h may be attributed in part to the short latent period (15 min) of the phage. During the second hour, the cell concentration of the culture with a MOI of 3 continued to decrease while the cell concentration of the culture with the MOI of 10 started to increase. In contrast, infection at the MOI of 0.3 initially caused a slow cell lysis (less than 0.5-log reduction) during the first hour, but a rapid cell lysis (3-log reduction) during the second hour. The data from statistical analysis showed that at 1 h after phage infection the cell concentrations from different MOIs were statistically different (P < 0.05) and every cell concentration was different from all other cell concentrations (α = 0.05). At 2 h after phage infection the cell concentration from the MOI of 0.3 was statistically different from all other cell concentrations while the cell concentrations from the initial MOIs of 3 and 10 were not statistically different. Similar rapid cell lysis caused by Φ241 in cucumber juice was also observed (preliminary data not shown).

Kudva et al. (1999) evaluated the lytic activity of three O157-specific phages in Luria-Bertani medium supplemented with 5 mM MgSO4 at 37°C. They reported that the significant (>4 log) decrease in *E. coli* O157:H7 concentration caused by those phages individually or in cocktail required much higher MOI (10⁴ PFU/CFU) and much longer incubation time (8 h) compared with those in our study. Figure 5 showed that the cultures with an initial MOI of 3 or 0.3 started to grow after 2 h. Interestingly, after 3 h of infection, all three phage-infected cultures, regardless of the initial MOI, reached the same cell concentration (10⁴ CFU/ml), which was 4.5-log lower than that of the control and 3-log lower than the initial cell concentration. As the incubation continued, the three cultures continued to grow at a similar rate, gradually approaching to the cell concentration of the control. After 12 h of infection, the phage titers in the cultures at the initial MOI of 10, 3, and 0.3 reached 4 × 10⁹, 5 × 10⁸, and 1.6 × 10¹⁰ PFU/ml, respectively. Apparently, the culture started with the lowest initial MOI (0.3) contained the highest phage titer (1.6 × 10¹⁰ PFU/ml) at the end of incubation.

The growth of phage-infected cultures after 1 or 2 h of infection indicated that phage-resistant mutants had emerged. The emergence of phage-resistant mutants during phage infection has been reported by many other studies (Kudva et al., 1999; O’Flynn et al., 2004; Park et al., 2012; Tomat et al., 2013). Phage resistance may result from mutation that alters cell surface receptors, restriction modification, or abortive infection associated with the presence of clustered regularly interspaced short palindromic repeats (CRISPRs) in the bacterial genome (Hill, 1993; Hashemhosseini et al., 1994; Allison and Klraehammer, 1998; Barrangou et al., 2007). A few studies found that certain phage resistant mutants of *E. coli* O157:H7 had altered OmpC expression or lost OmpC, suggesting the involvement of the major outer membrane protein in phage attachment (Yu et al., 2000; Morita et al., 2002; Mizoguchi et al., 2003). Some studies found that cell morphology and colony morphology of phage-resistant mutants differed greatly from those of the parent *E. coli* O157:H7 strains (Mizoguchi et al., 2003; O’Flynn et al., 2004). Phage-resistant mutant cells appeared coccoid and smaller. As a result, phage-resistant culture could not reach the same turbidity as that of the parent strain culture (O’Flynn et al., 2004). The frequency of phage-resistant mutation is generally around 10⁻⁹ CFU for *E. coli* O157:H7 (O’Flynn et al., 2004; Park et al., 2012; Tomat et al., 2013). With such a low mutation frequency and the low level of *E. coli* O157:H7 typically encountered in foods, phage resistance should not hinder the use of phages as biocontrol agents against the pathogenic bacteria (O’Flynn et al., 2004; Tanji et al., 2004). Some studies explored the potential of using a phage cocktail to minimize the development of phage-resistant mutants on meats and other foods.
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