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Isolation and Characterization of a Novel Broad-host-range Bacteriophage Infecting *Salmonella enterica* subsp. *enterica* for Biocontrol and Rapid Detection

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Bacteriophages have gained substantial attention as biocontrol and biorecognition agents, substituting antibodies. In this study, a *Salmonella* Enteritidis-specific bacteriophage, KFS-SE1, was isolated, identified, and characterized. This *Siphoviridae* phage infects *S.* Enteritidis with high specificity. This phage is highly stable under various pH (5–11), temperature (4–60°C), and organic solvent conditions. The KFS-SE1 genome consisted of 59,715 bp with 73 predicted open reading frames and 57.14% GC content; it had a complete set of genes required for phage reconstruction. Comparative phylogenetic analysis of KFS-SE1 revealed that it was very similar to the other *Salmonella* phages in the *Siphoviridae* family. These characteristics suggest that KFS-SE1 with its high specificity and host lysis activity toward *S.* Enteritidis may have various potential applications.

Keywords: Bacteriophage, *Salmonella* Enteritidis, biocontrol, rapid detection

Salmonella spp. have been recognized as some of the major foodborne pathogens that cause salmonellosis characterized by diarrhea, fever, abdominal cramps, nausea, occasional vomiting, or headache [1]. In the USA, 8,831 illnesses were reported with 1,156 hospitalizations and 14 deaths from 2010 to 2017. In addition, owing to the highly increased consumption of a wide variety of fresh fruits and vegetables, *Salmonella* outbreaks have recently occurred [2]. Therefore, the spread of *Salmonella* needs to be controlled for preventing its outbreaks. Bacteriophages have been recommended owing to their high specificity, low inherent toxicity, high safety to humans, adaptability to their host system, robustness against harsh environments, and relatively cheap and easy production [3, 4]. In addition, several bacteriophages have been recently developed and employed in amperometric, fluorescent, bioluminescent, impedimetric, and magneto-elastic biosensors, as well as quartz crystal microbalance and surface plasmon resonance. Therefore, phages could be developed as alternative natural food preservative agents against *Salmonella* or be used as a biosensor element for the rapid detection of this pathogen in foods.

A *Salmonella* Enteritidis-targeting bacteriophage (KFS-SE1) was isolated as described previously by Bandara *et al*. [5]. For observation of its morphology using TEM (H-7100; Hitachi, Japan), the bacteriophage KFS-SE1 was stained with phosphotungstic acid (Sigma-Aldrich Co., USA) for 30 sec and attached on a carbon-coated copper grid for 30 sec. The stained KFS-SE1 was observed by TEM at 100 kV. The host range test of KFS-SE1 was conducted using a dot assay using various bacterial strains as well as the isolates given in Table 1. Its lytic activity was observed by formation of clear plaques. Phage stability testing of KFS-SE1 (10^7 PFU/ml) was performed under various stress conditions, including temperature (4, 10, 22, 37, 50, and 60°C), pHs (3, 5, 7, 9, and 11), and presence of organic solvents (absolute ethanol, chloroform, and isopropanol). Finally, all the phage mixtures were incubated at room temperature for 1 h and the KFS-SE1 titer was determined by a plaque assay [6].

Table 1. Host range of KFS-SE1.

Bacterial strain	Plaque formation[a]	Source or reference[b]
Salmonella sp.		
S. Enteritidis ATCC 13076	++	ATCC
S. Typhimurium ATCC 19586	++	ATCC
ATCC 15812	++	ATCC
S. Hartford DPFS1	+	DPFS
S. Montevideo DPFS2	+	DPFS
S. Salamae DPFS3	+	DPFS
S. Heidelberg DPFS4	-	DPFS
S. Dublin DPFS5	-	DPFS
S. Panama DPFS6	-	DPFS
S. Senftenberg DPFS7	-	DPFS
S. Typhi DPFS8	-	DPFS
S. Mission DPFS9	-	DPFS
S. Arizonae DPFS10	-	DPFS
S. Mission DPFS11	-	DPFS
Gram-negative strains		
Aeromonas hydrophila ATCC 7996	-	ATCC
sobria ATCC 43979	-	ATCC
salmonicida ATCC 33658	-	ATCC
media ATCC 33907	-	ATCC
Escherichia coli ATCC 15144	-	ATCC
ATCC BAA-2196	-	ATCC
O157:H7 ATCC 43895	-	ATCC
Shigella flexneri 2a strain 2457T	-	IVI
sonnei ATCC 9290	-	ATCC
Vibrio parahaemolyticus ATCC 17802	-	ATCC
vulnificus DPFS12	-	DPFS
Klebsiella pneumoniae ATCC 13883	-	ATCC
Campylobacter jejuni DPFS13	-	DPFS
Pseudomonas aeruginosa ATCC 10145	-	ATCC
Gram-positive strains		
Bacillus cereus ATCC 13061	-	ATCC
ATCC 14579	-	ATCC
Listeria monocytogenes ATCC 19116	-	ATCC
innocua ATCC 33090	-	ATCC
Staphylococcus aureus ATCC 25923	-	ATCC
Yersinia enterocolitica ATCC 23715	-	ATCC
pseudotuberculosis ATCC 29833	-	ATCC

[a]++, clear plaque; +, turbid plaque; -, no susceptibility to KFS-SE1.

[b]ATCC, American Type Culture Collection; IVI, International Vaccine Institute; DPFS, Department of Plant and Food Sciences, Sangmyung University.

[c]All bacteria were grown in Tryptic Soy Broth at 37°C for 18–24 h with agitation.

A novel bacteriophage, KFS-SE1, infecting *S.* Enteritidis ATCC 13076 and forming clear plaques were isolated and purified from a chicken intestinal sample obtained from a poultry processing plant (Korea). Subsequent observation of its morphology by TEM revealed that KFS-SE1 belonged to the family *Siphoviridae*, and it had a head (75.0 ± 3.0 nm head length and 67.6 ± 1.7 nm head width) and a noncontractile tail (228.7 ± 13.4 nm tail length) (data not shown). To verify the host specificity, a host range test was conducted with 14 different *S. enterica* strains as well as other various gram-positive and gram-negative strains. Interestingly, KFS-SE1 infected *S.* Enteritidis and *S.* Typhimurium, forming a clear plaque, and *S.* Hartford, *S.* Newport, *S.* Montevideo, and *S.* Salamae, forming a turbid plaque, suggesting that it specifically infects and strongly inhibits the growth of *S.* Enteritidis and *S.* Typhimurium. However, it could not infect other various strains, indicating that this phage might have a highly specific and relatively narrow host range for *S. enterica* infection (Table 1). The phage stability may be important for applications in various stress conditions. To confirm the stability of KFS-SE1, it was tested under three different stress conditions (temperatures, pHs, and organic solvents); the results suggested that this phage was highly stable within the pH range of 5–11, temperature range of 4–60°C, and in the presence of ethanol and isopropanol (Fig. 1). However, its lytic activity against host was slightly low at 70°C (70% reduction), pH 3 (34% reduction), and in the presence of chloroform (23% reduction), suggesting that this phage may have potential applications under normal conditions.

The extracted genomic DNA of KFS-SE1 was sequenced by LabGenomics Co. (Korea) using the Illumina MiSeq platform (USA) and the qualified sequence reads were assembled with de novo assembler software (Platanus 1.2.4). The open reading frames (ORFs) were predicted using GeneMarkS [7], Glimmer [8], and FgenesV software (Softberry, Inc., USA), and the ribosomal binding sites (RBSs) were predicted using RBSfinder (J. Craig Venter Institute, USA). The predicted ORFs were annotated using BLASTP [9] and InterProScan programs [10]. The complete genome sequence and its annotation result were handled and edited using Artemis16 [11]. Phage virulence factor analysis was performed using Virulence Searcher [12]. For phylogenetic tree analysis of phage major capsid proteins (MCPs), MEGA6 was used with the neighbor-joining method with *P* distance values [13], and average nucleotide identity (ANI) analysis was performed using JSpecies program [14].

To understand the phage genomic characteristics as well

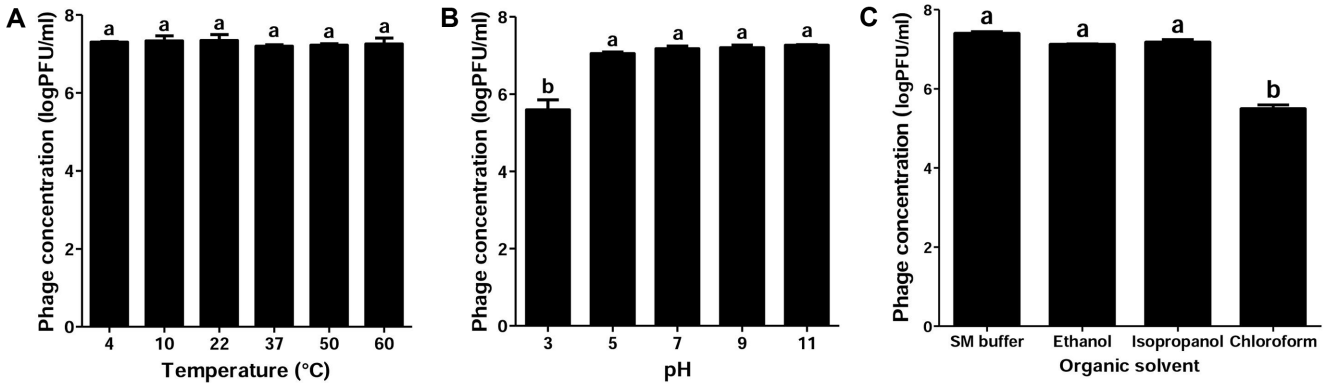

Fig. 1. Effects of (**A**) pH, (**B**) temperature, and (**C**) organic solvent on the stability of KFS-SE1.
Different letters (a, b) represent significant difference at $p < 0.05$ ($n = 3$).

Table 2. General genome characteristics of KFS-SE1 and other similar phages.

Characteristics	KFS-SE1	iEPS5	FSL SP-124	Utah
Morphology (family)	*Siphoviridae*	*Siphoviridae*	*Siphoviridae*	*Siphoviridae*
Genome size (bp)	59,715	59,254	59,245	59,024
G+C content (%)	57.14	56.3	56.5	56.4
Predicted ORFs	73	73	71	74
Tail structure proteins	4	3	5	7
Host lysis-related proteins	3	2	2	2
GenBank Accession No.	MG280946	KC677662	KC13915	KY014601

as safety verification for applications, the KFS-SE1 genome was completely sequenced and analyzed. The KFS-SE1 genome consists of a 59,715-bp double-stranded DNA containing 73 predicted ORFs with a GC content of 57.14% (Fig. 2A). Among the predicted ORFs, 21 (28.8%) were predicted to have specific functions and the functions of other ORFs are unknown, probably due to insufficient phage genome information in public databases. These functional ORFs were categorized into five functional groups: DNA replication/modification (DNA primase/helicase, DNA polymerase I, and terminase large/small subunits), structure and packaging (head-to-tail joining protein, capsid maturation protease, and capsid protein E), tail structure (tail assembly chaperone, tail measure protein, and tail assembly protein), host lysis (lysin protein and endolysin-like protein), and additional function (*N*-6-adenine-methyltransferase), suggesting that the KFS-SE1 genome has all the required core phage genes for its own replication, phage reconstruction, and host lysis. However, neither a virulence factor nor a toxin gene was detected in the genome, suggesting that KFS-SE1 may be stable and safe for use in further applications. To further understand the relationships among closely related *Salmonella* phages, their conserved MCP sequences were compared. Interestingly, *Salmonella* phages were grouped into three morphological families, suggesting that their relationship may be associated with the phage morphology (Fig. 2B). The phage KFS-SE1 belongs to the family *Siphoviridae*. To verify their taxonomical relationship, further ANI phylogenetic tree analysis with complete genome sequences of the *Siphoviridae* family phages infecting *Salmonella* was conducted, revealing that phages iEPS5, Utah, and FSL SP-124 are highly related to this phage (Fig. 2C). This ANI result showed more differential taxonomical relationships among them. Similar to the result of MCP comparison, these four phages have highly associated evolutionary relationships, suggesting that they might have evolved from a common ancestor.

Based on these results, KFS-SE1 of the *Siphoviridae* family was noted to be highly specific to the host bacterium and highly stable under a few stress conditions, indicating high applicability. This host specificity suggests the possibility of KFS-SE1 as a biorecognition element for use in rapid

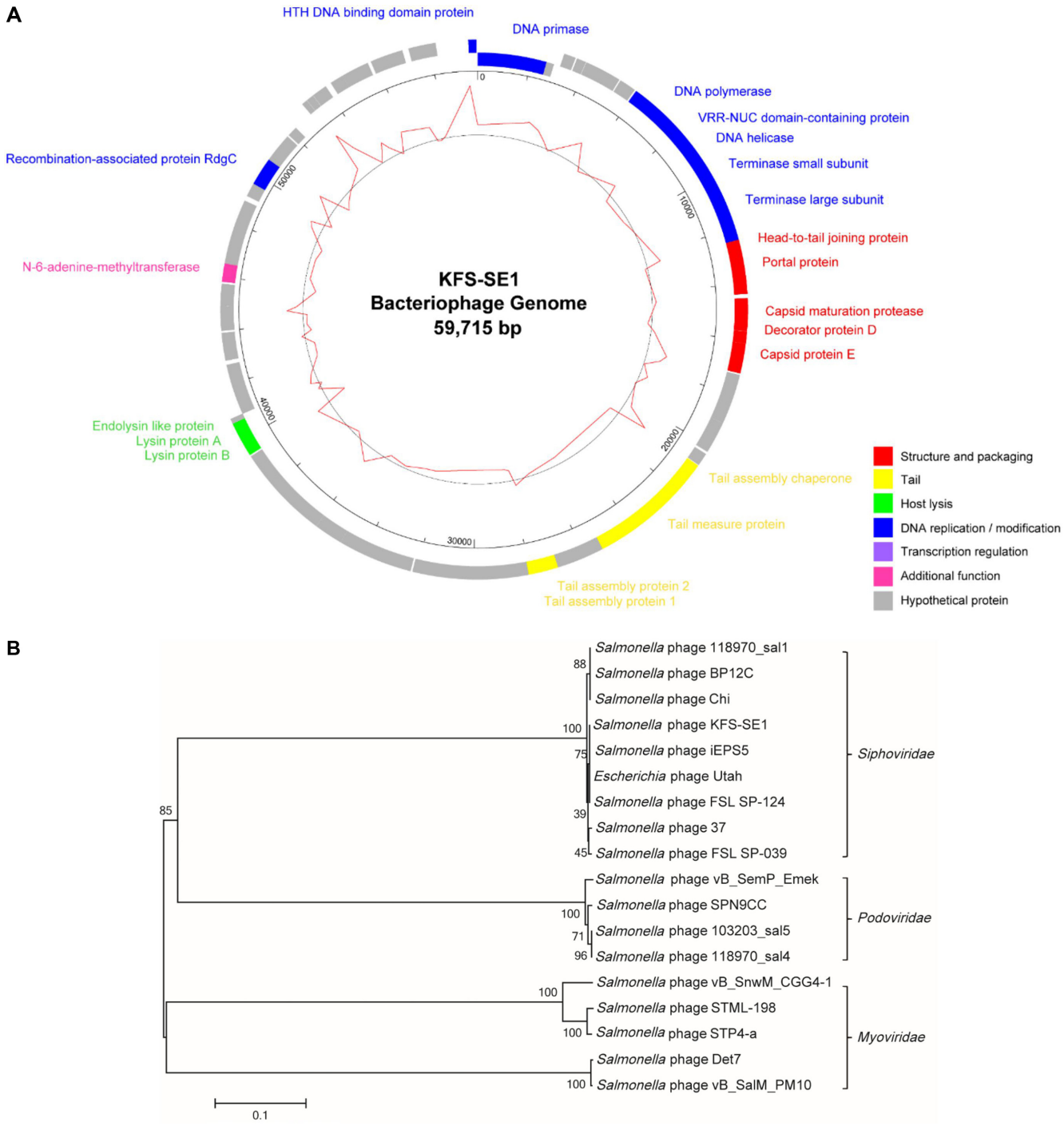

Fig. 2. Genomic studies of KFS-SE1.
(**A**) Genome map of KFS-SE1. Functional categories are indicated by specific colors in the legend. (**B**) Comparative phylogenetic analysis of major capsid proteins from various bacteriophages infecting *Salmonella*. (**C**) Comparative ANI phylogenetic analysis of complete genome sequences from various *Salmonella*-infecting bacteriophages in the *Siphoviridae* family.

detection methods. In addition, its lysis activity and lack of a virulence factor indicated that this phage could be used as a novel biocontrol agent for the prevention and control of *Salmonella* in foods. Therefore, KFS-SE1 may be a good candidate for various applications, with high specificity and high lysis activity toward *S*. Enteritidis. The complete

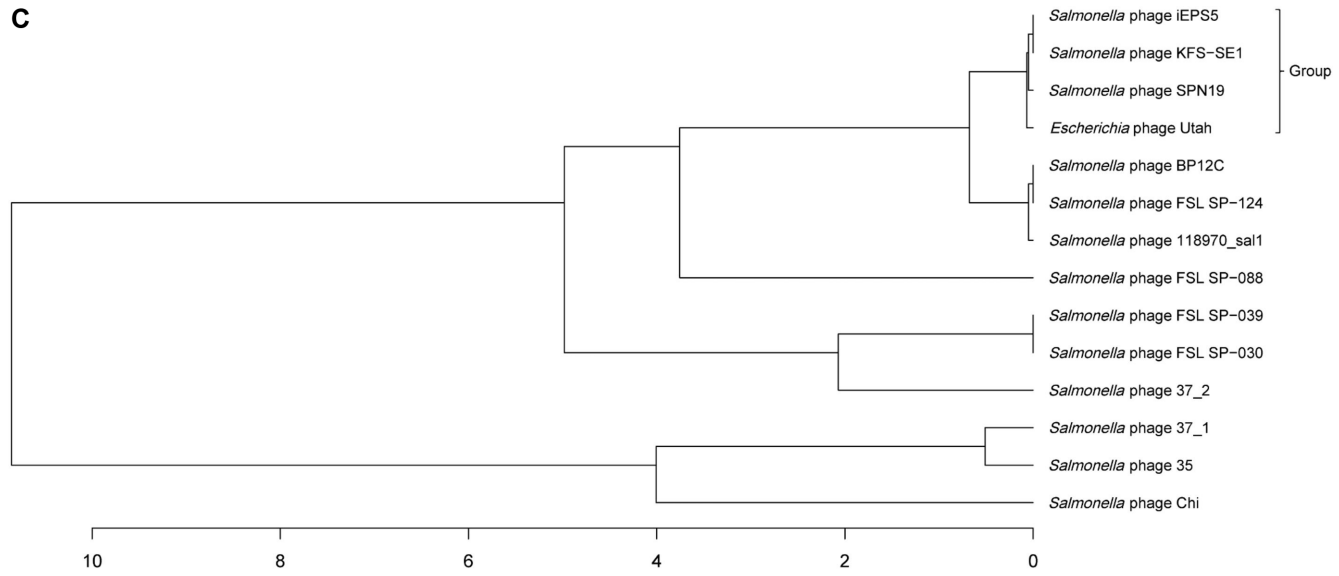

Fig. 2. Continued.

genome sequence and its annotation information have been deposited into the GenBank database under the accession number MG280946.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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