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Original Article

A Virulent *Salmonella enterica* Serovar Enteritidis Phage SE2 with a Strong Bacteriolytic Activity of Planktonic and Biofilmed Cells

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Salmonella enterica serovar Enteritidis is one of the major food borne pathogens. Utilizing lytic bacteriophages against this pathogen can be a new and effective approach for the prevention of food-contamination and food-borne infection. In this study, we isolated and characterized a *Salmonella* Enteritidis specific lytic bacteriophage (phage SE2). The bacteriolytic activity of planktonic and biofilmed cells against an antibiotic resistant strain of *Salmonella* Enteritidis was also evaluated. Phage SE2 revealed an efficient bacteriolytic effect with biofilm dispersing ability and could maintain its virulence even at extreme pH and temperature. It can be a potential biotherapeutic agent against *Salmonella* Enteritidis.

Key Words: Virulent, *Salmonella* Enteritidis, Bacteriophage, Drug resistance, Biofilm

INTRODUCTION

Salmonella enterica serovar Enteritidis (*Salmonella* Enteritidis) is one of the major food borne pathogens all over the world (1, 2). Resistance to nalidixic acid and reduced susceptibility to other quinolones by this pathogen have been increasing in many countries (3~6). This organism has the ability to form biofilm on food contact surfaces, which can be important implications in food safety (7~9). The adhesion and development of biofilm on food contact surfaces during food processing by this pathogen which has been transferred from infected food animals (poultry, livestock's) and their products may be the potential source and cause of infections (10). Like other biofilmed bacteria, biofilmed *Salmonella* also have been reported more resistant to many available disinfectants including chlorine than their planktonic counterparts (11).

In the recent years, utilizing lytic bacteriophages (phages) and their products as a safe and effective therapeutic and biocontrol agent is gaining interest to prevent infections (12~14). Bacteriophage had been successfully applied to reduce *Salmonella* and *Campylobacter* colonization *in vivo* and *in vitro* (15~17), in ready to eat food (18). Bacteriophage alone or combination with antibiotics had been applied to remove biofilm of different bacterial species (19~21). Furthermore, engineered phage enzymes had been shown effective to remove bacterial biofilms (22). However, the bacteriolytic activity and biofilm dispersion of phages against *Salmonella* Enteritidis had not been studied; utilizing lytic bacteriophage against this pathogen can be a new and effective approach. We isolated and characterized a lytic bacteriophage specific to *Salmonella* Enteritidis (phage SE2) in sewage samples from poultry farming

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vicinity at Anyang area in Republic of Korea. The genomic features of phage SE2 had been announced recently (23) and the genome sequenced annotations are available at GenBank database under accession number JQ007353. Here we report its other characteristics with bacteriolytic activity of planktonic and biofilmed bacteria against a nalidixic acid resistant and ciprofloxacin reduced susceptible clinical strain of *Salmonella* Enteritidis.

MATERIALS AND METHODS

Bacterial strains and determination of host range

Salmonella Enteritidis PT4 was used as propagating host. To determine host range of phage SE2, *Salmonella* strains of several serovars isolated from human and animal sources between the years 1999 to 2009 and non-*Salmonella* strains such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Staphylococcus aureus* were used. To assess the bacteriolytic and biofilm dispersion ability of phage SE2, a clinical strain of *Salmonella* Enteritidis, resistant to nalidixic acid and reduced susceptible to ciprofloxacin (MIC, 0.5 μg/ml), was used. All these bacterial pathogens were obtained from the frozen stock in our laboratory.

To determine the host susceptibility, spot test and plaque assay were performed; about 1 ml of the host bacterial culture at log phase was poured on the Luria Bertani (LB) agar plates (USB Corporation, Cleveland, OH, USA), excess liquid was withdrawn and allowed to dry at room temperature. A 7 μl of phage lysate was applied for spot test. All spot positive samples were further tested by plaque assay following double agar overlay technique as described below.

Phage isolation and purification

Phage isolation and purification was performed as described by Merabishvil *et al*. (24) with slight modifications. Sewage samples were collected from poultry farming vicinity at Anyang area in Republic of Korea and emulsified with about 45 ml of tap water. After keeping overnight at room temperature, a clear supernatant was obtained by centrifugation at 8,000 rpm for 10 minutes. A 4.5 ml of supernatant was aliquot in 14 ml conical tube in which 0.5 ml 10× LB and 0.5 ml host bacteria (in log phase) were incorporated and incubated overnight at 37℃ in shaking incubator. Samples were cooled to 4℃ for 2 hours after adding 200 μl of chloroform and centrifuged at 13,000 rpm at 4℃ for 10 minutes. Supernatants were filtered using 0.2 μm filters (Sartorius Stedim Biotech GmbH, Goettingen, Germany) and a 0.7 μl of filtrate was applied for spot test on the surface of LB agar lawn with host bacteria. All spot positive samples were further tested by plaque assay following double agar overlay technique. One hundred μl of host bacteria at log phase and 100 μl of phage lysate were added into 3 ml of soft agar (LB containing 0.6% agar) at 55℃ water bath and immediately poured onto LB agar plate with gentle mixing. Plates were incubated at 37℃ for overnight. Phage lysate was harvested after eluting with 4 ml of saline-magnesium-gelatin (SM) buffer by shaking over a plates shaker (Japan Servo Co. LTD, Tokyo Japan) for 30 min at room temperature and centrifuged at 13,000 rpm for 10 min at 4℃. The supernatant was filter sterilized by using 0.2 μm membrane filter and stored at 4℃ after adding 1/25 volume of chloroform. Phages were purified by isolating of three successive single plaques. The titer of final concentrated stock was determined by 10 fold serial dilution of the phage lysate and agar overlay method as described above.

Electron microscopy

Phage morphology was observed in high titer sample of phage SE2 lysate. Sample was deposited on carbon coated copper grids and stained negatively with 0.5% uranyl-acetate for 30 seconds then viewed by transmission electron microscope (HITACHI-700, Tokyo, Japan). The morphological features and average size of phage were estimated from 3 identical phage particles.

Determination of adsorption rate and burst size of phage SE2

To determine adsorption rate of phage SE2, *Salmonella* Enteritidis PT-4 was grown up to log phase in LB broth

and washed twice with phosphate buffer by centrifugation at 8,000 rpm for 5 min. The bacterial culture was adjusted to OD_{600} value of 0.4 (1.8×10^8 CFU/ml) and treated by approximately 0.0001 multiplicity of infection (MOI: ratio of phage number to bacteria number) of phage SE2. The mixtures were incubated at room temperature for phage adsorption followed by separation of unbound phages by centrifugation at 13,000 rpm in specified time points up to 27 minutes. Supernatants were immediately filtered by using 0.2 μm filters. A 100 μl of the filtrate from each tube was mixed with equal volume of host bacteria at log phase and performed for plaque forming unit (PFU) measurement by agar overlay technique.

To determine the burst size of phage SE2, *Salmonella* Enteritidis PT-4 was grown up to log phase in LB broth at 37℃ and adjusted to OD_{600} value of 1 (5.0×10^8 CFU/ml). The bacterial culture was mixed with 0.00001 MOI of phage SE2. The mixture was incubated at 4℃ for 30 minutes and then centrifuged at 13,000 rpm for 5 minutes. The pellet was again mixed with fresh LB broth, and incubated at 37℃ water bath. A 100 μl of samples was mixed in 3 ml of soft agar and seeded subsequently on LB agar in specified time points. Plates were incubated overnight at 37℃. The average burst size per host cell was calculated by subtracting the phage PFU value of latent time from the phage PFU value of log time, and dividing this resultant value from the value of latent time.

Assessment of phage's stability against temperature and pH

For thermal stability test, phage SE2 was treated at different temperature from 50~70℃ for an hour. A 100 μl of phage SE2 (1×10^7 PFU/ml) was added into 900 μl of SM buffer in heating blocks (FINEPCR Co. Gyenggi-do, Korea) in micro-centrifuge tubes and then cooled on ice for about 30 seconds before proceeding for plaque assay. The PFU of survival phage particles was estimated after overnight incubation at 37℃. To determine the stability of phage during storage, phage lysate was stored at -70℃ with DMSO (1/3, v/v), 4℃, and ambient temperature (18~24℃) with chloroform (1/25, v/v) for 1 year. Phage PFU was assessed every month up to 12 months. To determine the pH stability of phage SE2, SM buffer was adjusted at different pH (2~10) by adding either 0.1 N HCl or 0.1 N NaOH. A 100 μl of phage SE2 (1×10^7 PFU/ml) was added into 900 μl of SM buffer of different pH values in micro-centrifuge tubes and kept at room temperature for an hour. Phage PFU was measured from survival particles.

Assessment of phage ability to kill bacteria and produce progenies at different temperature

Salmonella Enteritidis strain JB-10-201 was grown at 37℃ up to log phase in LB broth. About 7.2×10^7 CFU/ml of bacterial culture was challenged with 7.2×10^{11} PFU/ml of phage SE2 and then incubated either at 37℃, ambient temperature, or 4℃ for 4 hours. Samples from each were taken for CFU counting on MacConkey agar plates (Becton, Dickinson and company, Spark, MD). Similarly, for phage PFU measurement, phage treated samples were centrifuged at 13,000 rpm for 10 minutes. After filtration by using 0.2 μm filter, PFU was determined by agar overlay method.

Assessment of phage SE2's ability to disperse biofilm

Salmonella Enteritidis JB-10-201 was grown at 37℃ overnight in tryptic soy broth (TSB, Becton, Dickinson and company, Spark, MD, USA), adjusted to OD_{600} value of 1, and diluted to 100 fold with TSB. About 3 ml of culture was aliquot to a sterile 6 well plates. Biofilms were developed on the surface of the glass coverslips as described elsewhere with modifications (25). Sterile glass coverslips (18 × 18 mm) were dipped into the culture and incubated up to 24 hours at 37℃. Coverslips in TSB without bacteria were considered as negative control. The biofilmed coverslips were carefully removed by using a sterile forceps and gently rinsed twice with sterile phosphate buffer (PBS, WelGENE, Daegu, Republic of Korea). They were placed over sterile piece of paper towel in slope position to remove excess liquid, transferred into a new 6 wells plate containing fresh TSB and treated by either phage SE2 (10^{11} PFU/ml) or sterile SM buffer for control. All samples were incubated for 4 hours at 37℃. After the treatment, biofilmed coverslips

Table 1. Host range of phage SE2 against *Salmonella enterica* serovars Enteritidis and Gallinarum.

Salmonella Serovars	Year of isolation	Plaques[1]	*Salmonella* Serovars	Year of isolation	Plaques[1]
S. Enteritidis 012	2000	++	*S.* Gallinarum I	?	++
S. Enteritidis 013	2000	++	*S.* Gallinarum II	?	++
S. Enteritidis 014	2000	++	*S.* Gallinarum 08vs-38	2008	++
S. Enteritidis 015	2002	++	*S.* Gallinarum 08vs-178	2008	++
S. Enteritidis 018	2002	++	*S.* Gallinarum 08vs-3	2008	++
S. Enteritidis 09	2005	++	*S.* Gallinarum 08vs-4	2008	++
S. Enteritidis 010	2005	++	*S.* Gallinarum 08vs-5	2008	++
S. Enteritidis 011	2005	++	*S.* Gallinarum 08vs-6	2008	++
S. Enteritidis 015	2006	++	*S.* Gallinarum 08vs-8	2008	++
S. Enteritidis 016	2006	++	*S.* Gallinarum 08vs-10	2008	++
S. Enteritidis 017	2006	++	*S.* Gallinarum 08vs-18	2008	++
S. Enteritidis 01	2009	++	*S.* Gallinarum 08vs-19	2008	++
S. Enteritidis 09	2009	++	*S.* Gallinarum 08vs-20	2008	++
S. Enteritidis PT-4	?	++	*S.* Gallinarum 08vs-21	2008	++
S. Enteritidis Jb -10-201	2010	++	*S.* Gallinarum 08vs-23	2008	++
S. Enteritidis 5-17-6	2010	++	*S.* Gallinarum 08vs-25	2008	++
S. Enteritidis 5-6-5	2010	+	*S.* Gallinarum 08vs-27	2008	++
S. Enteritidis 5-6-2	2010	+	*S.* Gallinarum 08vs-33	2008	++
			S. Gallinarum 08vs-34	2008	++
			S. Gallinarum 08vs-36	2008	+
			S. Gallinarum 08vs-38	2008	+
			S. Gallinarum 08vs-42	2008	+
			S. Gallinarum 08vs-43	2008	++
			S. Gallinarum 08vs-45	2008	++
			S. Gallinarum 08vs-51	2008	++
			S. Gallinarum 08vs-4	2008	++
			S. Gallinarum 08vs-52	2008	++
			S. Gallinarum 08vs-53	2008	++
			S. Gallinarum 08vs-56	2008	++
			S. Gallinarum 08vs-57	2008	++
			S. Gallinarum 08vs-165	2008	++
			S. Gallinarum 08vs-168	2008	++
			S. Gallinarum 08vs-173	2008	++
			S. Gallinarum 08vs-178	2008	++
			S. Gallinarum 08vs-188	2008	++
			S. Gallinarum 08vs-190	2008	++
			S. Gallinarum 08vs-191	2008	++

Table 1. Continued

Salmonella Serovars	Year of isolation	Plaques[1]	*Salmonella* Serovars	Year of isolation	Plaques[1]
			S. Gallinarum 08vs-200	2008	++
			S. Gallinarum 08vs-202	2008	++
			S..Gallinarum 08vs-236	2008	++
			S. Gallinarum 08vs-240	2008	++
			S. Gallinarum 08vs-251	2008	++
			S. Gallinarum 09vs-6	2009	+
			S. Gallinarum 09vs-7	2009	+
			S. Gallinarum 09vs-8	2009	+
			S. Gallinarum 09vs-9	2009	++

[1]++, large clear lysis, +, small clear lysis

were placed in 3 ml of TSB in 15 ml conical tube with sterile glass beads (3 mm) and were dispersed by vortexing for about 2 minutes as described elsewhere with modification (26). For CFU measurement, samples were seeded on MacConkey agar and incubated overnight at 37℃. For field emission-scanning electron microscopy (FE-SEM), phage-treated and control biofilmed coverslips were prepared as above. After washing and removing excess liquids, they were fixed in 50%, 70%, and absolute methanol (one minute in each). They were air dried, coated with platinum gold, and viewed under the scanning electron microscope (Hitachi S-4300, Tokyo, Japan). At least three independent experiments were carried for SEM analysis.

RESULTS AND DISCUSSION

Host range of phage SE2

Phage SE2 showed a strong lytic effect with large clear plaques to all tested strains including 18 clinical isolates of *Salmonella enterica* serovar Enteritidis and 47 animal isolates of *Salmonella enterica* serovar Gallinarum (Table 1). However, it did not form any plaque to other *Salmonella* serovars and non-*Salmonella* bacteria (*E. coli*, *K. pneumonia*, *P. aeruginosa*, *A. baumannii* and *S. aureus* (data not shown).

Morphology, adsorption rate and burst size of phage SE2

TEM observation revealed that phage SE2 has an icosahedral head with a long non contractile tail; the mean head diameter, 68 ± 5 nm; the mean tail length, about 110 ± 7 nm; and tail diameter, about 9 ± 2 nm. Based on the morphological studies, phage SE2 was assigned to the family Siphoviridae.

Phage adsorption showed that, approximately 70% of the phage particles adsorbed to *S.* Enteritidis within 6 minutes. More than 95% of the phage particles adsorbed in 15 minutes and nearly 100% of the phage particles adsorbed in 21 minutes (Fig. 1A). The one step growth curve revealed that the latent period of phage SE2 was approximately 10 minutes. The first burst out of phage SE2 was recorded at 15 minutes and the burst size was assumed to be approx. 155 PFU/host cell at 35 minutes (Fig. 1B).

Phage SE2 is morphologically similar to previously reported *Salmonella* phage FGcssa2 (27) and *Salmonella* Typhimurium phage10 (16). Phage SE2 differed from *Salmonella* phage FGcssa2 due to its larger burst size and shorter latent period; however, its adsorption time was slower than that of FGcssa2.

Stability of phage SE2 against temperature and hydrogen-ion concentration

Thermal stability of phage SE2 revealed that 100% and 85% of the phage particles were still capable to infect host cells after an hour of heat treatment at 50℃ and at 60℃ respectively. However, only about 18% of phage particles was survived after treatment for 20 minutes at 70℃. At

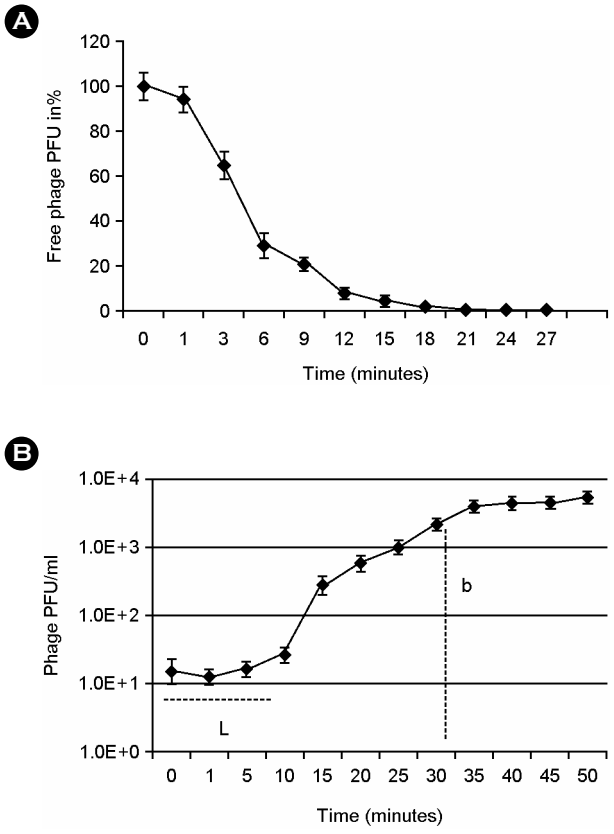

Figure 1. Adsorption rate and burst size of Phage SE2. (A) Adsorption rate of Phage SE2. (B) Burst size of Phage SE2. L, Latent time (10 minutes); b, average burst size (155 PFU/host cells at 35 minutes).

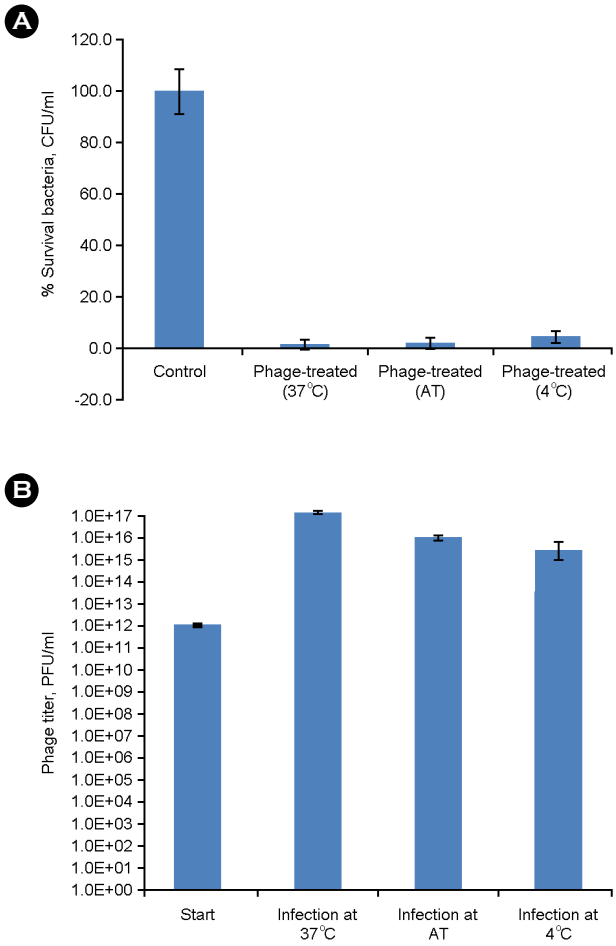

Figure 2. Ability of phage SE2 to kill bacteria and produce progenies at different temperature. (A) Ability of phage SE2 to kill bacteria at 37℃, ambient temperature and 4℃. (B) Ability of phage to produce progenies during infection at 37℃, ambient temperature (AT) and 4℃.

storage throughout the 1 year period, approximately 92%, 75% and 67% of phage particles were survived at -70℃, 4℃ and ambient temperature respectively. Phage SE2 showed almost no reduction at pH 4~9. Only about 9% and 14% of the phage particles were inactivated at pH 3 and pH 10 in an hour of treatment, whereas 100% of the phage particles were inactivated at pH 2. Therefore, Phage SE2 had revealed that its virulence could be maintained at extreme pH, high temperature and long term storage.

Bacteriolytic activity of the phage SE2 to planktonics and biofilmed cells

Bacteriolytic activity of Phage SE2 to planktonic cells of *Salmonella* Enteritidis showed 5, 4.2 and 2.5 logs decreased in viable bacteria at 4 hours at 37℃, ambient temperature and 4℃, respectively (Fig. 2A). At the same time, phage PFU was increased in approximately 6.8, 5.5 and 4 logs at 37℃, ambient temperature and 4℃, respectively (Fig. 2B). The rapid bacteriolytic activity of phage SE2 resembles with the virulent *Salmonella* phage st104a and st104b (28). The bacteriolytic ability of Phage SE2 to planktonic cells in different temperature revealed that phage SE2 could lyse and replicate not only in the optimum bacterial growth condition at 37℃ but also able to maintain its virulence and form progenies at 4℃.

Phage SE2 had lysed more than 97% of the biofilmed bacteria at 37℃ within 4 hours comparing with that of untreated control (Fig. 3A). FE-SEM images showed lysed

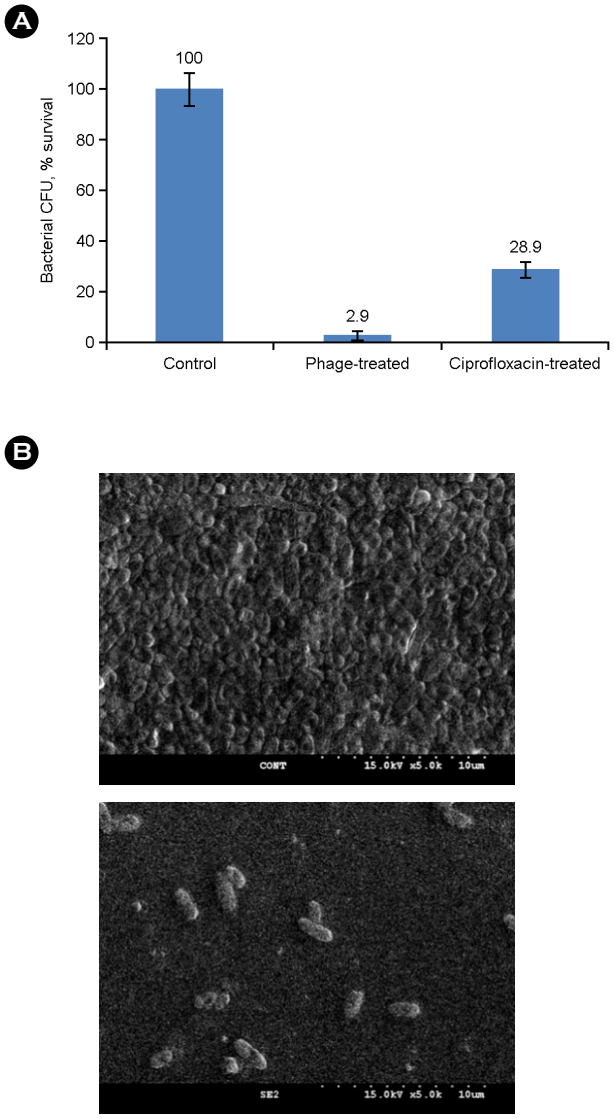

Figure 3. Bacteriolytic activity of the phage SE2 to biofilmed cells. (A) Biofilmed cells of *Salmonella* Enteritidis JB-201 were treated with tryptic soy broth (control), phage SE2 (10^{11} PFU/ml) or ciprofloxacin (0.5 μg/ml) for 4 hours at 37℃. Mean bacterial CFU was calculated from the triplicates. (B) Field emission scanning electron microscopic (FE-SEM) images showing biofilm dispersion activity of phage SE2. FE-SEM images showed that almost all the biofilmed cells were lysed with the dispersion of biofilm's matrix by phage SE2, very few cells, cell debris and ghost like bacteria can be seen on phage treated biofilms images (lower) compared that of phage untreated control (upper). At least three independent experiments were performed.

cells with cell debris and ghost like bacteria on phage treated biofilms (Fig. 3B, lower panel) comparing with that of phage untreated control (Fig. 3B, upper panel).

In the present study, phage SE2 treated *Salmonella* Enteritidis biofilms had shown a substantial decreased of viable cells almost below the detection limit in four hours comparing with that of phage untreated control. Moreover, lysed cell's debris and ghost like cells on the FE-SEM images of phage treated biofilms indicated the ability of phage to infiltrate and diffuse into the biofilm matrix with subsequent cell lysis. This result supported the previous findings by Briand *et al*. (29), who demonstrated the ability of phage diffusion and lysis within the biofilms. We also observed the biofilm removal activity at ambient temperature at different time points (4, 6, 9 hours and overnight), but only overnight treatment (16~20 hours) produced a significant bacteriolytic effect (data not shown). Although, phage SE2 did not lyse the *Salmonella* serovars other than Enteritidis and Gallinarum, it has shown a strong lytic effect to all tested *Salmonella* Enteritidis and serovar Gallinarum including the strain which is resistant to nalidixic acid and reduced susceptible to ciprofloxacin. Furthermore, phage SE2 could efficiently lysed (>99%) bacteria relatively in low dose (below 10^6 CFU/ml) even by using lower titer (MOI, 10~100) within 3 hours (data not shown). In order to lyse bacteria entrenched into the biofilms and relatively in high titer CFU of planktonic cells (above 10^7 CFU/ml), high titer (MOI, 10^3~10^4) phages were found effective. The result was similar to a previous report by Atterbury *et al*. (16) who found better efficacy by using high titer (MOI, 10^6) of phages *in vitro* against *Salmonella* serovars. All of these results revealed the potential of phage SE2 as a therapeutic agent against *Salmonella* Enteritidis.

In conclusion, this study characterized a newly isolated virulent phage specific to *Salmonella enterica* serovar Enteritidis and Gallinarum (phage SE2). It revealed a strong bacteriolytic ability against planktonic and biofilmed cells and maintained its virulence at extreme temperature and pH. These properties may enable its use asa therapeutic and biocontrol agent against *Salmonella* Enteritidis to prevent contamination of food and food-borne infection.

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