

Isolation and Characterization of a Lytic and Highly Specific Phage against *Yersinia enterocolitica* as a Novel Biocontrol Agent

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The aim of this study was to isolate and characterize a lytic *Yersinia enterocolitica*-specific phage (KFS-YE) as a biocontrol agent. KFS-YE was isolated and purified with the final concentration of (11.72 ± 0.03) log PFU/ml from poultry. As observed by transmission electron microscopy, KFS-YE consisted of an icosahedral head and a contractile tail, and was classified in the *Myoviridae* family. KFS-YE showed excellent narrow specificity against *Y. enterocolitica* only. Its lytic activity was stable at wide ranges of pH (4–11) and temperature (4–50°C). The latent period and burst size of KFS-YE were determined to be 45 min and 38 PFU/cell, respectively. KFS-YE showed relatively robust storage stability at -20, 4, and 22°C for 40 weeks. KFS-YE demonstrated a bactericidal effect *in vitro* against *Y. enterocolitica* and provided excellent efficiency with a multiplicity of infection as low as 0.01. This study demonstrated the excellent specificity, stability, and efficacy of KFS-YE as a novel biocontrol agent. KFS-YE may be employed as a practical and promising biocontrol agent against *Y. enterocolitica* in food.

Keywords: Bacteriophages (phages), *Yersinia enterocolitica*, biocontrol, lytic, storage

Introduction

Yersinia enterocolitica is a gram-negative, non-spore-forming, coccobacilli, psychrotrophic, and facultative anaerobe, which is one of three *Yersinia* species that are pathogenic to humans, along with *Y. pestis*, and *Y. pseudotuberculosis* [1, 2]. The most predominant natural hosts for *Y. enterocolitica* are animals (especially pigs). *Y. enterocolitica* also exists ubiquitously in water, soil, plant surfaces, and foods [3]. Although animals are the major source of *Y. enterocolitica*, many cases have recently been reported in which outbreaks of *Y. enterocolitica* were associated with fresh produce such as salad, bean sprouts and leafy vegetables [4, 5]. *Yersinia* infection, commonly known as yersiniosis, begins with some common symptoms such as fever, diarrhea (often bloody), and abdominal pain, which is sometimes confused with appendicitis. It is also associated with some severe complications such as skin rash, meningitis, mesenteric lymphadenitis, and sepsis [2, 3]. A European Union Summary Report [6] classified yersiniosis as the third most common

zoonosis in Europe [4]. Moreover, recent findings revealed that *Yersinia* species had developed resistance against penicillin, ampicillin, cephalosporin, and macrolides due to the production of beta-lactamases [7]. Thus, a safe, eco-friendly and effective “green” approach is required to control *Y. enterocolitica* to ensure food safety and public health [8].

Bacteriophages (phages) are the most abundant entities (10^{31} - 10^{32}) in nature and have recently gathered more attention as green biocontrol agents owing to several advantages, including excellent target specificity, the ability to multiply in the presence of hosts, preparation and cost efficiencies, stability in wide-ranging pH levels and temperatures, and harmlessness to humans, animals, and plants [9-11]. The necessity of novel biocontrol agents has prompted us to isolate numerous phages (mainly lytic phages) from various environments and foods [12]. Unlike lysogenic phages, the lytic phage can lyse the target bacteria by integrating their DNA into the bacterial chromosome and then replicating themselves inside the host, a trait that is preferred for their use as biocontrol agents [2, 13, 14].

Table 1. *Y. enterocolitica*-specific phages reported in the literatures.

Classification	Name	Sources	Length (nm)		Latent period (min)	Burst size (PFU/cell)	References
			Head	Tail			
<i>Myoviridae</i>	KFS-YE	Poultry	118	126	45	38	Present study
	8/C239	Human feces	58	140	-	-	[39]
	PY100	Pig manure	70	80	30	120	[40]
	PY95	<i>Y. enterocolitica</i> *	-	-	-	-	[19]
	PY68	<i>Y. enterocolitica</i> *	-	-	-	-	[19]
	PY44	<i>Y. enterocolitica</i> *	-	-	-	-	[19]
	PY20	<i>Y. enterocolitica</i> *	-	-	-	-	[19]
<i>Podoviridae</i>	ΦYeO3-12	Sewage	57	15	25	120	[1]
	AP5	Sewage	55	12	-	-	[41]
	PY96	<i>Y. enterocolitica</i> *	-	-	-	-	[19]
	PY31	<i>Y. enterocolitica</i> *	-	-	-	-	[19]
<i>Siphoviridae</i>	PY30	<i>Y. enterocolitica</i> *	-	-	-	-	[19]
	PY54	<i>Y. enterocolitica</i> *	-	-	-	-	[19]
-	fPS-8, 11, 16, 27, 67	Pig stools	-	-	-	-	[2]

Asterisk (*) means that *Y. enterocolitica*-specific phage was isolated from *Yersinia* strains by mitomycin C induction.

Although numerous phages have been isolated and even employed in various foods as biocontrol agents in previously published reports, the focus of these efforts was limited to *Salmonella*-, *Escherichia coli*- and *Listeria*-specific phages [12, 15–17]. Moreover, a few published reports are available today regarding *Yersinia*-specific phages as shown in Table 1, but these previous studies were primarily focused on isolation only rather than characterization and subsequent employment of phages as biocontrol agents in foods [1, 18, 19]. In addition, the FDA has recently permitted phages to be used as “food additives” with a Generally Recognized as Safe (GRAS) designation [17]. Therefore, a lot of phage research has been focused on the investigation of new phages as biocontrol agents. To investigate for an applicable and practical *Y. enterocolitica*-specific phage as a biocontrol agent, the phage first needs to be isolated and purified. The properties of a purified phage also need to be characterized for use in food since it could be affected by several environmental factors, such as pH, temperature, storage period, and chemicals [12, 20, 21]. Therefore, as an initial study, a lytic *Y. enterocolitica*-specific phage was isolated, purified, and characterized to demonstrate its potential as a novel biocontrol agent.

Materials and Methods

Bacterial Strains, Culture Media, and Growth Condition

Each bacterial strain listed in Table 2 was grown in 25 ml of tryptic soy broth (TSB, Difco, Sparks, USA) for 16 h at 37°C with

constant shaking at 110 rpm. After washing three times with sterilized phosphate-buffered saline (PBS, pH 7.4, Life Technologies Co., UK) by centrifugation at 4,000 ×g for 10 min at 4°C, the collected bacteria were suspended in PBS. The concentration of each bacterial suspension was adjusted to 8 log CFU/ml using a pre-constructed standard curve determined by optical density at 640 nm.

Isolation, Propagation, and Purification of Phages from Washing Water Used for Poultry and Carcasses

One liter of wash water was collected from each processing step in a poultry plant (Orpum Ltd., Sangju) and five slaughterhouses (Yeongcheon, Gumi, Gunwi, Daegu, and Andong) in Korea. Twenty-five milliliters of the water was mixed with 225 ml of TSB containing 1 ml of *Y. enterocolitica* ATCC 23715 as an indicator strain. After incubating for 16 h at 37°C with 160-rpm agitation, the mixture was centrifuged at 4,000 ×g for 10 min at 4°C. The supernatant was filtered using a 0.20-μm cellulose acetate filter (Advantec Toyo Ltd., Kaisha, Japan) and dot assay was then performed. Ten microliters of filtrate was spotted on the surface of TA soft agar (4 g/l agar, 8 g/l nutrient broth, 5 g/l NaCl, 0.2 g/l MgSO₄, 0.05 g/l MnSO₄, and 0.15 g/l CaCl₂) that was previously solidified with 200 μl of an overnight culture of *Y. enterocolitica* ATCC 23715. After incubation for 16 h at 37°C, the formation of plaque and its size were measured for further procedures.

For the isolation of a single phage against *Y. enterocolitica* ATCC 23715, 100 μl filtrate with 10-fold serial dilutions and 200 μl of an overnight culture of *Y. enterocolitica* ATCC 23715 were mixed into 4 ml of TA soft agar. After mixing and pouring onto a tryptic soy agar (TSA, Difco Laboratories Inc., Sparks, USA) plate (plaque assay), it was incubated for 16 h at 37°C. A single plaque was then

Table 2. Specificity of KFS-YE.

Bacteria	Plaque turbidity*	Plaque size (cm)
<i>Yersinia enterocolitica</i> ATCC 23715	++	1.27 ± 0.05 ^a
<i>Y. enterocolitica</i> ATCC 55075	+	0.95 ± 0.06 ^b
<i>Y. enterocolitica</i> ATCC 9610	+	0.79 ± 0.06 ^b
<i>Y. pseudotuberculosis</i> ATCC 29833	-	-
<i>Y. ruckeri</i> ATCC 29473	-	-
<i>Y. tuberculosis</i>	-	-
<i>Y. frederiksenii</i>	-	-
<i>Aeromonas hydrophila</i> ATCC 7966	-	-
<i>A. veronii</i> ATCC 9071	-	-
<i>A. salmonicida</i> ATCC 33658	-	-
<i>A. media</i> ATCC 33907	-	-
<i>A. sobria</i> ATCC 43979	-	-
<i>Bacillus cereus</i> ATCC 21768	-	-
<i>B. cereus</i> ATCC 13061	-	-
<i>B. subtilis</i> ATCC 6633	-	-
<i>Campylobacter jejuni</i>	-	-
<i>Escherichia coli</i> ATCC 15144	-	-
<i>E. coli</i> ATCC BAA-2196	-	-
<i>E. coli</i> O157:H7 ATCC 43895	-	-
<i>Klebsiella pneumoniae</i> ATCC 13883	-	-
<i>Listeria monocytogenes</i> ATCC 19116	-	-
<i>L. monocytogenes</i> ATCC 7644	-	-
<i>L. innocua</i> ATCC 33090	-	-
<i>Pseudomonas aeruginosa</i> ATCC 9027	-	-
<i>P. aeruginosa</i> ATCC 10145	-	-
<i>Salmonella</i> Enteritidis ATCC 13076	-	-
<i>S. Dublin</i>	-	-
<i>S. Hartford</i>	-	-
<i>S. Heidelberg</i>	-	-
<i>S. Mission</i>	-	-
<i>S. Montevideo</i>	-	-
<i>S. Newport</i>	-	-
<i>S. Panama</i>	-	-
<i>S. Salamae</i>	-	-
<i>S. Senftenberg</i>	-	-
<i>S. Typhi</i>	-	-
<i>S. Typhimurium</i> ATCC 19586	-	-
<i>S. Typhimurium</i> ATCC 15812	-	-
<i>Shigella flexneri</i> 2457T	-	-
<i>S. sonnei</i> ATCC 9290	-	-
<i>Staphylococcus aureus</i> ATCC 25923	-	-
<i>Vibrio parahaemolyticus</i> ATCC 17802	-	-
<i>V. vulnificus</i>	-	-

*++, clear plaque; +, turbid plaque; -, no plaque.

Different letters (a and b) represent significant differences at $p < 0.05$.

All bacterial strains with ATCC number and without ATCC number were provided from the American Type of Culture Collection and the laboratory in the Department of Plant and Food Sciences at Sangmyung University in Korea, respectively, except for *Yersinia tuberculosis* and *Yersinia frederiksenii*.

Yersinia tuberculosis and *Yersinia frederiksenii* were provided from the Korea Veterinary Culture Collection (KVCC) and Korea Centers for Disease Control and Prevention (KCDC), respectively.

eluted with sodium chloride-magnesium sulfate (SM) buffer (50 mmol/l Tris-HCl, 100 mmol/l NaCl, 10 mmol/l $MgSO_4$, pH 7.5) with vigorous agitation for 1 h at 22°C (plaque assay). For the propagation of the phage, 1% of the overnight culture of *Y. enterocolitica* ATCC 23715 was mixed with 3 ml of TA broth and incubated for 2 h at 37°C prior to adding 1 ml of the eluted single phage. After incubation for 2 h at 37°C, the mixture was centrifuged at 2,400 ×g for 10 min at 4°C and the supernatant was filtered through the 0.20 µm cellulose acetate filter. The above procedures were performed several times with increasing amounts of TA broth to increase the phage concentration. The final filtrate was precipitated using 10% polyethylene glycol (PEG) 6000 (Sigma-Aldrich, USA) and 10 ml of 1 mol/l NaCl. After precipitation, the PEG-precipitated filtrate was centrifuged at 7,200 ×g for 20 min at 4°C. The pellet was suspended in the SM buffer and subjected to CsCl gradient ultracentrifugation at 39,000 ×g for 2 h at 4°C. Finally, a bluish opalescent layer was dialyzed and stored in SM buffer, and the concentration of the purified phage (referred to as KFS-YE hereafter) was determined using plaque assay.

Morphological Analysis of KFS-YE

Ten microliters of KFS-YE (11 log PFU/ml) was placed on the top of a carbon-coated copper grid and stained with 2% phosphotungstic acid (Sigma-Aldrich Co., USA). The stained KFS-YE was observed using a transmission electron microscopy (TEM) (H-7100, Hitachi Ltd., Chiyoda, Japan) at 100 kV with 50,000 × to 200,000 × magnifications.

Specificity of KFS-YE

The specificity of KFS-YE was investigated by employing each overnight culture of bacteria (8 log CFU/ml), and a dot assay was performed following the procedures described for the methods of isolation, propagation, and purification of phages.

Effects of pHs, Temperatures, and Storage Conditions on the Stability of KFS-YE

The lytic activity of KFS-YE was investigated by exposing it to various pH levels and temperatures. To determine the pH effect on the lytic activity of KFS-YE, 100 µl of KFS-YE (9 log PFU/ml) was mixed with 900 µl of TSB at different pH levels (2-12) and incubated at 22°C for 1 h. To determine the temperature effect, 100 µl of KFS-YE (9 log PFU/ml) was mixed with 900 µl of SM buffer (pH 7) and incubated at various temperatures (4°C, 22°C, 37°C, 50°C, 60°C, 70°C, and 80°C) for 1 h. Finally, the plaque assay was performed for comparisons following the procedures described for the methods of isolation, propagation, and purification of phages. In addition, KFS-YE (9 log PFU/ml) in TA broth was stored at various temperatures (-80°C, -20°C, 4°C, and 22°C) to determine its stability during the storage period. To minimize damage from freezing, 20% glycerol was added to KFS-YE stored in TA broth at -80°C and -20°C. At 2-week intervals, the plaque assay was performed to measure the lytic activity of KFS-YE over the storage periods.

One-Step Growth Curve of KFS-YE

Equivalent ratios of overnight cultures of *Y. enterocolitica* ATCC 23715 (8 log CFU/ml) and KFS-YE (8 log PFU/ml) were mixed and adsorbed for 15 min at 22°C. After centrifugation at 11,400 ×g for 10 min at 4°C, a pellet containing infected bacteria was resuspended in 10-ml TSB prior to incubation at 37°C. At 5 min intervals, the plaque assay was performed to determine the latent period, rise period, and burst size of KFS-YE.

Bactericidal Effect of KFS-YE against *Y. enterocolitica*

Two percent of an overnight culture of *Y. enterocolitica* ATCC 23715 was inoculated into 50 ml of TSB and incubated at 37°C with agitation until it reached the early exponential growth phase (approximately, 8 log CFU/ml). KFS-YE was added to the bacterial suspension in TSB to reach a multiplicity of infection (MOI) of 0.01, 0.1, 1.0, and 10, respectively, for incubation during 20 h at 37°C. A negative control was incubated without KFS-YE. The viable bacterial number was measured at 1 h intervals using a TSA plate.

Statistical Analysis

The experiments were replicated three times and the experimental results are expressed as means ± standard deviations (SD). Student's paired *t*-test for two groups and a one-way analysis of variance (ANOVA) for more than two groups were used to compare the means using the GraphPad and InStatV.3 programs (GraphPad, USA). The significance level was determined at $p < 0.05$, $p < 0.01$ or $p < 0.001$.

Results

Isolation and Purification of KFS-YE

Twelve phages were isolated from 20 water samples collected from a poultry plant and five slaughterhouses by using *Y. enterocolitica* ATCC 23715 as an indicator strain. Among the 12 phages, one *Y. enterocolitica*-specific phage was selected for further purification procedures because of its production of the largest (1.25 ± 0.06 cm) and clearest plaque formation, which indicated relatively excellent lytic activity of the isolated phage among the others [22]. After propagation and purification of the *Y. enterocolitica*-specific phage, its final concentration was determined to be (11.72 ± 0.03) log PFU/ml, and it was named KFS-YE following the recommendation of Ackermann *et al.* [23].

Morphological Characteristics of KFS-YE

TEM images (Fig. 1) showed that KFS-YE consisted of an icosahedral head with a hexagonal image and a tail. The head length, head width, and tail length of KFS-YE were 118.4 ± 11.6 nm, 102.4 ± 6.1 nm, and 125.7 ± 15.8 nm, respectively. The head and tail were connected by a neck

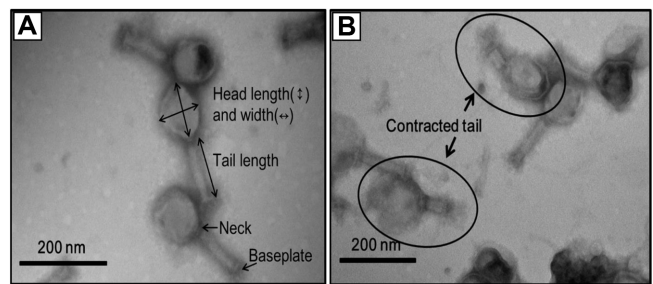


Fig. 1. TEM images of KFS-YE negatively stained with 2% phosphotungstic acid. The scale bars indicate 200 nm.

(10.6 ± 0.5 nm in length), and a baseplate was also observed at one end of the tail (Fig. 1A). Conversely, the other TEM image (Fig. 1B) showed a relatively short tail length of 48.2 ± 4.6 nm, which indicated the contractility of KFS-YE. Thus, KFS-YE was classified into the *Myoviridae* family because it possessed an icosahedral head and contractile tail unlike the *Podoviridae*, and *Siphoviridae* families [22, 24].

Specificity of KFS-YE

KFS-YE showed clear plaques against *Y. enterocolitica* ATCC 23715, *Y. enterocolitica* ATCC 55075, and *Y. enterocolitica* ATCC 9610 whereas it did not demonstrate any plaques against *Y. pseudotuberculosis* ATCC 29833, *Y. ruckeri* ATCC 29473, *Y. tuberculosis*, or *Y. frederiksenii* (Table 2). In addition, the plaque produced by *Y. enterocolitica* ATCC 23715 was significantly larger and clearer than those of *Y. enterocolitica* ATCC 55075 and *Y. enterocolitica* ATCC 9610 ($p < 0.05$). More importantly, KFS-YE did not show any cross-genus and cross-species specificities against 43 other bacteria. Thus, KFS-YE demonstrated a highly narrow specificity against *Y. enterocolitica* only.

Effects of pH Levels and Temperatures on the Stability of KFS-YE

As an effective biocontrol agent, KFS-YE should be stable when exposed to various pH levels and temperatures [12] (Fig. 3). KFS-YE was stable at wide pH ranges of 4–11 and there were no significant reductions in the phage titer ($p > 0.05$). However, the concentration of the phage decreased significantly to 6.4 ± 0.2 log PFU/ml at pH 3, and no plaques were observed at both pH 2 and 12 (Fig. 3A) ($p < 0.05$). Regarding the effect of temperature, the stability of KFS-YE was sustained at temperatures between 4°C and 50°C (Fig. 3B). Contrarily, the phage concentration decreased significantly to 5.2 ± 0.3 log PFU/ml and 2.7 ± 0.1 log PFU/ml at 60°C and 70°C, respectively ($p < 0.05$). At more extreme temperatures (*e.g.*, 80°C), phage stability was essentially lost.

Table 3. Stability of KFS-YE stored at various temperatures for 40 weeks.

Storage periods (weeks)	Storage temperatures (°C)			
	-80	-20	4	22
0	8.41 ± 0.02 ^{ax}	8.41 ± 0.02 ^{ax}	8.41 ± 0.02 ^{ax}	8.41 ± 0.02 ^{ax}
2	7.76 ± 0.05 ^{by}	8.39 ± 0.01 ^{ax}	8.40 ± 0.02 ^{ax}	8.40 ± 0.01 ^{ax}
4	7.78 ± 0.10 ^{by}	8.38 ± 0.03 ^{ax}	8.36 ± 0.09 ^{ax}	8.36 ± 0.06 ^{ax}
6	7.77 ± 0.15 ^{by}	8.36 ± 0.05 ^{ax}	8.37 ± 0.04 ^{ax}	8.37 ± 0.04 ^{ax}
8	7.75 ± 0.09 ^{by}	8.37 ± 0.04 ^{ax}	8.37 ± 0.05 ^{ax}	8.37 ± 0.02 ^{ax}
10	7.73 ± 0.06 ^{by}	8.37 ± 0.02 ^{ax}	8.38 ± 0.03 ^{ax}	8.38 ± 0.04 ^{ax}
12	7.75 ± 0.05 ^{bz}	8.37 ± 0.04 ^{ax}	8.39 ± 0.04 ^{ax}	8.39 ± 0.06 ^{by}
14	7.75 ± 0.05 ^{bz}	8.37 ± 0.04 ^{ax}	8.39 ± 0.02 ^{ax}	8.39 ± 0.05 ^{by}
16	7.74 ± 0.09 ^{bz}	8.38 ± 0.02 ^{ax}	8.37 ± 0.07 ^{ax}	8.37 ± 0.03 ^{by}
18	7.75 ± 0.02 ^{bz}	8.37 ± 0.04 ^{ax}	8.38 ± 0.05 ^{ax}	8.38 ± 0.03 ^{by}
20	7.52 ± 0.02 ^{bz}	8.25 ± 0.07 ^{bx}	8.22 ± 0.02 ^{bx}	8.22 ± 0.03 ^{by}
22	7.52 ± 0.03 ^{cz}	8.25 ± 0.05 ^{bx}	8.24 ± 0.01 ^{bx}	8.24 ± 0.01 ^{by}
24	7.51 ± 0.01 ^{cz}	8.26 ± 0.06 ^{bx}	8.22 ± 0.02 ^{bx}	8.22 ± 0.12 ^{cy}
26	7.51 ± 0.12 ^{cz}	8.26 ± 0.06 ^{bx}	8.19 ± 0.08 ^{bx}	8.19 ± 0.13 ^{cy}
28	7.52 ± 0.03 ^{cz}	8.23 ± 0.02 ^{bx}	8.22 ± 0.03 ^{bx}	8.22 ± 0.09 ^{cy}
30	7.50 ± 0.17 ^{cz}	8.21 ± 0.09 ^{bx}	8.24 ± 0.05 ^{bx}	8.24 ± 0.06 ^{cy}
32	7.51 ± 0.17 ^{cz}	8.26 ± 0.02 ^{bx}	8.24 ± 0.03 ^{bx}	8.24 ± 0.15 ^{cy}
36	7.45 ± 0.05 ^{cz}	8.26 ± 0.06 ^{bx}	8.23 ± 0.04 ^{bx}	8.23 ± 0.04 ^{cy}
40	7.30 ± 0.10 ^{dz}	8.08 ± 0.13 ^{bx}	8.19 ± 0.11 ^{cx}	7.87 ± 0.15 ^{dy}

Different letters (a, b, c, and d) in columns and (x, y, and z) in rows represent significant differences at $p < 0.05$.

Effects of Storage Conditions on the Stability of KFS-YE

Since previous studies [25, 26] have reported that a freeze-drying method was very damaging to most *Myoviridae* phages, KFS-YE was stored at freezing temperatures (-80°C and -20°C), refrigerator temperature (4°C), and room temperature (22°C) in this study. As shown in Table 3, phage titer declined slightly with time at all storage conditions. There were significant declines observed in phage titer beginning at 12 weeks, 20 weeks, and 20 weeks when stored at 22°C , 4°C , and -20°C , respectively ($p < 0.05$). However, there were no significant differences in phage titer when stored at -20°C and 4°C , except at 40 weeks ($p > 0.05$). Overall, phage titer was sustained with concentrations of 7.30 ± 0.10 log PFU/ml (87%), 8.08 ± 0.13 log PFU/ml (96%), 8.19 ± 0.11 log PFU/ml (97%), and 7.87 ± 0.15 log PFU/ml (94%) when stored at -80°C , -20°C , 4°C , and 22°C , respectively, for 40 weeks. Interestingly, phage titer stored at -80°C was significantly lower than those stored at other temperature conditions after the first 2 weeks ($p < 0.05$). In summary, KFS-YE showed a relatively

robust stability when stored at -20°C , 4°C , and 22°C rather than at -80°C for 40 weeks.

One-Step Growth Curve of KFS-YE

To elucidate the ability of KFS-YE to lyse *Y. enterocolitica*, the latent period and burst size were determined using a one-step growth curve analysis (Fig. 3). Because the definition of latent period is the time interval between the adsorption and the beginning of the first burst [27], it was determined to be 45 min. After the latent period, the number of liberated phages increased significantly for 65 min in what is called the rise period ($p < 0.05$). Afterwards, the number of liberated phages were sustained for 25 min during the entire plateau period. Since the burst size was determined as the ratio of the mean yield of phages that infected the bacteria cells to the mean phage particles liberated [28], the burst size of KFS-YE was determined to be 38 PFU/cell.

Bactericidal Effect of KFS-YE against *Y. enterocolitica*

The lytic property of KFS-YE at various concentrations was investigated for 20 h by the analysis of a bacterial growth curve pattern after KFS-YE infection (Fig. 4). The bacterial number of the control group (without introduction of phage) continued to increase during the entire incubation period, finally reaching 10.43 ± 0.32 log CFU/ml. In contrast, infection with KFS-YE inhibited bacterial growth significantly at all MOIs for 20 h in comparison to that of the control group at each incubation time ($p < 0.05$). After the number of *Y. enterocolitica* decreased abruptly and significantly at all MOI values at 2 h, the bacterial number was sustained up to 7 h ($p < 0.05$). Although there were significant differences in the bacterial numbers starting at 17 h between MOI of 10 and other MOIs ($p < 0.05$), there were no obvious and significant differences observed among different dosages at the MOI values. Therefore, KFS-YE was able to inhibit *Y. enterocolitica* efficiently and significantly even at the lowest MOI of 0.01.

Discussion

KFS-YE (*Y. enterocolitica*-specific phage) was isolated and purified among 12 phages and classified into the *Myoviridae* family because of its icosahedral head with contractile tail (Fig. 1) [22, 24]. To our knowledge, 12 *Yersinia*-specific phages, except those from the study of Salem et al. [2], have been reported and morphologically classified with six in *Myoviridae*, four in *Podoviridae*, and two in *Siphoviridae* (Table 1). Compared with these reported phages, the head

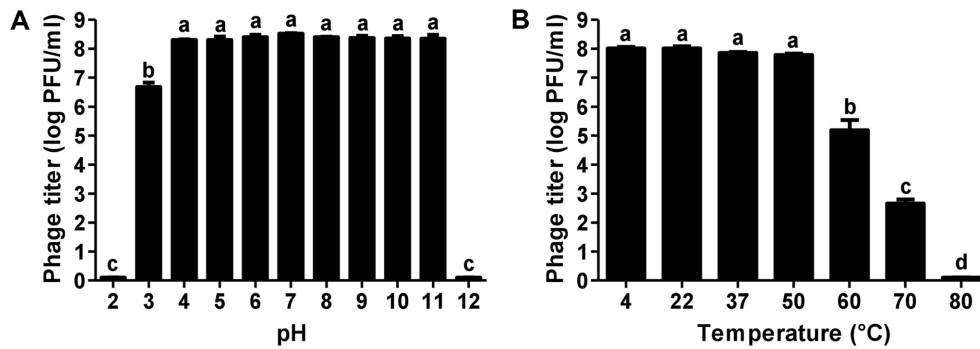


Fig. 2. Stability of KFS-YE at various (A) pHs and (B) temperatures. Different letters (a, b, c, and d) represent significant differences at $p < 0.05$ ($n = 3$).

length of KFS-YE was the longest, followed by that of the PY100 phage, and its tail length was second longest after that of the 8/C239 phage. Taken together, KFS-YE was assumed to be a novel *Myoviridae* phage against *Yersinia* because of the morphological differences in the length of head and tail. Thus, the morphological analysis supported the potential novelty of the isolated and purified KFS-YE.

The specificity of a phage is one of the most important factors determining its potential as a biocontrol agent [12, 29] (Table 2). KFS-YE demonstrated a highly narrow specificity against *Y. enterocolitica* only because it did not demonstrate any cross-genus and cross-species specificities against 43 other bacteria. Popp *et al.* [19] reported narrow host specificity for the PY44 phage and the PY96 phage against only *Y. enterocolitica* and *Y. frederiksenii*, respectively, among six *Yersinia* strains. Pajunen *et al.* [1] investigated the specificity of the ΦYeO3-12 phage, indicating its narrow specificity against *Y. enterocolitica*, *Y. frederiksenii*, and

Y. mollaretii among eight *Yersinia* strains. However, these two studies had a limitation because of the use of only *Yersinia* strains, and so their specificities against other genera were unclear. Conversely, Stevenson *et al.* [18] demonstrated that the YerA41 phage had a broad specificity against 9 bacterial strains, including *E. coli*, *Enterobacter cloacae*, *Erwinia herbicola*, *Klebsiella pneumoniae*, *Y. ruckeri*, and 4 strains of *Y. enterocolitica*. Taken together, the present study demonstrated that KFS-YE had an excellent narrow specificity against *Y. enterocolitica* only.

The broad pH and temperature stability of KFS-YE may be an advantageous characteristic because *Yersinia* in foods are exposed to various environmental conditions (Fig. 2). The FDA (2015) reported that the pH range of *Yersinia* growth was 4.2–10 and the broad pH stability of KFS-YE

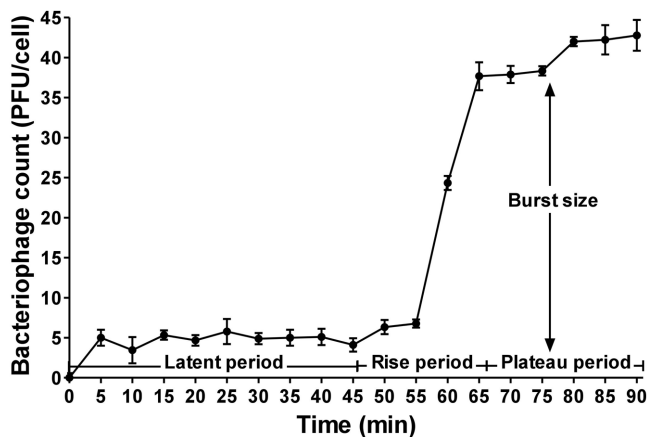


Fig. 3. One-step growth curve analysis of KFS-YE on *Y. enterocolitica* incubated in tryptic soy broth (TSB) at 37°C with gentle agitation.

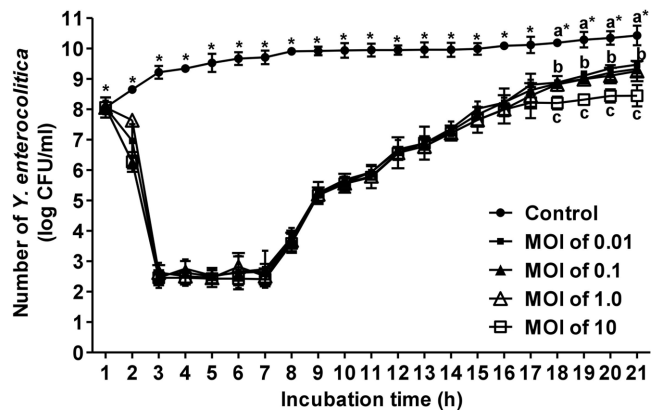


Fig. 4. Bactericidal effect of KFS-YE against *Y. enterocolitica*. *Y. enterocolitica* was infected by KFS-YE with MOIs of 0.01 (■), 0.1 (▲), 1.0 (△) or 10 (□). Control group was incubated without KFS-YE inoculation (●). Asterisk (*) means that there is a significant difference between the control group and the infection group with KFS-YE at $p < 0.05$. Different letters (a, b, and c) after 17 h incubation time represent significant differences at $p < 0.05$.

(pH 4–11) makes it suitable for its application. In addition, KFS-YE showed excellent stabilities at various temperatures between 4°C and 50°C, and significantly weak stabilities at both 60°C and 70°C. Interestingly, its robust stability at cold temperature (4°C) could enhance its advantage as a biocontrol agent because of the psychrotrophic property of *Y. enterocolitica*. When compared with other studies reporting the temperature stabilities of the *Klebsiella pneumoniae*-specific phage [22] and *Acinetobacter baumannii*-specific phage [30] at 25–40°C and 50–60°C, respectively, KFS-YE showed a relatively wide range of temperature stability. This suggests that the lytic activity of KFS-YE can occur both under relatively high as well as cold temperatures.

To guarantee phage quality, the phage needs to be sustained at its original quantity possessing viable status during certain storage periods. Although recent studies [26, 31] reported that phages can be inactivated or lost during storage presumably because of structural damage, lipid loss, and/or DNA structural changes, there have been very few studies performed regarding the storage stability of phages at different temperature conditions. As shown in Table 3, the overall trend of the KFS-YE titer declined very slightly with time such that the final phage titer was maintained in the range of 94–97% at –20°C, 4°C, and 22°C for 40 weeks. Unlike the findings of Capra *et al.* [32], wherein the *Siphoviridae* phage against *Lactobacillus* was more stable at –20°C and –80°C than at 4°C, our phage was not stable at –80°C. Considering the economic issue, KFS-YE showed robust stability for relatively long periods and this is further supportive of its practical use as a biocontrol agent.

Based on the one-step growth curve, the multiplication parameters of KFS-YE, such as the latent period and burst size, were analyzed (Fig. 3). The length of the latent period depends on the type of phage, phage species, physiological conditions, type of host, and the composition of the medium and incubation temperature [33]. The latent period of KFS-YE (45 min) was longer than those of both Φ YeO3-12 (25 min) and PY100 (30 min), whereas the burst size (38 PFU/cell) of KFS-YE was smaller than those of both Φ YeO3-12 (120 PFU/cell) and PY100 (120 PFU/cell). Conversely, Jun *et al.* [34] showed a relatively shorter latent period (30 min) and smaller burst size (16 PFU/cell) for the *Shigella*-specific phage as a new biocontrol agent. Since there have been no critical determinants for the latent period and burst size for its application as a biocontrol agent, these results showed that KFS-YE could lyse *Y. enterocolitica* effectively after a 45-min treatment with a

burst size 38 PFU/cell.

Furthermore, the bactericidal effect of KFS-YE against *Y. enterocolitica* was first investigated in this study (Fig. 4). As shown in Table 1, several phages have been reported, however, there have been no in vitro studies regarding the bactericidal effect of *Yersinia*-specific phages. The growth of *Y. enterocolitica* was decreased by KFS-YE and was sustained for 7 h even with MOI of 0.01, although there were no obvious and significant differences observed among different dosages at the MOI values. Depending on the phage characteristic and property, the pattern of bactericidal effect will follow dose-dependent or dose-independent manners. The presumable reason for the dose-independent manner of KFS-YE was derived from a phage-immune system that is induced by phages for refusing other phage infection after its saturation [35]. In addition, the incomplete lysis of KFS-YE for 7 h at any MOIs could be explained by the possibility that some proportion of bacteria were able to show resistance to the phage or by the rapid appearance of resistant mutants [36]. Afterwards, regrowth of *Y. enterocolitica* was initiated, which agreed with previous studies using the SFP 10 phage against *Salmonella enterica* and *E. coli* O157:H7, and the FSP1 phage against *Morganella morganii* [29, 37]. The reason for bacterial regrowth could be explained by the occurrence of spontaneous adaptation of the surviving bacteria during phage adsorption, phage DNA injection, cleavage of phage DNA, or abortive infection systems [9, 28, 37]. For the successful application of phages, this regrowth problem could be managed using phage cocktails rather than a single phage, and/or by increasing the MOI of the phages [37]. Although there are still many hurdles to address in the practical use of the KFS-YE phage, KFS-YE demonstrated excellent efficiency with the lowest MOI of 0.01. In addition, ORF analysis (data not provided) from whole genome sequencing of KFS-YE confirmed there were no toxin-related genes identified such as *yst*, *ttcC*, *yadA*, *virF*, *ysa*, and RTX-like toxins [38]. In future studies, KFS-YE will be directly employed to fresh produce for the reduction and prevention of *Y. enterocolitica* contamination for objective evaluation. In conclusion, this study demonstrated that KFS-YE was a potentially safe, effective, and promising biocontrol agent for the practical intervention of *Y. enterocolitica* in food.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

Reference

- Pajunen M, Kiljunen S, Skurnik M. 2000. Bacteriophage ϕ YeO3-12, specific for *Yersinia enterocolitica* serotype O: 3, is related to coliphages T3 and T7. *J. Bacteriol.* **182**: 5114-5120.
- Salem M, Virtanen S, Korkeala H, Skurnik M. 2015. Isolation and characterization of *Yersinia*-specific bacteriophages from pig stools in Finland. *J. Appl. Microbiol.* **118**: 599-608.
- Rahman A, Bonny TS, Stonsaovapak S, Ananchaipattana C. 2011. *Yersinia enterocolitica*: Epidemiological studies and outbreaks. *J. Pathog.* **2011**: 239391.
- Harris L, Farber J, Beuchat L, Parish M, Suslow T, Garrett E, et al. 2003. Outbreaks associated with fresh produce: incidence, growth, and survival of pathogens in fresh and fresh-cut produce. *Compr. Rev. Food Sci. Food Saf.* **2**: 78-141.
- MacDonald E, Einöder-Moreno M, Borgen K, Brandal LT, Diab L, Fossli, et al. 2016. National outbreak of *Yersinia enterocolitica* infections in military and civilian populations associated with consumption of mixed salad, Norway, 2014. *Euro Surveill.* **21**. doi: 10.2807/1560-7917.
- EFSA, 2012. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2010. *Euro Surveill.* **17**: 2597.
- Bottone EJ. 1997. *Yersinia enterocolitica*: the charisma continues. *Clin. Microbiol. Rev.* **10**: 257-276.
- Rashid MH, Revazishvili T, Dean T, Butani A, Verratti K, Bishop-Lilly KA, et al. 2012. A *Yersinia pestis*-specific, lytic phage preparation significantly reduces viable *Y. pestis* on various hard surfaces experimentally contaminated with the bacterium. *Bacteriophage* **2**: 168-177.
- Hwang S, Yun J, Kim KP, Heu S, Lee S, Ryu S. 2009. Isolation and characterization of bacteriophages specific for *Campylobacter jejuni*. *Microbiol Immunol.* **53**: 559-566.
- Park M-K, Oh J-H, Chin BA. 2011. The effect of incubation temperature on the binding of *Salmonella typhimurium* to phage-based magnetoelastic biosensors. *Sens. Actuators B: Chem.* **160**: 1427-1433.
- Byeon HM, Vodyanov VJ, Oh J-H, Kwon J-H, Park M-K. 2015. Lytic phage-based magnetoelastic biosensors for on-site detection of methicillin-resistant *Staphylococcus aureus* on spinach leaves. *J. Electrochem. Soc.* **162**: B230-B235.
- Hudson J, Billington C, Carey-Smith G, Greening G. 2005. Bacteriophages as biocontrol agents in food. *J. Food Prot.* **68**: 426-437.
- Owens J, Barton MD, Heuzenroeder MW. 2013. The isolation and characterization of *Campylobacter jejuni* bacteriophages from free range and indoor poultry. *Vet. Microbiol.* **162**: 144-150.
- Choi IY, Lee J-H, Kim H-J, Park M-K. 2017. Isolation and characterization of a novel broad-host-range bacteriophage infecting *Salmonella enterica* subsp. *enterica* for biocontrol and rapid detection. *J. Microbiol. Biotechnol.* **27**: 2151-2155.
- Sharma M. 2013. Lytic bacteriophages. *Bacteriophage* **3**: e25518.
- Cox N, Berrang M, Cason J. 2000. *Salmonella* penetration of egg shells and proliferation in broiler hatching eggs--a review. *Poult. Sci.* **79**: 1571-1574.
- Oh J-H, Park M-K. 2017. Recent trends in *Salmonella* outbreaks and emerging technology for biocontrol of *Salmonella* using phages in foods: a review. *J. Microbiol. Biotechnol.* **27**: 2075-2088.
- Stevenson R, Airdrie D. 1984. Isolation of *Yersinia ruckeri* bacteriophages. *Appl. Environ. Microbiol.* **47**: 1201-1205.
- Popp A, Hertwig S, Lurz R, Appel B. 2000. Comparative study of temperate bacteriophages isolated from *Yersinia*. *Syst. Appl. Microbiol.* **23**: 469-478.
- Pujato SA, Guglielmotti DM, Ackermann H-W, Patrignani F, Lanciotti R, Reinheimer JA, et al. 2014. *Leuconostoc* bacteriophages from blue cheese manufacture: long-term survival, resistance to thermal treatments, high pressure homogenization and chemical biocides of industrial application. *Int. J. Food Microbiol.* **177**: 81-88.
- Park M-K, Wickle III HC, Chai Y, Horikawa S, Shen W, Chin BA. 2012. The effect of incubation time for *Salmonella* Typhimurium binding to phage-based magnetoelastic biosensors. *Food Control.* **26**: 539-545.
- Verma V, Harjai K, Chhibber S. 2009. Characterization of a T7-like lytic bacteriophage of *Klebsiella pneumoniae* B5055: a potential therapeutic agent. *Curr. Microbiol.* **59**: 274-281.
- Ackermann H-W, DuBow M, Jarvis A, Jones L, Krylov V, Maniloff J, et al. 1992. The species concept and its application to tailed phages. *Arch. Virol.* **124**: 69-82.
- Bradley DE. 1967. Ultrastructure of bacteriophage and bacteriocins. *Bacteriol. Rev.* **31**: 230-314.
- Puapermpoonsiri U, Ford S, Van der Walle C. 2010. Stabilization of bacteriophage during freeze drying. *Int. J. Pharm.* **389**: 168-175.
- Golec P, Dąbrowski K, Hejnowicz MS, Gozdek A, Łoś JM, Węgrzyn G, et al. 2011. A reliable method for storage of tailed phages. *J. Microbiol. Methods* **84**: 486-489.
- Li L, Zhang Z. 2014. Isolation and characterization of a virulent bacteriophage SPW specific for *Staphylococcus aureus* isolated from bovine mastitis of lactating dairy cattle. *Mol. Biol. Rep.* **41**: 5829-5838.
- Haq IU, Chaudhry WN, Andleeb S, Qadri I. 2012. Isolation and partial characterization of a virulent bacteriophage IHQ1 specific for *Aeromonas punctata* from stream water. *Microb. Ecol.* **63**: 954-963.

29. Park M, Lee J-H, Shin H, Kim M, Choi J, Kang D-H, *et al.* 2012. Characterization and comparative genomic analysis of a novel bacteriophage SFP10 simultaneously inhibiting both *Salmonella* and *Escherichia coli* O157: H7. *Appl. Environ. Microbiol.* **78**: 58-69.
30. Yang H, Liang L, Lin S, Jia S. 2010. Isolation and characterization of a virulent bacteriophage AB1 of *Acinetobacter baumannii*. *BMC Microbiol.* **10**: 131.
31. Ackermann H-W, Tremblay D, Moineau S. 2004. Long-term bacteriophage preservation. *WFCC Newslett.* **38**: 35-40.
32. Capra M, Quiberoni AdL, Ackermann H-W, Moineau S, Reinheimer J. 2006. Characterization of a new virulent phage (MLC-A) of *Lactobacillus paracasei*. *J. Dairy Sci.* **89**: 2414-2423.
33. Weinbauer MG. 2004. Ecology of prokaryotic viruses. *FEMS Microbiol. Rev.* **28**: 127-181.
34. Jun JW, Kim HJ, Yun SK, Chai JY, Lee BC, Park SC. 2016. Isolation and comparative genomic analysis of T1-like *Shigella* bacteriophage pSf-2. *Curr. Microbiol.* **72**: 235-241.
35. Shen G-H, Wang J-L, Wen F-S, Chang K-M, Kuo C-F, Lin C-H, *et al.* 2012. Isolation and characterization of ϕ km18p, a novel lytic phage with therapeutic potential against extensively drug resistant *Acinetobacter baumannii*. *PLoS One* **7**: e46537.
36. Kim M, Ryu S. 2011. Characterization of a T5-like coliphage SPC35 and differential development of resistance to SPC35 in *Salmonella* Typhimurium and *Escherichia coli*. *Appl. Environ. Microbiol.* **77**: 2041-2050.
37. Yamaki S, Omachi T, Kawai Y, Yamazaki K. 2014. Characterization of a novel *Morganella morganii* bacteriophage FSP1 isolated from river water. *FEMS Microbiol. Lett.* **359**: 166-172.
38. Gnanasekaran G, Na EJ, Chung HY, Kim S, Kim Y-T, Kwak W, *et al.* 2017. Genomic insights and its comparative analysis with *Yersinia enterocolitica* reveals the potential virulence determinants and further pathogenicity for foodborne outbreaks. *J. Microbiol. Biotechnol.* **27**: 262-270.
39. Kasatiya S, Ackermann H-W. 1986. Morphology of *Yersinia enterocolitica* phages. *Ann. Inst. Pasteur. Virol.* **137**: 59-69.
40. Schwudke D, Ergin A, Michael K, Volkmar S, Appel B, Knabner D, *et al.* 2008. Broad-host-range *Yersinia* phage PY100: genome sequence, proteome analysis of virions, and DNA packaging strategy. *J. Bacteriol.* **190**: 332-342.
41. Leon-Velarde CG, Kropinski AM, Chen S, Abbasifar A, Griffiths MW, Odumeru JA. 2014. Complete genome sequence of bacteriophage vB_YenP_AP5 which infects *Yersinia enterocolitica* of serotype O: 3. *Virol. J.* **11**: 188.