Isolation and characterization of bacteriophages of *Salmonella enterica* serovar Pullorum

H. Bao, H. Zhang, and R. Wang[1]

Institute of Food Safety and Quality, Jiangsu Academy of Agricultural Science, Key Laboratory of Animal-Derived Food Safety of Jiangsu Province, and Key Laboratory of Food Safety Monitoring and Management, Ministry of Agriculture, Nanjing 210014, P. R. China

ABSTRACT In this study, 2 bacteriophages of *Salmonella* Pullorum were isolated using an enrichment protocol and the double agar layer method. They were named PSPu-95 and PSPu-4-116, respectively, against clinical isolates of *Salmonella* Pullorum SPu-95 and SPu-116. The host ranges of the 2 bacteriophages were determined by performing spot tests with 20 bacteria strains. Both bacteriophages had wide host ranges. Bacteriophage PSPu-95 had a lytic effect on 17 of the 20 isolates (85%), and PSPu-4-116 produced a lytic effect on 14 isolates (70%) and was the only bacteriophage that produced a clear plaque on enterotoxigenic *Escherichia coli* K88. Transmission electron microscopy revealed the bacteriophages belonged to the order *Caudovirales*. Bacteriophage PSPu-95 was a member of the family *Siphoviridae*, but bacteriophage PSPu-4-116 belonged to the family *Myoviridae*. Both had a double-stranded DNA, which was digested with *Hin*dIII or *Eco*RI, that was estimated to be 58.3 kbp (PSPu-95) and 45.2 kbp (PSPu-4-116) by 1% agar electrophoresis. One-step growth kinetics showed that the latent periods were all less than 20 min, and the burst size was 77.5 pfu/cell for PSPu-95 and 86 pfu/cell for PSPu-4-116. The bacteriophages were able to survive in a pH range between 4 and 10, and they were able to survive in a treatment of 70°C for 60 min. The characterizations of these 2 bacteriophages were helpful in establishing a basis for adopting the most effective bacteriophage to control bacteria in the poultry industry.

Key words: bacteriophage, one-step growth, pH and heat stability, *Salmonella enterica* serovar Pullorum, wide host range

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INTRODUCTION

Salmonella enterica serovar Pullorum (*Salmonella* Pullorum) can cause severe infective pullorum disease in domestic birds, thus bringing severe economic consequences in poultry rearing. Although *Salmonella* Pullorum is rare in the modern poultry industry of the developed countries because of the extensive testing and control of breeder birds, this *Salmonella* has been very common on poultry farms in Africa and Asia in recent years and has caused much economic loss (Orji et al., 2005; Prakash et al., 2005).

Several measures to control *Salmonella* Pullorum have been used, with the use of antimicrobial drugs being the dominant measure. But recently, a study by Pan et al. (2009) monitored the changes in antimicrobial resistance of *Salmonella* Pullorum isolated in China from 1962 to 2007; the results indicated that there were rapid increases in antimicrobial resistance in *Salmonella* Pullorum.

Bacteriophages are bacterial viruses. It is considered that they offer a great advantage over antibiotics. First, bacteriophages target only the pathogens of interest, so the normal gut microflora are not affected. Second, bacteriophages are self-replicating in the bacterial host and kill the bacteria. (Connerton and Connerton, 2005). Bacteriophages have been used against zoonotic pathogens in live animals (Smith and Huggins, 1983; Atterbury et al., 2003; Huff et al., 2005; Tanji et al., 2005). These instances demonstrated that phage therapy can be as efficient as antibiotics in treating bacterial infections. For this study, *Salmonella* Pullorum-specific bacteriophages were isolated from chicken lagoon effluent.

To our knowledge, the isolation and characterization of lytic bacteriophages of *Salmonella* Pullorum have not been documented, although McLaughlin et al. (2006) described the isolation of *Salmonella* Typhimurium and Enteritidis bacteriophages. In this study, we used 13 clinical isolates of *Salmonella* Pullorum as indica-

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[1]Corresponding author: wangran@jaas.ac.cn

tor hosts for the isolation of lytic bacteriophages. Two bacteriophages, designated PSPu-95 and PSPu-4-116, were isolated and characterized in terms of their morphology, heat and pH stability, host ranges, and one-step growth. This work will be the first to isolate and characterize lytic bacteriophages specifically infecting *Salmonella* Pullorum.

MATERIALS AND METHODS

Bacterial Strains and Growth Medium

The type strains of *Salmonella enterica* serovar Enteritidis (*Salmonella* Enteritidis) ATCC 13076, *Salmonella enterica* serovar Paratyphi A (*Salmonella* Paratyphi A) ATCC 50073, and *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium) ATCC 13311 were kindly donated by Dr. Cao (Chinese Academy of Agricultural Science, Yangzhou, China) and were originally obtained from the American Type Culture Collection (ATCC, Manassas, VA). The type strains of poultry *Salmonella* spp. CMCC 2184 and *Salmonella enterica* serovar Pullorum (*Salmonella* Pullorum) CMCC 533 were all purchased from the China Veterinary Culture Collection Center (CVCC, Beijing, China). A total of 13 *Salmonella* Pullorum isolates were recovered from chickens with clinical signs of pullorum disease in Jiangsu Province. Other non-*Salmonella* species, enterotoxigenic *Escherichia coli* K88 and K99, strains were obtained from Dr. Shao (Jiangsu Academy of Agricultural Science, Nanjing, China). All *Salmonella* Pullorum isolates were isolated according to the National Standard of China (GB/T 4789.4–2003). First, all samples were placed in buffered peptone water as preenrichment media and incubated at 37°C for 18 h. After incubation, 0.1 mL of the buffered peptone water was simultaneously added to Rappaport-Vassiliadis broth and selenite cystine broth and then respectively incubated at 42°C for 18 h and 37°C for 24 h. A swab of the Rappaport-Vassiliadis broth and a swab of the selenite cystine broth were inoculated onto brilliant green agar and *Salmonella-Shigella* agar selective media, respectively. The 2 kinds of plates were incubated at 37°C for 24 h. Up to 5 colonies were selected from each plate and streaked on triple sugar iron agar, motility-indole-lysine decarboxylase media, and urea agar to confirm *Salmonella.* Positive colonies were confirmed as *Salmonella* Pullorum by biochemical tests including fermentation of glucose, lactose, maltose, and sucrose; hydrogen sulfide production; urease activity; lysine decarboxylation; ornithine decarboxylation; and ducitol, citrate, methyl red, and indole tests and were serotyped with commercial antiserum (Difco, Detroit, MI). One isolate with the typical biochemical and serotype characteristics was then confirmed using specific PCR (Shah et al., 2005). Once identified, the *Salmonella* Pullorum isolates were resuspended in tryptic soy broth (**TSB**) containing 10% glycerol and stored at −70°C. In this experiment, the media were all purchased from Qingdao Hope Bio-Technology Co. Ltd., Qingdao, China.

Isolation of Bacteriophages

In this experiment, 13 *Salmonella* Pullorum clinical isolates were used for enrichment of bacteriophages. Samples (chicken excretion sewage) were obtained from local commercial chicken farms. A water sample (25 mL) was centrifuged (15,285 × *g*, 15 min at 4°C), and the supernatants were filtered through a 0.22-μm-pore-size filter. Five milliliters of each filtrate was combined with 5 mL of TSB (Qingdao Hope Bio-Technology Co. Ltd.) and 0.1 mL (~10^8 cfu) of each *Salmonella* Pullorum strain. The mixture was incubated with shaking at 37°C overnight. Following incubation, the bacteria were removed by centrifugation (15,285 × *g* for 10 min at 4°C), and the supernatants were filtered through a 0.22-μm-pore-size filter again. Bacteriophage activity in the supernatant was tested by a spot assay by placing 10 μL of the supernatant on tryptic soy agar (Qingdao Hope Bio-Technology Co. Ltd.) seeded with different *Salmonella* Pullorum strains. The plates were checked for plaques after 18 h at 37°C. Plaques of lysis-positive supernatants were isolated and purified using the double agar layered method of Adams (1959). Bacteriophage titers were also determined using the double agar layered method. The isolated bacteriophages were propagated on their respective host bacteria.

For inoculation, 1 mL of 10^9 pfu/mL bacteriophage was combined with an exponential grow-phase (~10^9 cfu) culture of host bacterium of 1 mL in 10 mL of TSB with 2 m*M* $CaCl_2$ (TSB) at 37°C. First, the mix was held without shaking for 15 min at 37°C, and then the mix was shaken for 6 h until visible lysis. Thus, the crude lysate was prepared. To prepare the bacteriophage particle, the crude lysate was centrifuged at 15,258 × *g* for 10 min at 4°C, and the supernatant was filtered through a 0.22-μm filter. Then NaCl and PEG 8000 (Amersco, Solon, OH) were added to the supernatant to reach final concentrations of 0.5 *M* and 10% (wt/vol), respectively. After centrifugation and pouring of the supernatant, the bacteriophage pellet was dissolved in SM buffer (5.8 g/L of NaCl, 2.0 g/L of $MgSO_4$, 50 mL/L of 1 *M* Tris, pH 7.5, 5 mL/L of presterilized 2% gelatin).

Bacteriophage Morphology

Two purified bacteriophage pellets in SM were used in this experiment. Negative staining of bacteriophages with 1% (wt/vol) phosphotungstic acid and transmission electron microscopy (H-7650, Hitachi High-Technologies Corporation, Tokyo, Japan) at an acceleration voltage of 80 kV were completed as described previously (Mc Grath et al., 2006). Bacteriophage morphologies and dimensions (head diameter, tail length) were recorded.

Determination of Host Ranges of Bacteriophages

Eighteen strains of *Salmonella* spp. and 2 strains of enterotoxigenic *E. coli* were used in this study. Host range of bacteriophages was determined by spotting 10 μL of bacteriophage preparation (~10^8 pfu/mL) on lawn cultures of the bacteria strains. The plates were observed for the appearance of clear zones after incubation at 37°C for 18 to 36 h.

Heat and pH Stability of Bacteriophages

Plastic tubes having a volume of 1.5 mL were used for the treatment experiments. First, the titer of original bacteriophage in this experiment was determined. For heat-stability testing, tubes that were full with bacteriophages were kept in a water bath at each temperature (30°C, 40°C, 50°C, 60°C, 70°C, 80°C, 90°C) for 30 and 60 min. For pH-stability testing, samples of bacteriophages were mixed in a series of tubes containing TSB of different pH (adjusted using NaOH or HCl) and incubated for 2 h at 37°C. Bacteriophage titers were all determined using the double-layer agar plate method.

One-Step Growth

A method was used with a 10-min adsorption. Cells of host (OD_{600} of 0.5) were infected with specific bacteriophages to give a multiplicity of infection of 10 to ~100 and incubated at 37°C for 15 min. Following centrifugation at 13,000 × *g* for 1 min at 4°C, the pellets containing (partially) infected cells were resuspended in 1 mL of prewarmed TSB. Samples were taken at 10-min intervals (up to 2 h) and immediately determined by the double-layer agar plate method. Repeat assays were carried out. Latent period was defined as the time interval between the adsorption (not including 15 min preincubation) and the beginning of the first burst, as indicated by the initial rise in bacteriophage titer. Burst size was calculated as the ratio of the final count of liberated bacteriophage particles to the initial count of infected bacterial cells during the latent period.

Analysis of Bacteriophage Nucleic Acid

Extraction of nucleic acid was conducted according to the method of Sambrook et al. (2002) with few changes. The crude lysate was centrifuged and filtered through a 0.22-μm filter, and the filtrate was used for extraction of bacteriophage nucleic acid. Ribonuclease A and DNase I (Takara Biotechnology, Darian, China; 1 mg/mL final concentration) were added to aliquots of bacteriophage preparation and allowed to incubate for 30 min at 37°C. Protease K (200 μg) and SDS (0.5% final concentration) were added, and the mixture was incubated at 56°C overnight. Proteins were removed by phenol:chloroform:isoamyl alcohol (25:24:1) extractions, and the nucleic acid was precipitated with alcohol. After washing in 70% ethanol, the pellets were resuspended in TE buffer (10 m*M* Tris, pH 8.0, 1 m*M* EDTA) and electrophoresed in 1% agarose gel. The nucleic acid of the isolated bacteriophage was digested with DNase I or RNase A at 37°C according to the manufacturer's instructions. Products of digested bacteriophage nucleic acid were separated by 1% agarose gel electrophoresis.

Two restriction enzymes were selected to digest genomes of the bacteriophages. The chosen enzymes were *Eco*RI and *Hin*dIII. The digestions were performed for 3 h at 37°C in 30-μL reaction volumes containing 10 μL of genome DNA solution, 3 μL of the commercially supplied incubation buffer, 16 μL of water, and 1 μL (10 U/mL) of the restriction enzyme. The DNA fragments were resolved in 1.0% (wt/vol) agarose gels with 1× Tris-acetate-EDTA buffer. The DNA was visualized by transillumination with UV light (Bio-Rad, Hercules, CA) after the gels were stained with ethidium bromide.

RESULTS

Bacteriophage Isolation and Purification

Ten *Salmonella* Pullorum bacteriophages were isolated, some of which exhibited efficiency at lysing several different *Salmonella* Pullorum clinical strains, whereas others had a narrow host range. According to the degree of transparency of plaques and the host range, we chose 2 typical bacteriophages to study further, named PSPu-95 and PSPu-4-116, propagating with specific host strain SPu-95 and SPu-116, respectively. In the plaque test, the PSPu-95 formed round and big zones, with a bit of turbidity, and the size was 4 to 5 mm (Figure 1A); PSPu-4-116 was found to form clear and transparent zones, the sizes of which were 1 to 2 mm in diameter (Figure 1B). According to the plaque morphology, these results indicated that the 2 bacteriophages were different bacteriophages. The titers of bacteriophages PSPu-4-116 and PSPu-95 against their host strain were 10^9 to 10^{11} pfu/mL.

Bacteriophage Morphology

To classify the bacteriophages into morphotype-specific groups, bacteriophage particles were examined by transmission electron microscopy. Bacteriophage PSPu-95 showed a thin tail (length, 103.57 nm) and a round head (diameter, 57.14 nm), a morphology characteristic of members of the family *Siphoviridae* (Figure 2A). Bacteriophage PSPu-4-116 possessed an icosahedral head (diameter, 74.3 nm) and a contractile tail (length, 114.2 nm), typical of the family *Myoviridae* (Figure 2B).

Host Ranges

The host ranges of the 2 bacteriophages were determined using several *Salmonella* spp. strains and *E. coli*

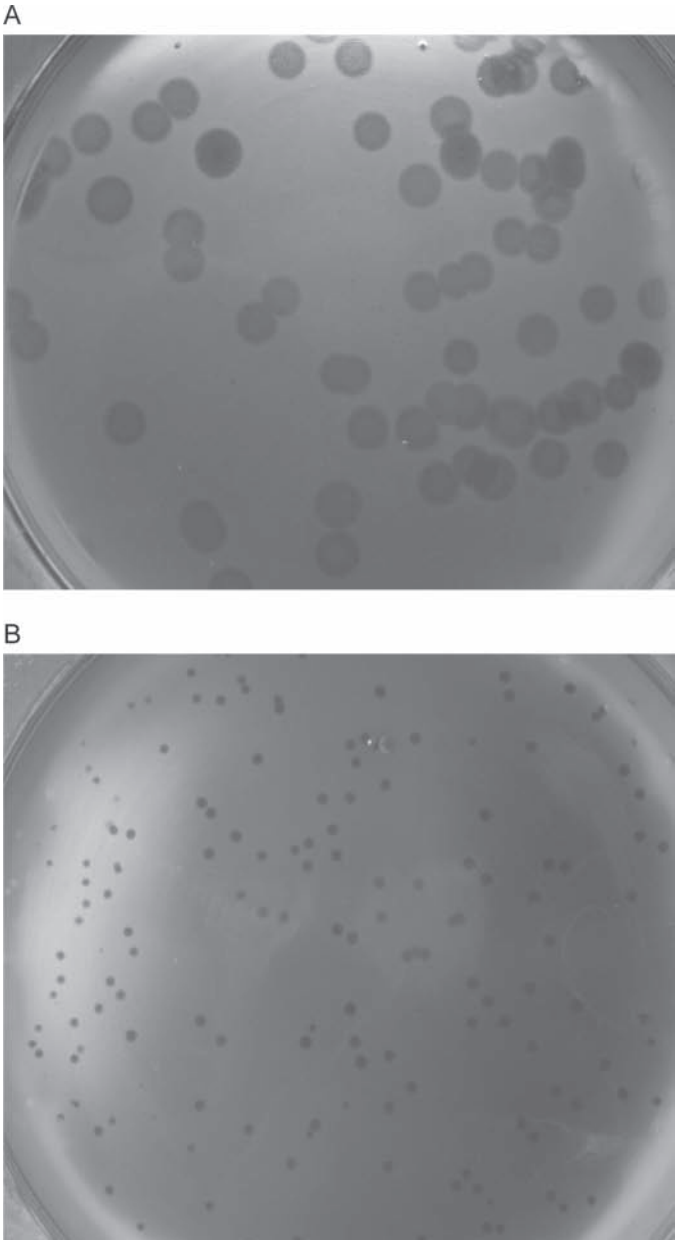

Figure 1. Plaque of bacteriophage: (A) PSPu-95, (B) PSPu-4-116.

(Table 1). The 2 bacteriophages both possessed wide host ranges. The results indicated that PSPu-95 had a lytic effect on 17 of the 20 isolates (85%), whereas PSPu-4-116 produced a lytic effect on 14 isolates (70%) and was the only bacteriophage that produced a clear plaque on enterotoxigenic *E. coli* K88.

Heat and pH Stability

For the experiment on pH stability, the titers of PSPu-95 and PSPu-4-116 were adjusted to 5×10^7 pfu/mL and 2×10^6 pfu/mL, respectively. The activity of bacteriophages PSPu-4-116 and PSPu-95 was relatively stable at pH 6 to 9 and declined dramatically under lower or higher pH conditions (Figure 3). For the experiment on heat stability, the titers of PSPu-95 and PSPu-4-116 were all adjusted to 5×10^9 pfu/mL. The titers of the 2 bacteriophages were all stable at 60°C for 30 min and for 60 min, and there was only a 0.05-log reduction for 30 min and a 0.15-log reduction for 60 min at 70°C. At 80°C, the 2 bacteriophages could not be detected (Figure 4).

One-Step Growth

One-step growth curves for bacteriophages PSPu-95 and PSPu-4-116 showed a latent period of about 20 min. The average burst sizes were about 77.5 and 86 pfu/cell (Figure 5).

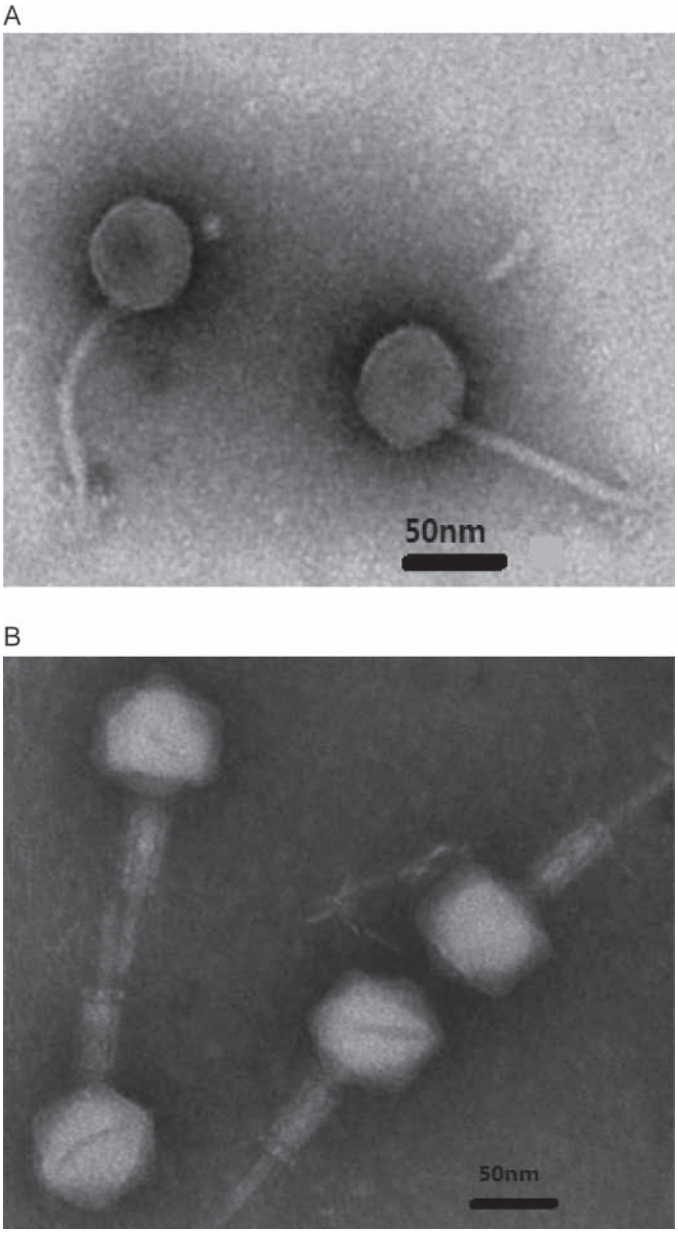

Figure 2. Electron micrograph of bacteriophage PSPu-95 (A) and PSPu-4-116 (B). The bar represents 50 nm.

Table 1. Host ranges of the 2 bacteriophages[1]

Strain[2]	Genus	PSPu-95	PSPu-4-116
SPu-115	*Salmonella* Pullorum	+	+
SPu-905	*Salmonella* Pullorum	+	+
SPu-116	*Salmonella* Pullorum	+	+
SPu-27	*Salmonella* Pullorum	+	+
SPu-95	*Salmonella* Pullorum	+	+
SPu-01	*Salmonella* Pullorum	−	−
SPu-49	*Salmonella* Pullorum	+	−
SPu-109	*Salmonella* Pullorum	+	±
SPu-85	*Salmonella* Pullorum	+	±
SPu-103	*Salmonella* Pullorum	+	±
SPu-13	*Salmonella* Pullorum	+	−
SPu-45	*Salmonella* Pullorum	+	±
SPu-102	*Salmonella* Pullorum	+	−
ATCC 13311	*Salmonella* Typhimurium	+	+
CMCC 2184	*Salmonella*	±	±
ATCC 13076	*Salmonella* Enteritidis	+	−
CMCC 533	*Salmonella* Pullorum	+	±
ATCC 50073	*Salmonella* Paratyphimurium A	+	+
K88	Enterotoxigenic *Escherichia coli*	−	+
K99	Enterotoxigenic *Escherichia coli*	−	−

[1](+) clear spot the diameter of the inoculated area; (±) faint spot within the inoculated area; (−) no spot formation.

[2]ATCC = American Type Culture Collection; CMCC = China Veterinary Culture Collection.

DNA Characterization

The genomic nucleic acids of 2 bacteriophages could be digested with DNase I but not with RNase A, indicating that they consist of DNA (Figure 6A). The restriction patterns of bacteriophage PSPu-95 obtained with enzymes *Eco*RI or *Hin*dIII were absolutely different from PSPu-4-116 (Figure 6B). When summing up the restriction fragment size, the bacteriophage genome was estimated to be 58.3 kbp in length for PSPu-95 and 45.2 kbp in length for PSPu-4-116.

DISCUSSION

In this work, 2 bacteriophages from chicken lagoon effluent were specifically isolated and characterized for *Salmonella* Pullorum. Bacteriophages that have killed *Salmonella* have been isolated from sewage water and poultry litter (Berchieri et al., 1991; Sklar and Joerger, 2001), which demonstrates their natural occurrence in the environment. To our knowledge, this is the first study to isolate *Salmonella* Pullorum bacteriophages from poultry sewage samples, because *Salmonella* Pullorum is a common pathogen on Chinese poultry farms but is rarely found in developed countries.

Host specificity is a common property of bacteriophages; however, it is a limiting factor in therapeutic treatment of bacterial infections (Bielke et al., 2007). In our study, phages PSPu-95 and PSPu-4-116 showed wide host ranges. Some known *Salmonella* bacteriophages also have wide host ranges, for example, bacteriophages SSP5 and SSP6 have broad host ranges of over 65% of the 41 *Salmonella* strains tested (Kocharunchitt et al., 2009). Furthermore, the bacteriophage Felix 01, which has a broad host range, has been validated to produce a 2-log reduction in *Salmonella* Typhimurium DT104 inoculated onto chicken legs (Kostrzynska et al., 2002) and chicken frankfurters (Whichard et al., 2003).

Resistance to heat and pH was investigated to determine the efficacy of these bacteriophages to prevent and treat *Salmonella* Pullorum infection. The 2 bacteriophages both had certain resistance to heat and pH. Although the 2 bacteriophages were relatively stable at pH 6 to 9, we hypothesized that they would have been resistant to pH <2. This would have allowed them to survive during the digestive process and would have made their use to control *Salmonella* Pullorum more efficacious.

The latent period is the time from adsorption to the lysing of the host cell and release of viral progeny, and the burst size is the number of new virus particles that are liberated from a single bacterial cell. Bacteriophages PSPu-95 and PSPu-4-116 not only showed a short latent period of about 20 min, but also had a large burst size (77.5 pfu/cell for PSPu-95 or 86 pfu/cell for PSPu-4-116). Their burst sizes were close to that of bacteriophage SMP (77 pfu/cell; Ma and Lu, 2008) but much smaller than that for *Acinetobacter baumannii* bacteriophage Φ AB2 (200 pfu/cell; Lin et al., 2010). In fact, if the latent period is shorter and the burst size is larger, the bacteriophages will be replicated more quickly and these new virus particles could be released more efficiently.

For the therapeutic treatment, the small genome of the 2 bacteriophages might decrease the risk of transferring long chromosomal DNA fragments carrying virulent genes into the animal body (Merril et al., 2006). The estimated genome sizes of PSPu-95 (58.3 kbp) and PSPu-4-116 (45.2 kbp) were smaller than that of known *Salmonella* bacteriophage Felix 01 (80 kbp; Kuhn et

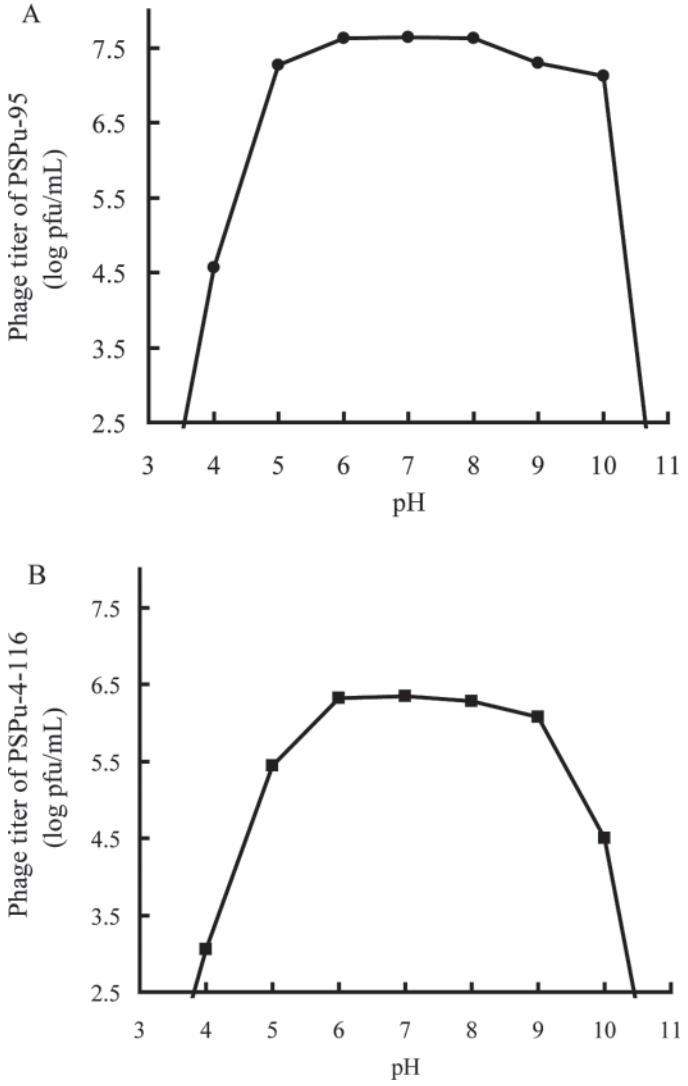

Figure 3. Effect of pH on the stability of PSPu-95 (A) and PSPu-4-116 (B). At pH 3 and pH 11, the titers of the 2 bacteriophages were zero (not shown). All data were repeated in triplicate.

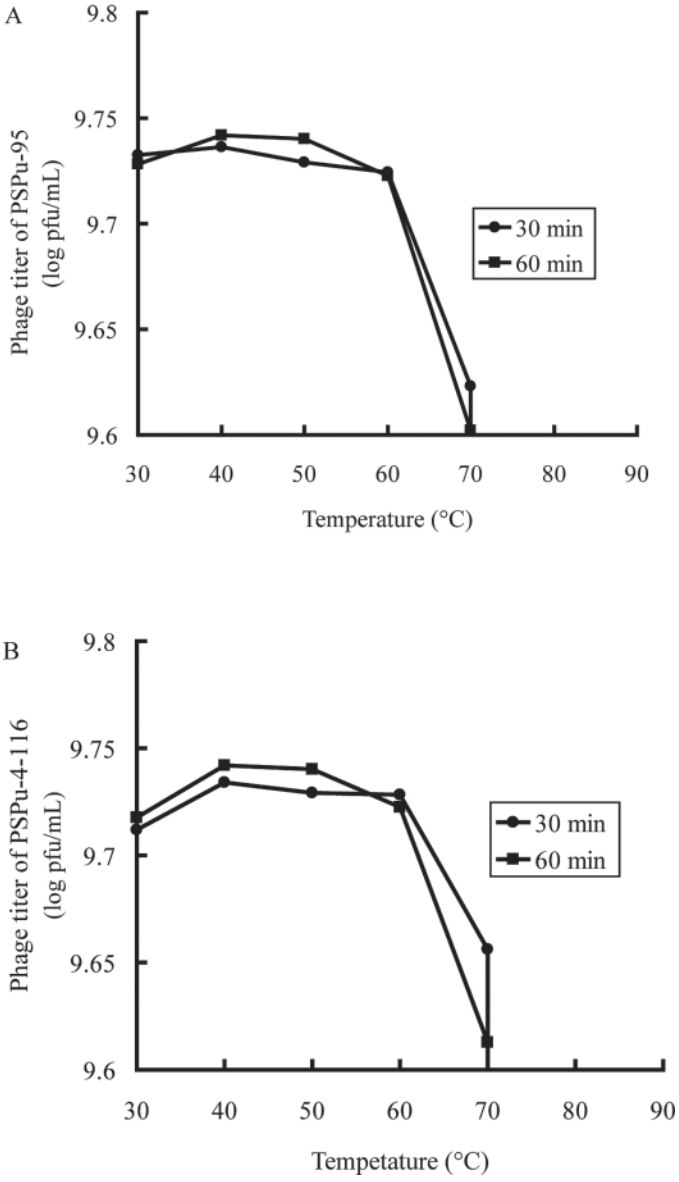

Figure 4. The heat stability of PSPu-95 (A) and PSPu-4-116 (B). At 80 and 90°C, the titers of the 2 bacteriophages were zero (not shown). All data were repeated in triplicate.

al., 2002) and were similar to *Salmonella* bacteriophage P22 (41,724 bp; Vander Byl and Kropinski, 2000).

As early as 1991, the study by Berchieri et al. (1991) showed that a bacteriophage, when inoculated into newly hatched chickens simultaneously with any of the 3 strains of *Salmonella enterica* serovar Typhimurium, produced a considerable reduction in mortality in the birds. Thereafter, the use of bacteriophages of *Salmonella* spp. as therapeutic and prophylactic agents was reported more and more. In the study of Fiorentin et al. (2005), bacteriophages, as a therapeutic agent, reduced the concentration of *Salmonella enterica* serovar Enteritidis bacteriophage type 4 (*Salmonella* Enteritidis PT4) in the ceca of broilers. In 2007, Atterbury and colleagues explored the possibilities of using *Salmonella* bacteriophages to treat commercial broiler chickens challenged with specific *Salmonella* strains. Their results demonstrated that these bacteriophages significantly reduce the cecal colonization of *Salmonella enterica* serotypes Enteritidis and Typhimurium (At-

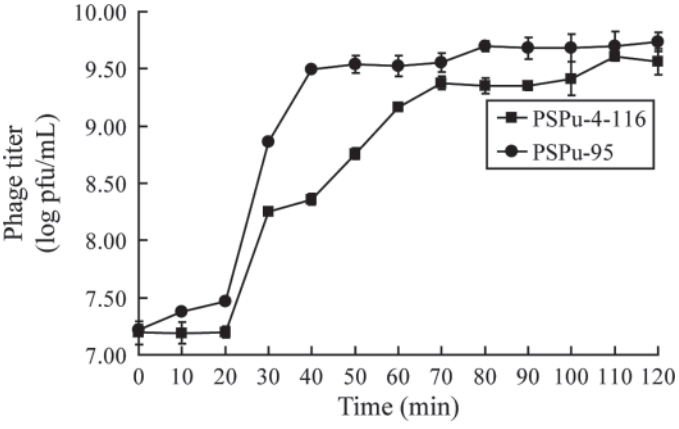

Figure 5. One-step growth curves of bacteriophages PSPu-95 and PSPu-4-116 in their host at 37°C. Values are the mean of 3 determinations.

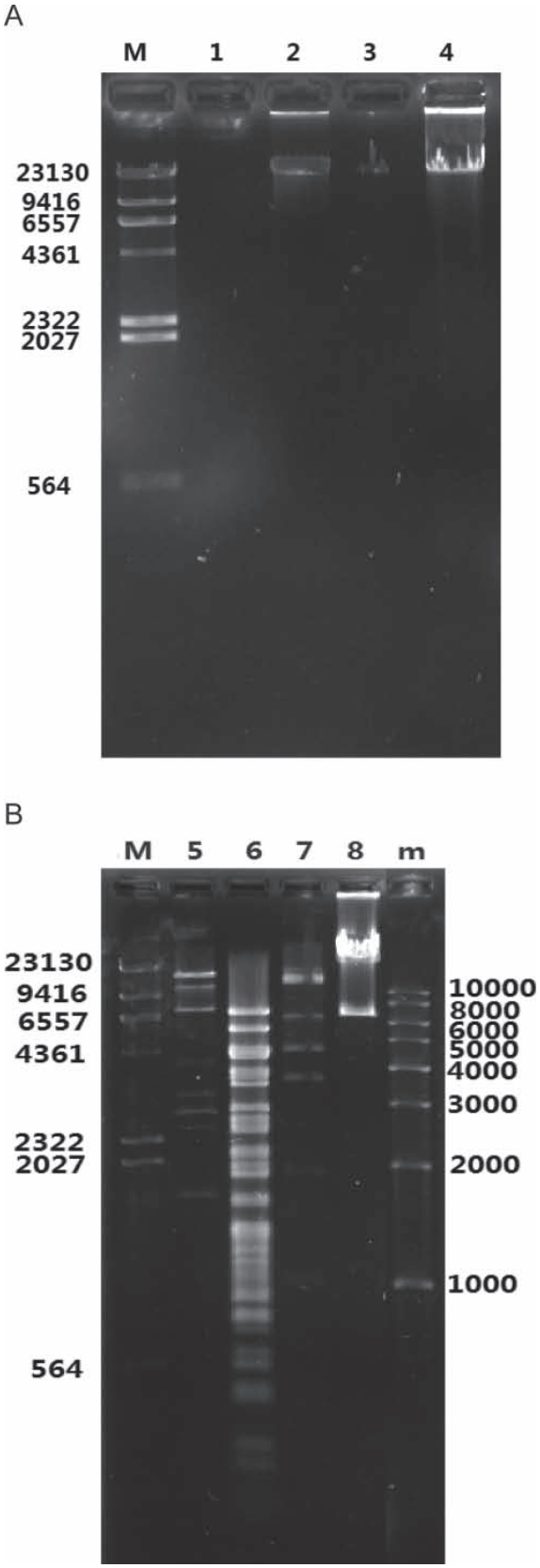

Figure 6. Agarose gel electrophoresis analysis of genomic nucleic acid digested with DNase I and RNase A (A) and restriction analysis of bacteriophages DNA digested with *Eco*RI or *Hin*dIII (B). Lanes contained the following: 1, digested DNA of PSPu-95 with DNase I; 2, undigested DNA of PSPu-95 with RNase A; 3, digested DNA of PSPu-4-116 with DNase I; 4, undigested DNA of PSPu-4-116 with RNase A; 5, *Hin*dIII + PSPu-95; 6, *Hin*dIII + PSPu-4-116; 7, *Eco*RI + PSPu-95; 8, *Eco*RI + PSPu-4-116; M, 23-kb DNA ladder; m, 1-kb DNA ladder.

terbury et al., 2007). Considering these advantages of bacteriophages and the application of *Salmonella* bacteriophages, the bacteriophages isolated in this study may have the potential to be good therapeutic and prophylactic agents for controlling the bacterial infections of chicken caused by *Salmonella* Pullorum.

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