

Detection and Characterization of a Lytic *Pediococcus* Bacteriophage from the Fermenting Cucumber Brine

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Abstract Of the twelve lytic bacteriophages recovered from five different fermenting cucumber tanks that were inoculated with *Pediococcus* sp. LA0281, a lytic phage, ϕ ps05, was characterized in the present study. The plaques were mostly clear and round-shaped on the lawn of starter strain, indicating lytic phage. Overall appearance indicated that it belongs to the *Siphoviridae* family or Bradley's group B1, with a small isometric head and a flexible noncontractile tail with swollen base plate. The average size was found to be 51.2 nm in head diameter and 11.6 nm wide \times 129.6 nm long for the tail. The single-step growth kinetics curve showed that the eclipse and the latent period were 29 min and 34 min, respectively, and an average burst size was calculated to be 12 particles per infective center. The optimum proliferating temperature (35°C) was slightly lower than that of cell growth (35 to 40°C). The structural proteins revealed by SDS-PAGE consisted of one main protein of 33 kDa and three minor proteins of 85, 58, and 52 kDa. The phage genome was a linear double-stranded DNA without cohesive ends. Based on the single and double digestion patterns obtained by EcoRI, HindIII, and Sall, the physical map was constructed. The overall size of the phage genome was estimated to be 24.1 kb. The present report describes the presence of a lytic phage active against a commercial starter culture *Pediococcus* sp. LA0281 in cucumber fermentation, and a preliminary study characterizes the phage on bacterial successions in the process of starter-added cucumber fermentation.

Key words: *Pediococcus* bacteriophage, lytic, detection, characterization, cucumber

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Members of genus *Pediococcus* are nonmotile, Gram-positive lactic acid bacteria (LAB), occurring mostly in pairs or in tetrads, which include species primarily important for meat and vegetable fermentations [18]. They produce lactic acid from glucose *via* the homolactic fermentative pathway, but are unable to ferment lactose [7, 18]. Of the currently known eleven *Pediococcus* members, *P. pentosaceus*, *P. dextrinicus*, *P. acidilactici*, and *P. ethanodurans* are industrially important organisms. *P. pentosaceus* and *P. dextrinicus* are reported to be actively involved in vegetable fermentations. *P. acidilactici* is commonly used in the manufacture of fermented sausage [33], and *P. halophilus* (presently *Tetragenococcus halophilus*) is responsible for soy sauce fermentation under relatively high salt concentrations [35]. In recent years, there have been several reports concerning bacteriocin productions from *Pediococci* and their characterizations [20–21, 29, 33]. Although this group of organisms has been considered to be one of the desirable lactic and flora most frequently associated with cucumber fermentation [3, 9], they have not been extensively studied in terms of ecological roles that affect the final qualities of several fermented products. Ever since a procedure of controlled fermentation was introduced in the pickle industry in 1973, some *Pediococcus* strains including *P. pentosaceus* have been commercially available for production of cucumber pickles in the United States [12, 14]. Needless to say, more fundamental knowledge on bacteriophage susceptibility is required for the technology to be exploited industrially in cucumber fermentations. Even though a number of LAB infecting bacteriophages have been isolated from various foods [19], neither *Pediococcus* phages from the vegetable origins nor their impacts on vegetable fermentation have been investigated.

Recently, while studying the fate of an added-starter in cucumber fermentation, we found surprisingly that some lytic bacteriophages, which were able to attack and lyse the inoculated *Pediococcus* starter culture, occurred from the brines within 48 h after inoculation. In order to establish a controlled fermentation technology, it is imperative to evaluate the impacts of these bacteriophages that infect the inoculated starters. Such phage prevalence encouraged us to explore a possible role of bacteriophage in cucumber fermentation. This report describes the isolation and characterization of a lytic *Pediococcus* phage specifically active against strain LA0281, which is a salt- and acid-tolerant starter currently used in the industry.

MATERIALS AND METHODS

Bacteria, Bacteriophage, and Culture Conditions

Eleven strains of LAB were obtained from the USDA/ARS culture stock, Department of Food Science, North Carolina State University: Four *P. pentosaceus*, three *P. acidilactici*, two *Leuconostoc mesenteroides*, two *Lactobacillus plantarum*, one *Lactococcus lactis*, and one strain of *L. brevis*. All the strains except for *L. brevis* were grown aerobically at 30°C in MRS broth medium. *L. brevis* was cultivated in MRS broth containing 0.05% cysteine-HCl or on MRS agar in an anaerobic jar (BBL Gaspak, Cockeysville, MD, U.S.A.). M17 broth [34] was used for cultivation of *Lactococcus lactis* LA0119. Cells were stored in the appropriate frozen medium containing 16% glycerol. Prior to test for phage sensitivity, they were subcultured twice in the relevant broths at 30°C. Soft agar plate (MRS + 0.7% agar) for plaque assays contained 10 mM CaCl₂. All the broth media were supplemented with 10 mM CaCl₂·6H₂O (MRS-Ca²⁺), unless otherwise specified. The phages obtained were stored at 4°C within 6 months in MRS broth containing 0.1% (v/v) chloroform. For a long-term storage, phage suspensions in MRS broth containing 16% (w/v) glycerol were frozen at -80°C.

Determination of Phage Titer

Plaque forming units (PFU/ml) were determined according to the method described by Foschino *et al.* [16]. After suitable dilutions of phage-containing samples, 0.1-ml aliquots were added to 0.1 ml of logarithmic culture (OD₆₆₀ = 0.2) of LA0281. The suspension was incubated for 15 min at 37°C, mixed with 3 ml of soft agar at 50°C and poured onto the surface of a MRS agar plate. The plate was incubated at 37°C, and the plaques formed after overnight incubation were counted.

Phage Detection

For phage detection, 50 ml of five brine samples were collected from each of the five cucumber fermenting tanks

and were kept in a refrigerator. After being transferred to the laboratory every other day under refrigerated condition, bacteriophages were enriched by addition starter culture as described above, by a modification of the method described by Reddy [28]. After a brief centrifugation, 1 ml of a mid-log-phase bacterial culture (OD₆₆₀ = 0.2–0.3) was added to each 5 ml sample and mixed thoroughly, followed by incubating at 37°C overnight. A plaque assay was carried out by the agar overlay method [2], in which 0.1 ml of phage-enriched sample was mixed with 3 ml of soft agar (0.7%, prewarmed to 50°C), 0.1 ml of 100 mM CaCl₂, and 0.1 ml of freshly grown cells. Alternatively, phage activity was determined by the spot-on-lawn assay [2]. If necessary, cycloheximide (50 µg/ml, Sigma) was supplemented into soft agar in order to suppress yeast growth prior to top agar plating. All the bacteriophages isolated were purified by replaques on MRS agar.

Single-Step Growth Kinetics

Experiments were conducted according to the modification of the method of Ellis and Delbrück [11]. Ten ml of MRS broth was inoculated with 0.3 ml of host overnight culture and incubated for three to five hours at 37°C to reach the exponential phase (A₆₆₀ = 0.2). The cells were then centrifuged for five minutes at 3,000 ×g, resuspended in 10 ml of fresh warm MRS, and then infected with the bacteriophage at a multiplicity of infection (MOI) between 0.05 and 0.1. After ten minutes of incubation, the adsorption was stopped by diluting the phage-host suspension ten times with MRS broth (pH 6.5) and the cells were centrifuged for 10 min at 8,500 ×g. The supernatants were carefully decanted, and the infected pellet was thoroughly resuspended in 10 ml of fresh and warm MRS broth (30°C, pH 6.5), followed by incubation in a water bath at 37°C. Aliquots were removed periodically and assayed for phages after suitable dilution. For intracellular phage count [10] during the latent period of 30 min, the phage-host suspension was treated with 0.01 M KCN for artificial cell lysis, and incubated at 37°C for at least 20 min.

Preparation of High-Titer Phage Lysate

The overlay plates of each phage were prepared to give confluent lysis of the indicator strain. The plates were then flooded with 5.0 ml of MRS broth (pH 5.5) and left to stand for 10 min at room temperature. A sterile bent glass rod was used to scrape the top agar layer into a slurry for easy collection into a sterile tube, followed by centrifugation (5,000 ×g for 10 min) at 15°C. One ml of this supernatant was added to 100 ml of an early-log-phase culture of LA0281 in MRS broth, followed by incubating the mixture for 6 h at 37°C. After centrifugation for 15 min at 10,000 ×g, high-titer phage lysates (*ca.* 10⁹ PFU/ml) was obtained by filtration through a 0.45-µm membrane (Gelman Science, Ann Arbor, MI, U.S.A.).

Large-Scale Phage Purification and Preparation of Phage DNA

For large-scale preparation of phage, a 1-l culture of the host strain was infected with a high-titer lysate of phage ϕ ps05 at an optical density of 0.1–0.2 at 660 nm. In this case, the MOI was adjusted to about 0.1 and followed by incubation at 30°C. About 3 h after challenging, 3 ml of chloroform and final concentration of 0.5 M NaCl were added simultaneously into the cultured broth, and the culture left on ice for 1 h. Cell debris was removed by centrifugation (Sorvall, Newtown, CT, U.S.A.) at 10,000 $\times g$ for 15 min and filtered through a 0.45- μ m membrane (Nalgene, Rochester, NY, U.S.A.). Then, 1 M NaCl and 10% (w/v) PEG 8,000 were added into the supernatant as described by Yamamoto *et al.* [37], and they were kept overnight at 4°C. After carefully pouring off the supernatant after centrifugation, the precipitate was dissolved in 100 mM Tris-HCl buffer (pH 8.0) containing 10 mM MgCl₂ and dialyzed against the same buffer for at least 6 h. Phage genomic DNA was extracted at this stage by using the phenol/chloroform/ethanol precipitation protocol described by Santos *et al.* [31]. For preparation of EM specimen, this preparation was further purified by CsCl density-gradient ($d = 1.70, 1.50, 1.40$) ultracentrifugation (Sorvall M150 GX, Newtown, CT, U.S.A.) at 600,000 $\times g$ (S100 AT6 rotor) for 6 h at 15°C. After centrifugation, the faint bluish band was carefully recovered according to standard protocol [30].

Electron Microscopy [23]

The phages obtained by the above purification methods were fixed in 0.1 M glutaraldehyde (Sigma) in sodium cacodylate buffer, pH 7.2, post-fixed in 1% osmium tetroxide (Sigma) in the same buffer, and then dehydrated in a graded series of ethanol to 100%. Samples were negatively stained with 4% uranyl acetate (pH 4.0) followed by Reynold's lead citrate, and viewed by an electron microscope (EM; Jeol 100S, Japan) at 80 kV. Electron micrographs were taken at 85,000 \times magnification. The phage size was determined from the average of five to seven independent measurements.

Host Range Determination

Four strains of *P. pentosaceus*, three strains of *P. acidilactici*, two strains of *L. mesenteroides*, two strains of *L. plantarum*, one *L. lactis* strain, and one *L. brevis* strain were used as indicators for the determination of phage sensitivity by an agar spotting method [27]. Ten high-titer bacteriophage samples (10 μ l) were placed on each lawn of indicator cells and left to stand for 30 min. Plaque formation was examined for lysis after 24 h of incubation at 30°C and 37°C. Based on plaque formation, results were recorded as turbid (+/-), clear (+), or no plaque (-).

Effect of Incubation Temperature

To determine the incubation temperature on phage proliferation, growth media were maintained at four different

temperatures: 25, 30, 35, and 40°C. After challenging with a diluted phage preparation ($2\text{--}3 \times 10^2$ PFU/ml) against 10 ml of MRS broth inoculated with overnight LA0281 culture at the level of 0.1% (v/v), changes of cell growth and phage titer were examined during 6 h of incubation. The medium pH was determined by using an IQ200 pH meter (IQ Scientific, San Diego, CA, U.S.A.), and cell growth was measured at 660 nm with a spectrophotometer (Model Novaspec II, Pharmacia LKB, Sweden). For the samples diluted appropriately with 0.85% (w/v) physiological saline, phage titers were assayed as described above.

SDS-PAGE of Structural Proteins

Phage proteins obtained by ultracentrifugation was subjected to SDS-PAGE according to the recommendation of the NuPAGE electrophoresis system (Novex, San Diego, CA, U.S.A.). Proteins were boiled in loading buffer (Novex NP0007, NuPAGE LES, pH 8.5) and separated on a 4–12% Bis-Tris polyacrylamide gel at 200 V with a constant current of 125 mA for 35 min and stained with staining solution (0.1% Coomassie TM R-250 in 40% ethanol, 10% acetic acid). The two molecular mass standards contained myosin (188 kDa), phosphorylase B (98 kDa), bovine serum albumin (62 kDa), glutamic dehydrogenase (49 kDa), alcohol dehydrogenase (38 kDa), carbonic anhydrase (28 kDa), myoglobin (23 kDa), lysozyme (16 kDa), aprotinin (7 kDa), and insulin (3 kDa). The size of the proteins was calculated by the proper calibration line.

Restriction Endonuclease Digestion

Phage DNA was digested with restriction enzymes: BamHI, EcoRI, HindIII, and SalI (Promega, Madison, WI, U.S.A.) according to the supplier's instructions. DNA fragments were separated on 0.8% agarose (Fischer, Fair Lawn, NJ, U.S.A.) gels in 1 \times TAE buffer (0.04 M Tris acetate, 0.02 M EDTA, pH, 8.0). HindIII/ λ DNA digests and a 1 kb DNA ladder (Promega, Madison, WI, U.S.A.) were used as molecular weight standards.

RESULTS

Isolation and Plaque Morphology

Phages were isolated on a lawn of *Pediococcus* sp. LA0281 on MRS agar by spotting brine samples and overlaying soft agar, and many clear plaques were formed on the lawn of LA0281, indicating that these phages are lytic as an intrinsic attribute. Twelve lytic bacteriophages were recovered at different time points from the five cucumber fermenting tanks, all of which were inoculated with *Pediococcus* sp. LA0281 at the time of brining. Cleared lysate was made against *Pediococcus* sp. LA0281 by challenging with this phage. Each phage was purified, concentrated, and taken for EM imaging. Based on their

Table 1. Host spectrum of the *Pediococcus* bacteriophage ϕ ps05.

Indicators	Source	Lysis	Remarks
<i>Pediococcus</i> sp. LA 0281	This study	+	Pickle starter, Aferm ®772
<i>Pediococcus pentosaceus</i> LA 0073	USDA/FFL	-	Gonzales <i>et al.</i> AEM46:81,1983
<i>Pediococcus pentosaceus</i> LA 0076	USDA/FFL	-	ATCC 33316
<i>Pediococcus pentosaceus</i> LA 0061	USDA/FFL	-	ATCC 43201, plasmid bac+
<i>Pediococcus acidilactici</i> LA 0054	USDA/FFL	-	PC 135, NCSU
<i>Pediococcus acidilactici</i> LA 0056	USDA/FFL	-	PC SP4
<i>Pediococcus acidilactici</i> LA 0074	USDA/FFL	-	ATCC 33314
<i>Lactobacillus plantarum</i> LA 0280	KCCM	-	KCCM 11322
<i>Lactobacillus plantarum</i> LA 0070	USDA/FFL	-	ATCC 14917, type strain
<i>Leuconostoc mesenteroides</i> LA 0010	USDA/FFL	-	FFL C-33
<i>Leuc. mesenteroides</i> ssp. <i>dextranicum</i> 4G12	USDA/FFL	-	FFL, sauerkraut isolate after 10-day fermentation
<i>Lactobacillus brevis</i> LA 0036	USDA/FFL	-	ATCC 14839
<i>Lactococcus lactis</i> LA 0119	USDA/FFL	-	ATCC 11454, Nisin producer

ATCC, American Type Culture Collection; USDA/FFL, Food Fermentation Laboratory, Department of Food Science, North Carolina State University, KCCM, Korean Culture Collection of Microorganisms; AEM, Applied and Environmental Microbiology, American Society for Microbiology.

morphological similarity, all phages, ϕ ps01 to ϕ ps012, were assigned to three groups (data not shown). Since ϕ ps05 occurred more than the others, ϕ ps05 was subjected to further characterization in this study. When each phage lysate was spotted on the lawn of LA0281, many clear plaques were produced, indicating that these phages are lytic. The diameter of the phage ϕ ps05 plaque, once visible on agar surface, did not increase with incubation time, showing an average 1.55-mm diameter (data not shown) after 24 h incubation. These plaques were mostly clear and round-shaped.

Host Range Determination

The phage ϕ ps05 was examined for the ability to infect other LAB. Thus, a phage suspension (10^8 PFU/ml) was

spotted on lawns of test LAB strains on MRS soft agar. As shown in Table 1, none of the strains other than the original host LA0281 was lysed by this phage at 30 or 37°C, indicating that the phage could not lyse other *Pediococcus* strains or related species of LAB.

Latent Period and Burst Size

Based on the single-step growth kinetics curve against LA0281, the eclipse and the latent period were determined to be 29 min and 34 min, respectively, and an average burst

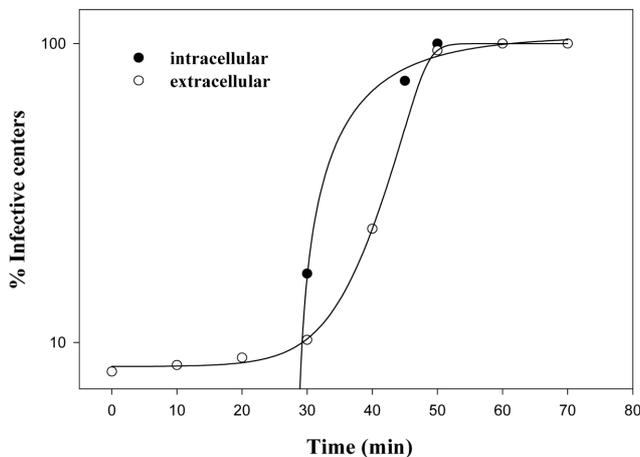


Fig. 1. Single-step growth kinetics of *Pediococcus* phage ϕ ps05 in MRS broth at 37°C. Intracellular phage count was determined after artificial lysis by treating with 0.01 M KCN.

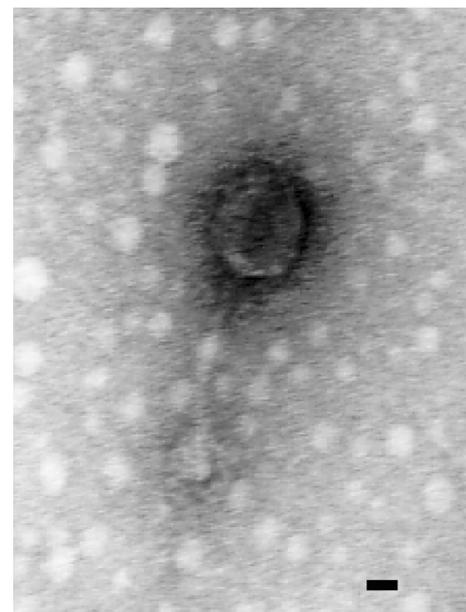


Fig. 2. Electron micrograph of *Pediococcus* phage ϕ ps05. CsCl-purified phage preparation from a lysed culture was negatively stained with 2% uranyl acetate, pH 4.0. Magnification, 85,000 \times (bar = 100 nm).

size was calculated to be 12 particles per infective center (Fig. 1). These results indicate that intracellular development started shortly after infection. The burst size was found to be smaller than that of *P. halophilus* phages, which typically have a burst size of 27 to 28 particles per cell [35].

Morphology

EM pictures showed that ϕ ps05 belongs to the *Siphoviridae* family [1, 24] or Bradley’s group B1 [5], because of a small isometric head and a noncontractile tail (Fig. 2). The average phage dimensions were 51.2 nm for head diameter, and 11.6 nm wide \times 129.6 nm for tail length. No collar or tail sheath was observed on the tail, and a swollen structure appeared at the end of the tail, presumably indicating a rosette-like base plate [1]. No tail fibers were visible under EM examination.

Effect of Incubation Temperature

To determine the effect of incubation temperature on phage proliferation, the phage was propagated on LA0281 at four different temperatures: 25, 30, 35, and 40°C. The results are shown in Fig. 3. Both phage titers and cell growth were shown to gradually increase at each incubation temperature. Medium pHs for all the test samples decreased to below pH 5.5 in 6 h. It was noted that the optimum temperatures were 35°C, and 35 to 40°C, for phage proliferation and growth of host cell, respectively. These data also suggest that phage proliferation favored slightly lower temperature than that of optimal cellular growth, which is consistent with a previous paper [36]. The data obtained at 25°C were almost the same as those at 30°C (data not shown).

Structural Protein Compositions

The structural proteins were analyzed by SDS-PAGE (Fig. 4), showing one main protein with calculated

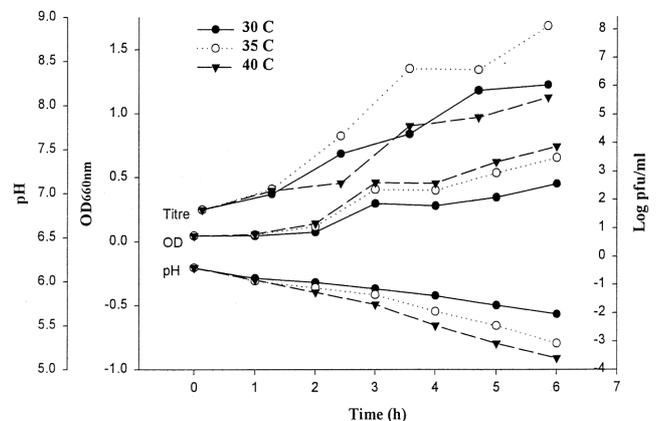


Fig. 3. Effect of temperature on growth and phage proliferation of *Pediococcus* sp. LA0281 in MRS-CaCl₂ (10 mM) broth. Bacterial growth was determined by measuring optical density at 660 nm.

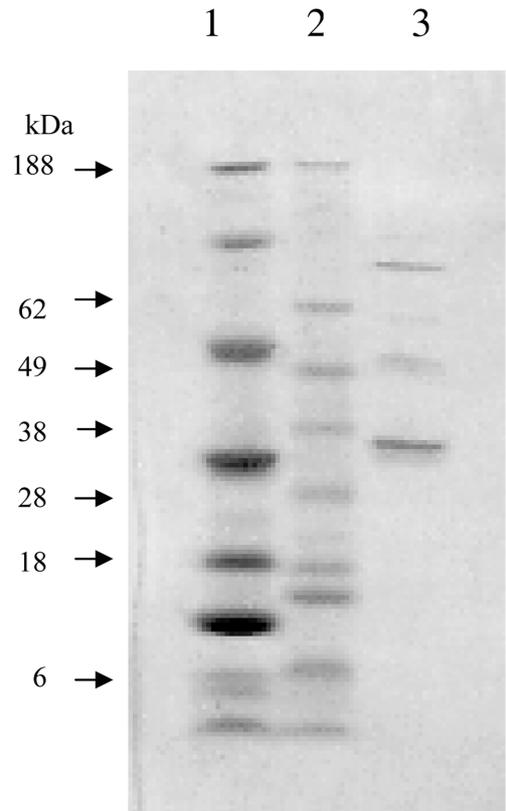


Fig. 4. SDS-PAGE of structural proteins of *Pediococcus* bacteriophage ϕ ps05 (lane 3) and two molecular mass standards. Marker 1 (lane 1): myosin (188 kDa), phosphorylase B (98 kDa), glutamic dehydrogenase (52 kDa), carbonic anhydrase (31 kDa), myoglobin (15 kDa), lysozyme (11 kDa), aprotinin (6 kDa); lane 2 (the marker 2): myosin (188 kDa), bovine serum albumin (BSA, 62 kDa), glutamic dehydrogenase (49 kDa), alcohol dehydrogenase (38 kDa), carbonic anhydrase (28 kDa), myoglobin (18 kDa), lysozyme (14 kDa), aprotinin (7 kDa), insulin (3 kDa).

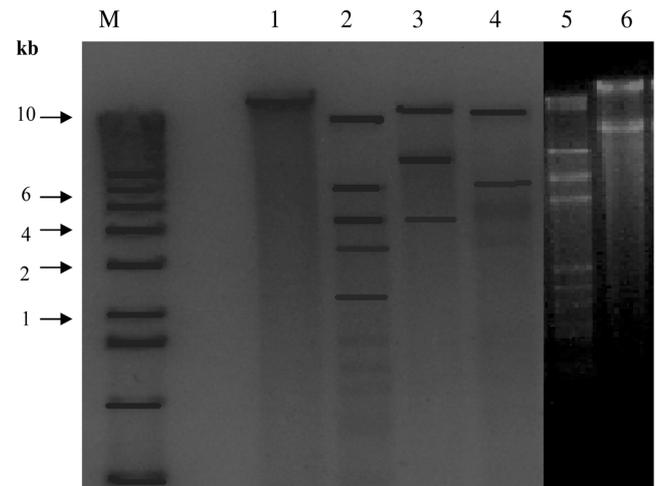


Fig. 5. Restriction patterns of the DNA of *Pediococcus* bacteriophage ϕ ps05 in a 0.8% agarose gel. Lanes: M, kb DNA step ladder (Promega); 1, Bam H1; 2, HindIII; 3, Sall; 4, EcoR1; 5, HindIII+Sall; 6, Bam H1+Sall.

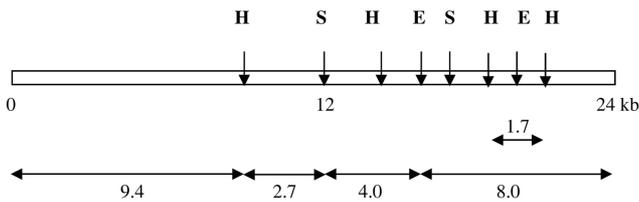


Fig. 6. Restriction endonuclease map of the DNA of *Pediococcus* phage ϕ ps05. B, E, H, and S refer to BamHI, EcoRI, HindIII, and Sall, respectively.

molecular masses of 33 kDa and three minor proteins of 85, 58, and 52 kDa.

Phage Genome

The DNA obtained from purified phage particles was found to be linear and double-stranded. Isolated DNA was digested with several restriction endonucleases and analyzed on 0.8% agarose gels (Fig. 5). BamHI has no cutting site and HindIII has three on the phage DNA. Because no subfragments [15] were detected after treating the restriction digestion of DNA at 70°C for 10 min, the phage genome was considered to be linear and double-stranded without cohesive ends. Based on the single and double digestion patterns using EcoRI, HindIII, and Sall, the physical map was constructed, and is shown in Fig. 6. The size of the phage DNA was estimated to be 24.1 kb.

DISCUSSION

Fleming *et al.* [13] observed in 1988 that the added starter culture, *L. plantarum* WSO, grew actively immediately after brining, but its populations dropped rapidly from 3 days of fermentation: It is possible that bacteriophages active against the inoculated WSO culture may have been responsible for the rapid reduction of viable counts of WSO strain during fermentation. However, the possibility of bacteriophage affecting starter culture performance for cucumber fermentation has not previously been investigated. We were very much intrigued by the rapid drop of the added starter, *L. plantarum* WSO, during the fermentation described above, and thus searched for bacteriophages in some fermented vegetables such as sauerkraut, kimchi, and cucumber pickle [38]. Surprisingly, we detected *Pediococcus*-specific phages more frequently in the brine samples withdrawn from the commercially fermenting cucumber tanks inoculated with a *Pediococcus* starter culture than in the uninoculated ones. Therefore, we believe that these phages play a critical role in the control of complex microflora in cucumber fermentation, as indicated by the fluctuations of phage titers in a cheese factory [6]. Fermentation by the selected culture may be interrupted, delayed, or completely inhibited by phage. In addition to

destruction of the starter culture, phages may allow less-desirable LAB to ferment for the rest of the fermentation period, eventually leading to quality deterioration. We first suspected the commercial starter culture for phage presence. Thus, an experiment was conducted according to the method of Gautier *et al.* [17] to determine whether it was contaminated with phage or not. However, phage particles against *Pediococcus* sp. LA0281 were not recovered in the commercial starter culture. On the other hand, a number of plaques were formed on the lawn of *Pediococcus* sp. LA0281 by the fermenting brines where the strain was inoculated.

In cucumber fermentation, the brine salt concentration (preferably 5–8%) is a critical factor for obtaining a normal fermentation and to maintain structural integrity of the product [12, 14]. Because of increasing environmental concerns, the industry is faced with an immediate challenge to reduce the salt content in the brines; the salt concentration in the wastewater discharged is under strict regulation in the United States [13, 14]. In this context, starter-added technology with low salt concentration is considered as a feasible alternative in various vegetable fermentation industries. Unfortunately, one of the disadvantages of starter-added technology is the presence of phage active against the starter culture, and the potential for significant economic losses, as is often experienced in the dairy industry. Comprehensive measures on bacteriophages in vegetable fermentations may lead to success with introduction of starter-added technology [19, 22]. To the best of our knowledge, there are only two previous reports regarding bacteriophages active against the genus *Pediococcus*. *P. halophilus* phage was isolated from fermenting soy sauce [35]. More recently, three temperate bacteriophages of *P. acidilactici* have been isolated [8]. The present work is the first study on a lytic *Pediococcus* phage from fermenting cucumber brines. In order to obtain more detailed information on a prevalent phage type from the brine samples, ϕ ps05 was chosen for characterization. As described above, the plaques were relatively small, mainly due to a small burst on the indicator *Pediococcus* sp. LA0281. UV irradiation experiment on *Pediococcus* sp. LA0281 was carried out to induce temperate bacteriophage against the plasmid cured *Pediococcus* strains, but we failed to recover any phage particles therefrom.

On the other hand, morphological characteristics shown by an electron microscopy have been the most common criteria in phage taxonomy [25]. Mostly, the bacteriophages active against lactobacilli, either isolated as virulent from the different environments or induced from the lysogenic cultures, belong to the *Siphoviridae* or Ackermann's group B1 [32]. Uchida and Kanabe [35] reported two lytic *P. halophilus* phages, ϕ 7116 and ϕ D-86, from fermenting soy sauce. The phage ϕ 7116 was 87–96 nm in head diameter and tail with a contractile sheath of 200 nm in length, and

belongs to Ackermann's group A1 [1]. Phage ϕ D-86 was found to be the morphotype B1, having a 67-nm isometric head and 300-nm noncontractile tail. Latent period and burst sizes of these two phages were 5 h and 27–28 particles per cell, respectively. More recently, three temperate bacteriophages [8], pa97, pa40, and pa42, were induced from *P. acidilactici* using mitomycin C. Electron microscopy showed that these phages have small isometric heads with noncontractile heads in common. Similarly, the ϕ ps05 possessed isometric heads with dimensions of about 51.2 nm and their base plates were seemingly rosette-like without tail fibers. In this respect, ϕ ps05 seems to have a morphology similar to the temperate phages above.

Developing phages in the process of vegetable fermentations are exposed to a brine solution of salts for an extended period of time, which means that salts can affect the phage stability during storage. When ϕ ps05 was treated with deionized water and some salt solutions, as shown in Table 2, the phage titers significantly decreased. Three buffer solutions also resulted in varying degrees of drop of phage titers, compared with deionized water. However, it was noted that addition of divalent cations, Mg^{2+} and Ca^{2+} , in Tris buffer (pH 8.0) maintained the survival rate over 80%, strongly suggesting the protection of phage particle from inactivation. Specifically, Mg^{2+} exerted better protection than Ca^{2+} did.

For structural proteins, Santos *et al.* [31] analyzed the 17 *Leuconostoc oenos* phages by SDS-PAGE and found an overall similarity: a major protein of 33–34.5 kDa was present in all the phages and about half had a second major protein of 25.5 kDa. Neve *et al.* [26] reported two major structural proteins with molecular weights of 30.9 and about 24 kDa for *Streptococcus salivarius* subsp. *thermophilus* phages. Of the above described three temperate *P. acidilactici* phages [8], pa97 and pa40 possess one major protein with an estimated mass of 34–37 kDa, and pa42 contains the second major protein of approximately 47 kDa. The structural

protein of our isolate, ϕ ps05, had a major protein similar in size to those of the temperate *Pediococcus* phages, pa97 and pa40.

It is generally believed that two types of phage genomes, *cos*-type (cohesive ends) and *pac*-type (packaging sites), exist for organization of phage genome [4]. For *cos*-type phage, the phage packaging sequence starts at the *cos* site and continues to the next *cos* site, where the DNA is nicked by a specific phage gene-encoded nuclease called terminase. The presence of cohesive ends (*cos*-type) could be demonstrated by heat treatment at 70°C: Immediately after heat treatment, DNA digests with restriction enzymes are loaded on agarose gel. If a DNA fragment dissociates into two subfragments, then a cohesive end circularly redundant and terminally permuted is strongly indicated [4, 15]. For *pac*-type phages, packaging of DNA starts at the *pac* site and continues until the empty phage capsid is filled (headful mechanism) [15]. In the present study, an approximate overall size of 24.1 kb was calculated based on DNA fragments of the phage genome with a few restriction enzymes. When ϕ ps05 and three *Pediococcus* temperate phages [8] are compared by genomic size, ϕ ps05 is much smaller than those of *Pediococcus*, whose genomes have been reported to be linear without cohesive ends and 33–37 kb in size. The genomic size of *Lactobacillus* phages has been reported to range from 30 to 40 kb, and is 20 to 40 kb for *Lactococcus* phages [19]. For some unknown reason in this study, half of the ϕ ps05 genome could not be readily digested with the restriction enzymes used.

As shown in Fig. 1, judging from the small burst size, which is not typical for lysogenic phages but for the lytic ones, it is presumed that ϕ ps05 most likely evolved from the temperate phages derived from *Pediococcus* starter culture. Further experiments are needed to determine whether ϕ ps05 is lysogenic or not. This will be the subject of future research.

Table 2. Effect of salts on the stability of *Pediococcus* phage ϕ ps05 under refrigerated storage.

Salt solutions	Phage counts (PFU/ml)	Survival, %
Control (0 h incubation) ^a	520,400×10 ⁷	100
dH ₂ O	224,340×10 ⁷	61.3
0.85% saline (pH 6.8)	350,280×10 ⁷	68.4
0.85% saline+MgSO ₄ (10 mM)	400,400×10 ⁷	86.9
0.85% saline+CaCl ₂ (10 mM)	525,363×10 ⁷	78.9
0.1 M phosphate buffer (pH 8.0)	174,150×10 ⁷	35.2
0.1 M phosphate buffer (pH 8.0)+MgSO ₄ (10 mM)	526,210×10 ⁷	80.0
0.1 M phosphate buffer (pH 8.0)+CaCl ₂ (10 mM)	289,160×10 ⁷	48.9
0.1 M Tris buffer (pH 8.0) ^b	390,212×10 ⁷	65.4
0.1 M Tris buffer (pH 8.0)+MgSO ₄ (10 mM)	530,280×10 ⁷	88.0
0.1 M Tris buffer (pH 8.0)+CaCl ₂ (10 mM)	285,220×10 ⁷	55.0

^aInitial titer was adjusted to 520,400 PFU/ml×10⁷ for each salt solution.

^b0.1 M Tris(hydroxymethyl)aminomethane was dissolved in deionized water.

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