J. Microbiol. Biotechnol. (2016), 26(12), 2060–2065
http://dx.doi.org/10.4014/jmb.1609.09039

jmb

Virulence Factors and Stability of Coliphages Specific to *Escherichia coli* O157:H7 and to Various *E. coli* Infection

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Received: September 22, 2016
Revised: September 28, 2016
Accepted: September 30, 2016

First published online
October 6, 2016

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pISSN 1017-7825, eISSN 1738-8872

Characteristics of *E. coli* O157:H7-specific infection bacteriophages (O157 coliphages) and broad-host-range bacteriophages for other *E. coli* serotypes (broad-host coliphages) were compared. The burst sizes of the two groups ranged from 40 to 176 PFU/infected cell. Distributions of the virulence factors *stx1*, *stx2*, *ehxA*, and *saa* between the two groups were not differentiated. Broad-host-range coliphages showed lower stability at 70°C, in relation to O157 coliphages. However, O157 coliphages showed high acid and ethanol tolerance by reduction of only 22% and 11% phages, respectively, under pH 3 and 70% ethanol for 1 h exposure. Therefore, these results revealed that the O157 coliphages might be more stable under harsh environments, which might explain their effective infection of the acid-tolerant *E. coli* O157:H7.

Keywords: *E. coli* O157:H7, bacteriophage, host spectrum, virulence gene, stability

Shiga toxin-producing *Escherichia coli* (STEC) is considered as an important group of pathogenic *E. coli*, whose cytotoxin produced is identical at the genetic and protein levels of the Shiga toxin (Stx) produced by *Shigella dysenteriae* [18, 19]. There are at least 100 different *E. coli* serotypes of STEC, including *E. coli* O157, which is the most well-known serotype [15]. STEC produces only one or both types of Stx, and several variants of both Stx1 and Stx2 have been identified [3]. Specifically, s*tx1* and s*tx2* are encoded by phage in the genome of STEC and considered to have a major virulence factor role with transmission to nonpathogenic *E. coli* [3, 28]. In addition to Stx, the other potential virulence factors of STEC strains causing human disease are known as enterohemolysin, intimin, and outer membrane protein. Most STEC strains have a large virulence plasmid that encodes *ehxA* for enterohemolysin protein [26]. Recently, Paton *et al*. [22] also reported that *saa*, which is located on the large plasmid of certain LEE-negative STEC, encodes an outer membrane protein that appears to function in autoagglutinating adhesion.

E. coli O157:H7 is a highly acid-resistant foodborne pathogen that causes several large outbreaks of human illness, including hemorrhagic colitis and hemolytic uremic syndrome [7, 13]. *E. coli* O157:H7 can survive and grow at pH 4.70 in organic acids, making it an important potent foodborne pathogen [1]. Not only *E. coli* O157:H7 but also non-O157 STEC have globally emerged as significant causes of human diseases. Hence, some recent studies have reported comparisons between *E. coli* O157 and non-O157 STEC, including physiological characteristics, genomics, and virulence factors [7, 8]. Genetic transfer may be suggested as the cause for the emergence of multidrug-resistant pathogens [27], which causes resistance to antibiotics through plasmids, transposons, and bacteriophages by transduction, transformation, and conjugation [14]. Bacteriophages are present in all habitats on Earth, and their high stability makes them more suitable than free DNA for the transfer of genes among bacteria. However, the importance of bacteriophages in gene transfer is still underestimated, unlike the other transfer mechanisms like transformation and conjugation. There have been few studies on the characteristics or stability of bacteriophages specific for *E. coli* O157:H7, although numerous different phages targeting *E. coli* have been isolated and studied. Furthermore, previous researchers have not reported characteristic studies of bacteriophages with a broad host range for various *E. coli* serotypes. Therefore, the objective of the present study was to analyze characteristics of *E. coli*

O157:H7-specific bacteriophages and broad-host-range bacteriophages for other *E. coli* serotypes, where one-step growth, virulence factor profile, and stability test of the two bacteriophage groups were conducted.

Seven bacteriophages that infected *E. coli* O157:H7 specifically (O157 coliphages) and other seven bacteriophages that showed a broad host range for other *E. coli* serotypes (broad-host coliphages) were used in this study as follows: ECP3, ECP7, ECP13, ECP1, ECP4, ECP6, and ECP9 for O157 coliphages; ECP19, BECP4, NOECP16, ECP15, ECP20, BECP3, and NOECP10 for broad-host coliphages. The strains inhibited by these phages and the host strains used for isolation and propagation are indicated in previous reports [11, 12].

Each of the host strains was grown in LBC broth (Luria Bertani broth (Difco Laboratory, USA) with 10 mM $CaCl_2$ (Sigma Aldrich, USA)) at 37°C for overnight in a shaking incubator [16], and propagation was conducted as described by Sambrook and Russell [24]. After the host strain was grown in LBC broth at 37°C until mid-exponential phase, 1 ml of the culture cell harvested by centrifugation (10,000 ×*g* for 5 min) was mixed with 0.1 ml of bacteriophage lysate (MOI of 0.01) and incubated at 37°C for 10 min. The mixture was centrifuged at 10,000 ×*g* for 10 min, and the supernatant was discarded to remove the excess phage particles. The cell pellets were resuspended in 10 ml of LBC and incubated at 37°C. Samples were collected every 5 min during the subsequent incubation of the resuspension, and plaque assay was conducted with the collected samples. Plaques were enumerated after 24 h of incubation at 37°C, and the latent period and burst size were determined based on the number of PFU per infected cell [16, 20]. All the tests were performed in triplicates. The burst periods of O157 coliphages were within the range of 45–65 min, and the burst sizes were estimated at 54–172 PFU/infected cell, depending on bacteriophages. Meanwhile, the burst periods of broad-host coliphages were within the range of 45–70 min, and the burst sizes were estimated at 40–176 PFU/infected cell, depending on bacteriophages. Similar to our results, Park *et al*. [20] reported that the latent period and burst size of SFP10 was 25 min and 100 PFU/infected cell respectively. However, Li *et al*. [17] reported that the burst size of EEP was estimated at 375 ± 43 PFU/infected cell, which was bigger than those of the two coliphage groups.

Horizontal gene transfer and the acquisition of virulence factors by several mobile genetic elements, including bacteriophage, plasmid, and pathogenicity islands, are known as a major driving force in the emergence and evolution of pathogenic isolates [5]. One of the most significant groups is the bacteriophages, and Boyd and Brüssow [5] reported that the bacteriophages encoding virulence factors could convert their bacterial host. It has been reported that many bacteriophage-encoded virulence factors included extracellular toxins, such as Stx and enterohemolysin, effector proteins involved in invasion, and enzymes required for intracellular survival [5]. Toxins are the classical examples of bacteriophage-encoded virulence factors, and O'Brien *et al*. [19] and Huang *et al*. [9] have reported *E. coli* bacteriophage H-19B, which encoded *stx1* and *stx2*. Beutin *et al*. [4] also have reported bacteriophage ΦFC3208, which encoded *ehxA*. Since the bacteriophages would play a key role in pathogenesis and genomic diversity as described above, the virulence factor profile of the two coliphage groups was confirmed using specific primers. To confirm the virulence factor profile on bacteriophages, PCR was performed using *stx1*, *stx2* (shiga toxin-encoding genes), *ehxA* (enterohemolysin-encoding gene), and *saa* (STEC autoagglutinating adhesion-encoding gene) specific primers. The primer sequences and PCR conditions have been reported by Amisano *et al*. [2], Gerrish *et al*. [6], Sánchez *et al*. [25], Paton and Paton [21], and Schmidt *et al*. [26]. As the result of O157 coliphages (Table 1), four virulence factors were confirmed on only ECP7, but not confirmed on ECP1 and ECP4 at all. The two virulence factors *stx1* and *stx2* were confirmed on ECP3 and ECP13, and three virulence factors except *saa* were

Table 1. Virulence gene profiles of two coliphage groups by PCR.

		Virulence genes			
		stx1	*stx2*	*ehxA*	*saa*
E. coli O157-infecting coliphages	ECP3	+	+	-	-
	ECP7	+	+	+	+
	ECP13	+	+	-	-
	ECP1	-	-	-	-
	ECP4	-	-	-	-
	ECP6	+	+	+	-
	ECP9	+	+	+	-
Various *E. coli*-infecting coliphages	ECP19	+	+	+	-
	BECP4	+	-	-	-
	NOECP16	+	+	+	-
	ECP15	-	-	-	-
	ECP20	+	+	+	-
	BECP3	-	-	-	-
	NOECP10	+	-	+	-

+, detected; -, not detected.

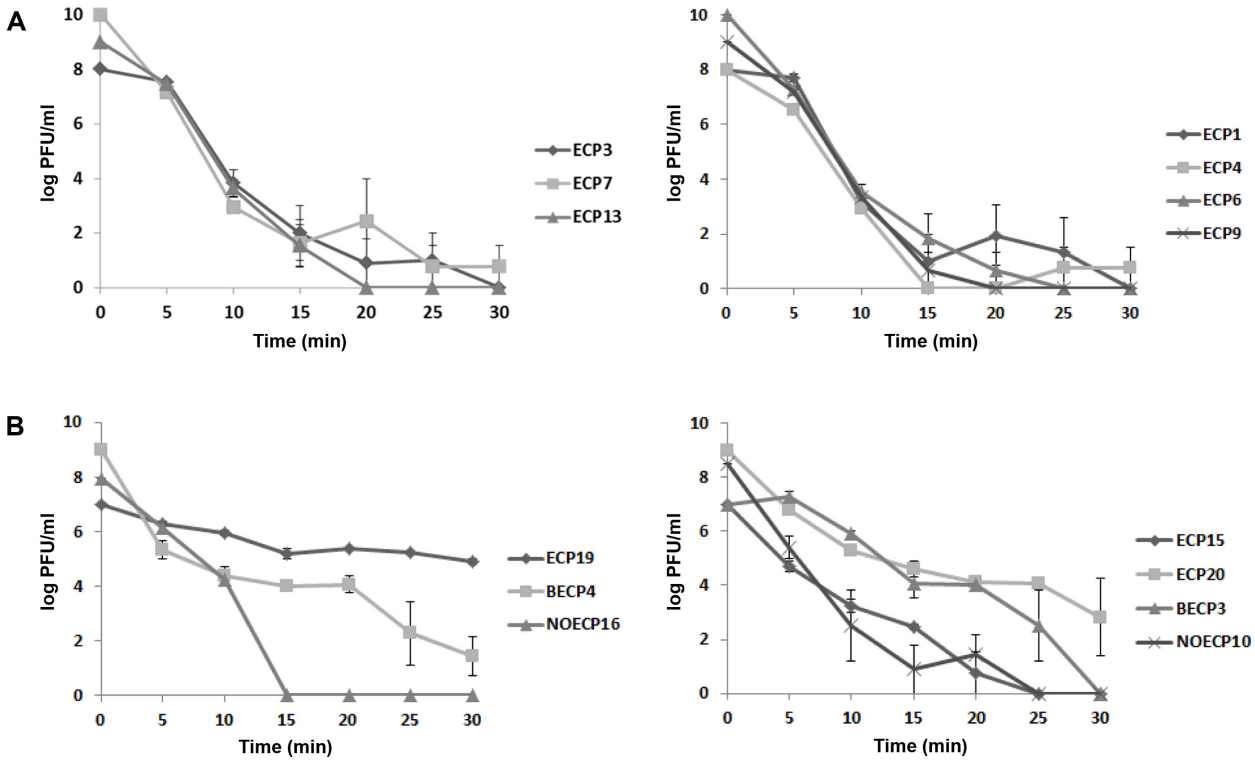

Fig. 1. The stability of *E. coli* O157-infecting coliphages (**A**) and various *E. coli*-infecting coliphages (**B**) at 70°C.

confirmed on ECP6 and ECP9. Meanwhile, the four virulence factors were not confirmed on ECP15 and BECP3 of broad-host coliphages. ECP19, NOECP16, and ECP20 were positive for three virulence factors except *saa*. NOECP10 was positive for *stx1* and *ehxA*, and BECP4 was positive only for *stx1*. These results revealed that the virulence factor profile of the two coliphage groups was diverse regardless of their host infection specificities. As an application aspect, the four phages ECP1, ECP4, ECP15, and BECP3 showed no signals in virulence gene profiling PCR assay and could be considered good candidates as biocontrol agents.

To study the thermal tolerance of the bacteriophages, 100 µl of phage lysate was incubated at 65°C and 70°C for 30 min using a heat block, respectively. Samples were collected every 5 min during heat treatment of the phage, and the samples were immediately 10-fold serially diluted and spotted on the plates overlaid with the cultural host bacteria. After incubation at 37°C for 24 h, log reduction of bacteriophage was determined by counting the surviving phages. The results of the two coliphage groups for thermal tolerance are shown in Fig. 1. At 70°C, most of the O157 coliphages were reduced and not detected after 20 min, and ECP7 and ECP4 were also reduced by 7 $\log_{10}$ PFU/ml after 30 min (Fig. 1A). However, most of the broad-host coliphages were reduced to a non-detectable level at 70°C after 15–30 min (Fig. 1B). ECP20 and BECP4 also showed 6 $\log_{10}$ PFU/ml reduction after 30 min, but ECP19 was more stable than the other broad host coliphages, showing only 2 $\log_{10}$ PFU/ml reduction after 30 min. Although broad-host coliphages showed various stability degrees at 70°C according to the characteristics of each coliphage, heat resistance might be moderate and similar to previous bacteriophages reported from Park *et al*. [20] and Li *et al*. [17]. Park *et al*. [20] reported that SFP10 was stable at 60°C for 1 h, but the phage count was reduced by 54% at 70°C and completely inactivated at 75°C. Li *et al*. [17] reported only 2 and 3 $\log_{10}$ PFU/ml reduction of EEP at 63°C and 72°C for 30 min, respectively.

To study the stability at various pH values, 10 µl of phage lysate was mixed with 990 µl of high or low pH-adjusted SM buffer. After the mixture was incubated at room temperature for an hour, spot assay was conducted as described for the thermal tolerance test procedure. Three O157 and three broad-host coliphages were randomly selected from each coliphage group and the effect of pH on these coliphages is shown in Fig. 2. None of the two coliphage groups could be detected after incubation at pH

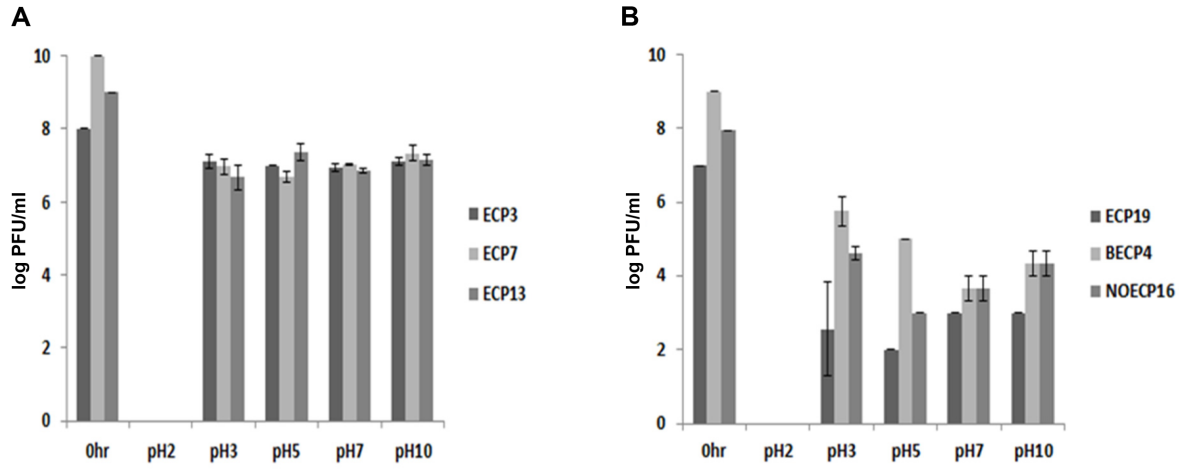

Fig. 2. The stability of *E. coli* O157-infecting coliphages ECP3, ECP7, and ECP13 (**A**), and *E. coli*-infecting coliphages ECP19, BECP4, and NOECP16 (**B**) at various pH values for 1 h.

2 for 1 h. At the range of pH 3–10 for 1 h, O157 coliphages showed slight decrease to less than 3 $\log_{10}$ PFU/ml (Fig. 2A), while broad-host coliphages were significantly inactivated, showing high reduction from 3 $\log_{10}$ PFU/ml to maximum 5 $\log_{10}$ PFU/ml (Fig. 2B). These results revealed that the O157 coliphages showing slight decrease of phage titers were more stable than the broad-host coliphages showing large decrease of 3–5 $\log_{10}$ PFU/ml at low and high pH conditions. Similar to our results for O157 coliphages, Park *et al*. [20] reported that SFP10 was highly stable between pH 4 and 10, and SFP10 was completely abolished under strong acidic pH (pH < 2). Some studies showed similar results to ours for broad-host coliphages. Li *et al*. [17] reported that EEP was very sensitive to strong acidic pH 3 and was not detected after 30 min. Rode *et al*. [23] reported that infectious particles of recombinant Stx phage (Δ*stx*::cat) was not present after 10 min at pH 3. Jamal *et al*. [10] also confirmed that there were no active infectious particles of *Myoviridae* bacteriophage WZ1 at pH 3.

To study the stability under various concentrations of ethanol, phage lysate was mixed with ethanol. After the mixture was incubated at room temperature for 1 h, spot assay was conducted as described for the thermal tolerance test procedure. When the three O157 coliphages were treated with various concentrations of ethanol for 1 h, the phage titers were not affected or were slightly reduced to less than 1–2 $\log_{10}$ PFU/ml (Fig. 3A). However, the three broad-host coliphages showed higher sensitivity with reduction over 3 $\log_{10}$ PFU/ml under the same conditions (Fig. 3B). Specifically, 70% ethanol reduced a large amount of infective phages by more than 4 $\log_{10}$ PFU/ml. These results revealed that O157 coliphages were more stable

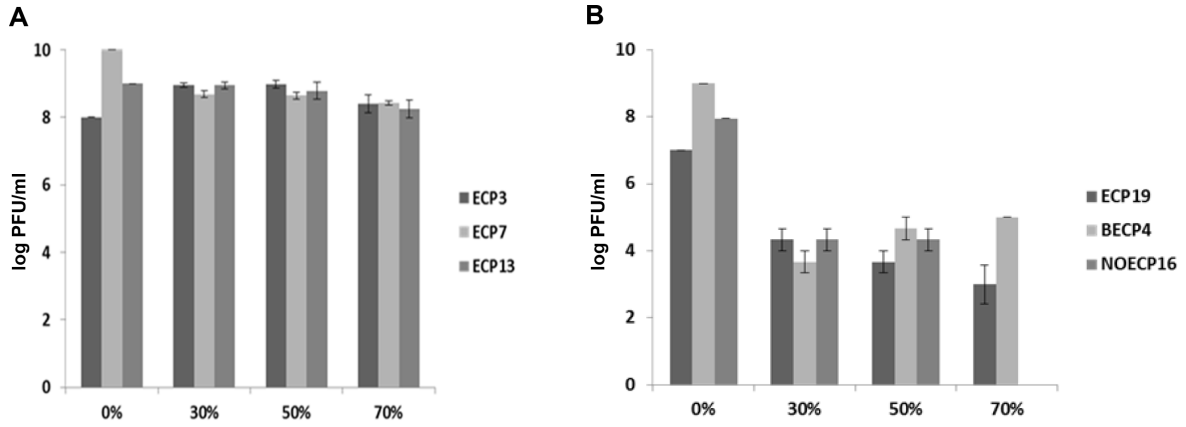

Fig. 3. The stability of *E. coli* O157-infecting coliphages ECP3, ECP7, and ECP13 (**A**) and *E. coli*-infecting coliphages ECP19, BECP4, and NOECP16 (**B**) under various concentrations of ethanol for 1 h.

than broad-host coliphages under various concentrations of ethanol, especially 70% ethanol. Li *et al*. [17] showed similar results to ours for O157 coliphages, reporting that EEP was slightly decreased in the presence of ethanol (25%, 50%, and 75%). Moreover, Rode *et al*. [23] reported similar results to ours for broad-host coliphages, in which Stx phage was very sensitive and not detected after 30 min of exposure to 70% ethanol.

In conclusion, this study suggests individual characteristics and different degrees of stability of *E. coli* O157:H7-specific bacteriophages (O157 coliphages) and broad-host-range bacteriophages for other *E. coli* serotypes (broad-host coliphages). The burst size of the two coliphage groups were varied regardless of the coliphage group, and the virulence factor profile of the two groups was also diverse according to the characteristics of each coliphage. However, interestingly, the results of acidic and ethanol stability suggest that O157 coliphages might be more stable than broad-host coliphages under harsh conditions of acidic pH and high concentration of ethanol. The high pH and ethanol stability of O157 coliphages might be related to the size of *Siphoviridae*, which are smaller than broad-host coliphages of *Myoviridae* [12]. Again, such high stability of O157 coliphages to pH and ethanol would provide better understanding on their infection of acid-tolerant *E. coli* O157:H7.

Acknowledgments

This research was financially supported by the National Research Foundation of Korea (Grant# # 2013R1A1A2062065) and Korea Food Research Institute.

References

1. Abdul-Raouf UM, Beuchat LR, Ammar MS. 1993. Survival and growth of *Escherichia coli* O157:H7 in ground, roasted beef as affected by pH, acidulants, and temperature. *Appl. Environ. Microbiol.* **59:** 2364-2368.
2. Amisano G, Fornasero S, Migliaretti G, Caramello S, Tarasco V, Savino F. 2011. Diarrheagenic *Escherichia coli* in acute gastroenteritis in infants in North-West Italy. *New Microbiol.* **34:** 45-51.
3. Bergan J, Dyve Lingelem AB, Simm R, Skotland T, Sandvig K. 2012. Shiga toxins. *Toxicon* **60:** 1085-1107.
4. Beutin L, Stroehe UH, Manning PA. 1993. Isolation of enterohemolysin (Ehly2)-associated sequences encoded on temperate phages of *Escherichia coli*. *Gene* **132:** 95-99.
5. Boyd EF, Brüssow H. 2002. Common themes among bacteriophage-encoded virulence factors and diversity among the bacteriophages involved. *Trends Microbiol.* **10:** 521-529.
6. Gerrish RS, Lee JE, Reed J, Williams J, Farrell LD, Spiegel KM, Shields MS. 2007. PCR versus hybridization for detecting virulence genes of enterohemorrhagic *Escherichia coli*. *Emerg. Infect. Dis*. **13:** 1253-1258.
7. Gould LH, Mody RK, Ong KL, Clogher P, Cronquist AB, Garman KN, *et al*.; the Emerging Infections Program FoodNet Working Group. 2013. Increased recognition of non-O157 Shiga toxin–producing *Escherichia coli* infections in the United States during 2000–2010: epidemiologic features and comparison with *E. coli* O157 infections. *Foodborne Pathog. Dis.* **10:** 453-460.
8. Hedican EB, Medus C, Besser JM, Juni BA, Koziol B, Taylor C, Smith KE. 2009. Characteristics of O157 versus non-O157 Shiga toxin-producing *Escherichia coli* infections in Minnesota, 2000–2006. *Clin. Infect. Dis*. **49:** 358-364.
9. Huang A, Friesen J, Brunton JL. 1987. Characterization of a bacteriophage that carries the genes for production of Shiga-like toxin 1 in *Escherichia coli*. *J. Bacteriol*. **169:** 4308-4312.
10. Jamal M, Chaudhry WN, Hussain T, Das CR, Andleeb S. 2015. Characterization of new *Myoviridae* bacteriophage WZ1 against multi-drug resistant (MDR) *Shigella dysenteriae*. *J. Basic Microbiol.* **55:** 420-431.
11. Kim EJ. 2015. Characterization and infection receptor analysis of bacteriophages for *Escherichia coli* O157:H7 and non-O157 Shiga toxin-producing *E. coli*. Master's dissertation, Gachon University, Sungnam, South Korea.
12. Kim EJ, Lee H, Lee JH, Ryu S, Park JH. 2016. Morphological features and lipopolysaccharide attachment of coliphages specific to *Escherichia coli* O157:H7 and to a broad range of *E. coli* hosts. *Appl. Biol. Chem*. **59:** 109-116.
13. Koodi L, Dhople AM. 2001. Acid tolerance of *Escherichia coli* O157:H7 and its survival in apple juice. *Microbios* **104:** 167-175.
14. Lacey RW. 1984. Mechanisms of resistance to beta-lactam antibiotics in *Staphylococcus aureus*. *Scand. J. Infect. Dis*. **42:** 64-71.
15. Law D. 2000. Virulence factors of *Escherichia coli* O157 and other Shiga toxin-producing *E. coli*. *J. Appl. Microbiol*. **88:** 729-745.
16. Lee YD, Kim JY, Park JH. 2013. Characteristics of coliphage ECP4 and potential use as a sanitizing agent for biocontrol of *Escherichia coli* O157: H7. *Food Control* **34:** 255-260.
17. Li S, Liu L, Zhu J, Zou L, Li M, Cong Y, Hu F. 2010. Characterization and genome sequencing of a novel coliphage isolated from engineered *Escherichia coli*. *Intervirology* **53:** 211-220.
18. Nataro JP, Kaper JB. 1998. Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev*. **11:** 142-201.
19. O'Brien AD, Newland JW, Miller SF, Holmes RK, Smith HW, Forma, SB. 1984. Shiga-like toxin-converting phages from *Escherichia coli* strains that cause hemorrhagic colitis or infantile diarrhea. *Science* **226:** 694-696.
20. Park M, Lee JH, Shin H, Kim M, Choi J, Kang DH, Ryu S.

2012. Characterization and comparative genomic analysis of a novel bacteriophage, SFP10, simultaneously inhibiting both *Salmonella enterica* and *Escherichia coli* O157:H7. *Appl. Environ. Microbiol*. **78:** 58-69.

21. Paton AW, Paton JC. 2002. Direct detection and characterization of Shiga toxigenic *Escherichia coli* by multiplex PCR for *stx1*, *stx2*, *eae*, *ehxA*, and *saa*. *J. Clin. Microbiol*. **40:** 271-274.
22. Paton AW, Srimanote P, Woodrow MC, Paton JC. 2001. Characterization of Saa, a novel autoagglutinating adhesin produced by locus of enterocyte effacement-negative Shiga-toxigenic *Escherichia coli* strains that are virulent for humans. *Infect. Immun*. **69:** 6999-7009.
23. Rode TM, Axelsson L, Granum PE, Heir E, Holck A, Trine ML. 2011. High stability of Stx2 phage in food and under food-processing conditions. *Appl. Environ. Microbiol*. **77:** 5336-5341.
24. Sambrook J, Russell DW. 2001. *Molecular Cloning: A Laboratory Manual*, Vol. 999. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.
25. Sánchez S, García-Sánchez A, Martínez R, Blanco J, Blanco JE, Blanco M, Rey J. 2009. Detection and characterisation of Shiga toxin-producing *Escherichia coli* other than *Escherichia coli* O157: H7 in wild ruminants. *Vet. J*. **180:** 384-388.
26. Schmidt H, Beutin L, Karch H. 1995. Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL 933. *Infect. Immun*. **63:** 1055-1061.
27. Skippington E, Ragan MA. 2011. Lateral genetic transfer and the construction of genetic exchange communities. *FEMS Microbiol. Rev*. **35:** 707-735.
28. Wagner PL, Waldor MK. 2002. Bacteriophage control of bacterial virulence. *Infect. Immun*. **70:** 3985-3993.