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International Journal of Food Microbiology 84 (2003) 225–235
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Isolation and characterization of a *Lactobacillus plantarum* bacteriophage, ΦJL-1, from a cucumber fermentation ☆

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Accepted 14 February 2003

Abstract

A virulent *Lactobacillus plantarum* bacteriophage, ΦJL-1, was isolated from a commercial cucumber fermentation. The phage was specific for two related strains of *L. plantarum*, BI7 and its mutant (deficient in malolactate fermenting ability) MU45, which have been evaluated as starter cultures for controlled cucumber fermentation and as biocontrol microorganisms for minimally processed vegetable products. The phage genome of ΦJL-1 was sequenced to reveal a linear, double-stranded DNA (36.7 kbp). Sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE) profiles indicated that ΦJL-1 contains six structural proteins (28, 34, 45, 50, 61, and 76 kDa). Electron microscopy revealed that the phage has an isometric head (59 nm in diameter), a long non-contractile tail (182 nm in length and 11 nm in width), and a complex base plate. The phage belongs to the Bradley group B1 or Siphoviridae family. One-step growth kinetics of the phage showed that the latent period was 35 min, the rise period was 40 min, and the average burst size was 22 phage particles/infected cell. Phage particles (90%) adsorbed to the host cells 20 min after infection. Calcium supplementation (up to 30 mM $CaCl_2$) in MRS media did not affect the first cycle of phage adsorption, but promoted rapid phage propagation and cell lysis in the infection cycle subsequent to adsorption. The *D* values of ΦJL-1 at pH 6.5 were estimated to be 2.7 min at 70 °C and 0.2 min at 80 °C by a thermal inactivation experiment. Knowledge of the properties of *L. plantarum* bacteriophage ΦJL-1 may be important for the development of controlled vegetable fermentations.

Keywords: Bacteriophage; *Lactobacillus plantarum*; Lactic acid bacteria; Vegetable fermentation; Cucumber fermentation

1. Introduction

Lactobacillus plantarum completes the final stage of natural fruit and vegetable fermentations due to its higher acid tolerance than other lactic acid bacteria (LAB; Fleming, 1982; Pederson and Albury, 1969). The growth and fermentative activity of *L. plantarum* in cucumber and cabbage fermentations greatly affects the quality and microbial stability of the final product. Many physical, chemical, and biological factors,

☆ Paper no. FSR01-38 of the Journal Series of the Department of Food Science, NC State University, Raleigh, NC 27695-7624. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or North Carolina Agricultural Research Service, nor does it imply approval to the exclusion of other products that may be suitable.

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0168-1605/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0168-1605(03)00111-9

including bacteriophage (phage), influence the fermentative behavior of *L. plantarum*. While most commercial cucumber fermentations rely on epiphytic LAB (Fleming, 1984), the use of starter cultures has been investigated (Etchells et al., 1973). Current commercial cucumber fermentation and storage procedures may use 10–15% NaCl. Although brine recycling is a common practice, waste chloride production remains a problem in this industry. Significant reductions in salt concentration (to 4% or less) may be possible with fermentation technology under development, using blanched cucumbers to reduce the initial microflora present on the cucumbers (H.P. Fleming, unpublished). With these fermentations, a *L. plantarum* starter culture may be required to ensure quality. Therefore, the potential of phage infection, causing starter culture failure, needs to be investigated.

Bacteriophages are ubiquitous in nature. About 96% of phage investigated in the last 45 years are tailed phage belonging to the Siphoviridae, Myoviridae, or Podoviridae families (Ackermann, 1996, 1999). Siphoviridae is by far the most frequent phage group (61.7%), followed by the Myoviridae (24.5%) and Podoviridae (13.9%) (Ackermann, 1999). Most *Lactobacillus* phages, including several reported *L. plantarum* phages, such as phage B2 of *L. plantarum* ATCC 8014 (Nes et al., 1988), ΦLP1, and ΦLP2 (Caso et al., 1995), and phage SC921 (Yoon et al., 2001), belong to the Siphoviridae family. They have isometric heads with non-contractile tails (Sechaud et al., 1988). To our knowledge, phage fri is the only reported *L. plantarum* phage having a contractile tail and belonging to the Myoviridae family (Trevors et al., 1983). *L. plantarum* phages have been isolated from a variety of fermentation sources: phage fri from a commercial meat starter culture (Trevors et al., 1983); ΦLP-1 from corn silage (Caso et al., 1995); phage B2 from anaerobic sewage sludge (Douglas and Wolin, 1971); phage ΦLP-2 from a homemade cheese whey (Caso et al., 1995); and phage SC921 from Kimchi (Yoon et al., 2001).

The objective of this study was to isolate and characterize phages specific for *L. plantarum* MU45, which has been evaluated for use in low-salt, controlled vegetable fermentation. This culture has also been evaluated for use as a biocontrol organism to inhibit the growth of pathogenic organisms such as *Listeria monocytogenes* in a non-acidified, refrigerated pickle product and in mixed culture cucumber juice fermentations (Romick, 1994). This is the first report of isolation and characterization of a *L. plantarum* phage from a cucumber fermentation.

2. Materials and methods

2.1. Bacterial strains and culture media

L. plantarum strain MU45 was used as the primary host for the isolation, propagation, and characterization of the bacteriophage named ΦJL-1. Strains (32) of LAB (Table 1) were tested for phage sensitivity. All strains were obtained from the USDA-ARS Food Fermentation Laboratory Culture Collection (Raleigh, NC). All bacterial stock cultures were stored at −84 °C in MRS broth (Difco Laboratories, Detroit, MI) containing 16% (v/v) glycerol. When needed, frozen cultures were plated onto MRS agar (Difco), and fresh overnight cultures were prepared from isolated colonies. Bacteria and phages were propagated in MRS broth (de Man et al., 1960). For phage lysate preparation, MRS broth was supplemented with 10 mM $CaCl_2$ (Sigma-Aldrich, St. Louis, MO) unless otherwise stated. Soft agar was prepared with MRS broth supplemented with 0.7% agar.

2.2. Phage isolation and enrichment

Brine samples were obtained from a commercial cucumber fermentation tank containing size no. 1 (2.4–2.7-cm diameter) cucumbers. The samples were adjusted to pH 6.5 with 3 N NaOH and centrifuged ($5000 \times g$ for 15 min) to remove bacterial cells and debris. The supernatant was filtered through a 0.45-μm pore size syringe filter (Pall Corporation, Ann Arbor, MI). The filtrate was added to equal amounts of double strength MRS broth supplemented with 10 mM $CaCl_2$ and inoculated with an early log-phase host culture. After incubation at 30 °C for 16–18 h, the medium was centrifuged at $4000 \times g$ for 10 min. This enrichment procedure was repeated twice. The supernatant obtained from the final enrichment step was filter-sterilized and tested for the presence of phage active against *L. plantarum* MU45.

Table 1
Host range of *L. plantarum* phage JL-1[a]

Strain	Lysis[b]	FFL ID[c]
Lactobacillus plantarum MOP3	+	BI7
Lactobacillus plantarum MOP3-M6	+	MU45
Lactobacillus plantarum ATCC 14917	–	LA70
Lactobacillus plantarum WSO	–	LA23
Lactobacillus plantarum	–	LA287
Lactobacillus pentosaceus ATCC 8041	–	LA136
Lactobacillus curvatus ATCC 25601	–	LA223
Lactobacillus brevis ATCC 14869	–	LA228
Lactobacillus pentosus ATCC 8041	–	LA233
Lactobacillus coryniformis coryniformis ATCC 25602	–	LA252
Lactobacillus fructivorans ATCC 8288	–	LA255
Lactobacillus fructosus ATCC 13162	–	LA256
Lactobacillus gasseri ATCC 33323	–	LA257
Lactobacillus hilgardii ATCC 8290	–	LA258
Lactobacillus jensenii ATCC 25258	–	LA259
Lactobacillus mali ATCC 27053	–	LA260
Lactobacillus salivarius ATCC 11741	–	LA263
Lactobacillus curvatus curvatus ATCC 25601	–	LA272
Lactobacillus reuteri	–	LA273
Lactobacillus paraplantarum	–	LA274
Lactobacillus gramminis	–	LA276
Lactobacillus paracasei paracasei ATCC 25598	–	LA278
Lactobacillus casei casei ATCC 393	–	LA284
Lactococcus lactis ATCC 11454	–	LA119
Leuconostoc paramesenteroides ATCC 33313	–	LA225
Leuconostoc lactis ATCC 19256	–	LA265
Leuconostoc mesenteroides cremoris ATCC 19254	–	LA266
Leuconostoc mesenteroides dextranicum ATCC 19255	–	LA267
Leuconostoc mesenteroides mesenteroides ATCC 8293	–	LA268
Leuconostoc mesenteroides	–	LA10
Leuconostoc fallax ATCC 700006	–	LA283
Pediococcus dextrinicus ATCC 33087	–	LA224
Pediococcus pentosaceus	–	PS 772

[a] All strains were obtained from the culture collection in USDA-ARS Food Fermentation Laboratory (Raleigh, NC). ATCC = American Type Culture Collection, Rockville, MD.
[b] + = Plaques formed; – = no plaque formed.
[c] FFL ID = identification number in the culture collection of USDA-ARS Food Fermentation Laboratory (Raleigh, NC).

2.3. Phage detection and host range

The spot test method (Chopin et al., 1976) was used as an initial test for the presence of phage by measuring lytic activity. Soft agar in 3 ml (MRS broth with 0.7% agar) was seeded with 0.1 ml of culture (10^9 colony-forming units, cfu/ml), mixed gently, and poured onto an MRS agar plate. After solidification, 10 μl of phage lysate was spotted on the lawn of *L. plantarum* MU45. After drying, the plate was incubated at 30 °C overnight. A clear zone in the plate, resulting from the lysis of host cells, indicated the presence of phage. Spot tests were also used for host range studies, and, in all cases, positive tests were confirmed by plaque assay.

2.4. Plaque purification, lysate preparation, and bacteriophage tittering

Phage ΦJL-1 was purified by successive single-plaque isolation using the propagating strain MU45. A single plaque was picked from the MU45 lawn, inoculated into an early log phase MU45 culture and the lysate plaqued again. After repeating the cycle three additional times, a single plaque was picked and transferred into a tube containing 5 ml of MRS broth, 0.1 ml of 1 M $CaCl_2$, and an early log phase host culture (10^8 cfu/ml). The tube was then incubated at 30 °C for 7 h. The phage lysate was centrifuged at $4000 \times g$ for 10 min at 4 °C (Sorvall RC-5B centrifuge, Wilmington, DE). The pH of the supernatant was adjusted to 6.5 with 3 N NaOH and filtered using a 0.45-μm pore size syringe filter. Phage stock was stored with chloroform (5% by volume) at 4 °C, and an aliquot was frozen at −84 °C in MRS broth containing 16% glycerol. Phage titer was determined as plaque-forming units (pfu/ml) using the double-layer agar plate method similar to that of Adams (1959). After appropriate dilution with saline, 0.1 ml of phage-containing sample and 0.1 ml of actively growing host culture (10^9 cfu/ml) were added to a tube containing 3 ml of soft agar (maintained at 50 °C in a water bath) and 0.1 ml of 0.3 M $CaCl_2$. The mixture was overlaid onto the surface of an MRS agar plate and incubated overnight at 30 °C to enumerate plaques.

2.5. Determination of optimal multiplicity of infection (MOI)

Multiplicity of infection was defined as the ratio of virus particles to potential host cells (Birge, 2000).

MU45 was grown in MRS broth at 30 °C to an absorbance at 630 nm of 0.08, measured in a spectrophotometer (Novaspec II, Pharmacia LKB, Piscataway, NJ). This corresponding to a cell count of approximately 1×10^8 cfu/ml. The early log phase cells were infected with ΦJL-1 at four different ratios (0.01, 0.1, 1, and 10 pfu/cfu). After incubation for 3.5 h at 30 °C, the phage lysate was centrifuged at $9000 \times g$ for 3 min. The supernatant was filtered (0.45-μm pore size syringe filter) and assayed to determine the phage titer. Viable cell counts were determined by using a spiral plater (Autoplate 4000; Spiral Biotech, Bethesda, MD) for plating samples on MRS agar and an automated counter (Protos Plus: Bioscience International, Rockville, MD) for colony enumeration. Phage-free cultures (containing only bacteria) and cell-free cultures (containing only phage) were used as controls in all experiments. All assays were performed in duplicate. The MOI resulting in highest phage titer within 3.5 h was considered as an optimal MOI and used in subsequent large-scale phage production.

2.6. Large-scale phage production

One liter of pre-warmed (30 °C) MRS broth was inoculated with an overnight *L. plantarum* MU45 culture to an initial cell level of approximately 8×10^7 cfu/ml. The cells were grown to approximately 2×10^8 cfu/ml at 30 °C before 10 ml of 1 M $CaCl_2$ was added into the broth. The host cells were then infected with phage at a predetermined optimum MOI of 0.01–0.02. The incubation was continued until complete lysis was observed (about 4.5 h after infection).

2.7. Concentration and purification of phage lysates

A 1-l phage lysate was centrifuged at $8000 \times g$ for 20 min and the supernatant filtered through a 0.45-μm pore size, bottle-top filter. The filtrate was treated with 0.5 ml of nuclease solution containing DNAse I, 3 mg/ml and RNAse A, 3 mg/ml (Sigma-Aldrich), at 30 °C for 2 h. Phages were then precipitated using modification of a method described by Yamamoto et al. (1970). Polyethylene glycol (Sigma-Aldrich) 8000 and NaCl were added to final concentrations of 10% (w/v) and 0.5 M, respectively. After gentle mixing, the phage preparation was incubated overnight at 4 °C. The phages were pelleted by centrifugation at $10{,}000 \times g$ for 20 min, then resuspended in 6 ml of 10 mM Tris–HCl buffer (pH 7.4, Sigma-Aldrich). The phage preparation was overlaid on a CsCl (Sigma-Aldrich) step gradient ($d = 1.7$, 1.5, 1.4 g/ml, 1 ml each step) in 5-ml centrifuge tubes (tube #45248, Sorvall, Newtown, CT) and centrifuged at $600{,}000 \times g$ for 6 h at 15 °C (Sorvall micro-ultracentrifuge with rotor S100AT6, RC-M150 GX). The phage band (between $d = 1.7$ and $d = 1.5$) was drawn through the wall of the centrifuge tube using a syringe. The purified phage preparation was dialyzed against 2 l 10 mM Tris buffer for 24 h with three to four changes of buffer with a 6000–8000-Da pore size membrane (Spectrum, Houston, TX).

2.8. Electron microscopy

A CsCl-purified and concentrated phage sample was negatively stained with 2% (w/v) aqueous uranyl acetate (pH 4.0) on a carbon-coated grid and examined by transmission electron microscopy (JEOL JEM-100S, Japan Electronics and Optics Laboratory, Tokyo, Japan) at an accelerating voltage of 80 kV. Electron micrographs were taken at a magnification of 50,000 × and printed at 85,000 × (V. Knowlton, Center for Electron Microscopy, NC State University, Raleigh, NC). The phage size was determined from the average of five independent measurements.

2.9. Phage DNA extraction, sequencing, and restriction analyses

Phage DNA was extracted essentially as described by Durmaz and Klaenhammer (2000). Briefly, 3 ml of CsCl-purified phage suspension was extracted twice with 3 ml of phenol and 200 μl of chloroform-isoamyl alcohol (23:1, vol/vol, Sigma-Aldrich). This was followed by three extractions with 1.5 ml of phenol (pH 8.0, Sigma-Aldrich) and 1.5 ml of chloroform-isoamyl alcohol, and two extractions with 3 ml of chloroform-isoamyl alcohol. The nucleic acids were precipitated with 0.1 volume of 3 M sodium acetate and 3 volumes of 95% cold ethanol (-20°C) and pelleted with a microcentrifuge ($13{,}000 \times g$). The final pellet was washed twice with 10 ml of 70% ethanol, air dried, and then resuspended in 400 μl of

TE buffer containing 10 mM Tris–HCl and 0.1 mM EDTA (pH 7.6). DNA sequencing was performed at the Department of Energy Joint Genome Institute (JGI) sequencing facility (Walnut Creek, CA). Open reading frames (ORFs) were identified using sequence analysis software (Clone Manager 6 and Plasmid Map Enhancer v. 3, Scientific Educational Software, Durham, NC). For restriction analyses, the phage DNA was digested with restriction endonucleases (*Ava*I, *Bam*HI, *Bgl*I, *Bgl*II, *Eco*RI, *Eco*RV, and *Xba*I) according to the supplier's recommendations (Promega, Madison, WI). The DNA fragments were separated by agarose (0.8%) gel electrophoresis in Tris–acetate–EDTA buffer at constant voltage (150 V) for 3 h and visualized by UV light (300 nm) after staining with ethidium bromide (1 μg/ml).

2.10. Phage adsorption

The adsorption experiments were carried out as described by Foschino et al. (1995) and Ellis and Delbruck (1939), except that unadsorbed phages were obtained by filtration instead of centrifugation. A host strain culture ($\approx 10^8$ cfu/ml) in MRS broth supplemented with 0, 5, 10, 15, 20, 25, or 30 mM $CaCl_2$ was infected by a phage suspension to give an MOI of 0.01, and incubated at 30 °C. Aliquots of 0.6 ml were taken at 0, 3, 6, 10, 15, 20, 25, and 30 min after infection and immediately filtered through a 0.45-μm pore size syringe filter. Filtrates were tittered for unadsorbed phages by the double-layer agar plate method. MRS broth containing only phage was used as a control. Percent adsorption of the phage was calculated as [(control titer − residual titer)/control titer] × 100% (Durmaz, 1992). A separate experiment was carried out in *N*, *N*,-bis(2-Hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer instead of MRS broth to study calcium effect on phage adsorption. BES buffer was prepared at a concentration of 50 mM and pH 7.2. After filter-sterilization, the buffer was supplemented with $CaCl_2$ to final concentration of 0–20 mM. The ionic strength of BES buffer was adjusted with NaCl so that the ionic strength resulting from $CaCl_2$ plus NaCl was 0.145. One milliliter of an early log phase culture of the host cells (2×10^8 cfu/ml) was harvested by centrifugation (12,000 × *g* for 4 min) and washed twice with saline (0.85% NaCl). The cell pellet was resuspended with 1 ml of BES buffer containing $CaCl_2$ (0, 0.01, 0.1, 1, 2, 5, 10, or 20 mM). The cell suspension was infected with phage stock (1×10^9 pfu/ml) at an MOI of about 0.02. After incubation at 30 °C for 30 min, the mixture was filtered through a 0.45-μm pore size syringe filter. The filtrate was tested for unadsorbed phages by the double-layer technique.

2.11. Calcium effect on phage propagation

Calcium effect on phage propagation was determined in five 15-ml tubes. Ten milliliters of early log-phase host culture ($\approx 1 \times 10^8$ cfu/ml) in MRS broth was transferred into each of the five 15-ml tubes containing 0, 1, 10, 20, or 30 mM supplemented $CaCl_2$. After the final volume was adjusted with distilled water, each tube was infected with the phage at an MOI of about 0.03. All tubes were incubated at 30 °C. An aliquot (0.5 ml) was taken from each tube at selected intervals and filtered immediately. pfu was determined by the double-layer agar plate method.

2.12. One-step growth

For one-step growth experiments, a modification of the methods of Leuschner et al. (1993) and Foschino et al. (1995) was used with a 10-min adsorption. Following centrifugation at 13,000 × *g* for 30 s (Fisher model 16KM Marathon microcentrifuge, Fisher Scientific, Pittsburgh, PA), the pellet containing (partially) infected cells was resuspended in 1 ml of pre-warmed MRS broth. Samples were taken at 5- or 10 min-intervals (up to 2 h) and immediately tittered by the double-layer agar plate method. Assays were carried out in triplicate. Latent period was defined as the time interval between the adsorption (not including 10 min pre-incubation) and the beginning of the first burst, as indicated by the initial rise in phage titer (Adams, 1959; Ellis and Delbruck, 1939). Burst size was calculated as the ratio of the final count of liberated phage particles to the initial count of infected bacterial cells during the latent period (Adams, 1959). A four-parameter sigmoidal model was fit to the one-step growth curve. The NLIN procedure of SAS (SAS Institute, Cary, NC) was used to estimate the parameters of the model.

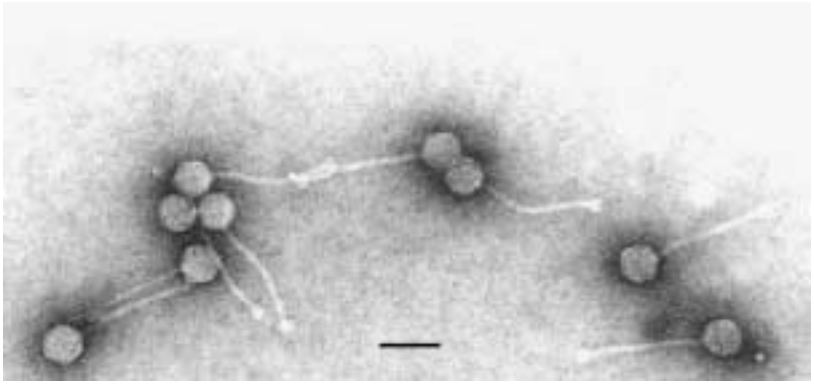

Fig. 1. Electron micrograph of *L. plantarum* phage ΦJL-1. CsCl-purified bacteriophage preparation was negatively stained with 2% uranyl acetate (pH 4.0). Magnification: 85,000×. Bar: 100 nm.

2.13. Sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE)

An aliquot (26 μl) of CsCl-purified ΦJL-1 sample was mixed with 10 μl of buffer and 4 μl of reducing agent (NuPAGE LDS system, Novex, San Diego, CA). The mixture was heated in boiling water for 10 min and then subjected to electrophoresis on a 4–12% Bis–Tris gel at 200 V and 120 mA for 35 min. The protein bands were stained with Coomassie blue G-250 (Pharmacia), followed by destaining with a solution containing 50% methanol and 10% acetic acid. The reported molecular weight values were obtained using molecular weight standards (Mark 12, Novex), and are averages of results from three electrophoresis runs.

2.14. Thermal inactivation

A temperature-controlled water bath (M3 Lauda, Brinkman Instrument, Westbury, NY) was used to determine the *D* values of phage ΦJL-1. A 1.5-ml microcentrifuge tube containing 900 μl of sterile, deionized water was preheated to a desirable temperature, ranging from 70 to 100 °C. Phage solution in 100 μl (10^6 pfu/ml in water) was added to the tube.

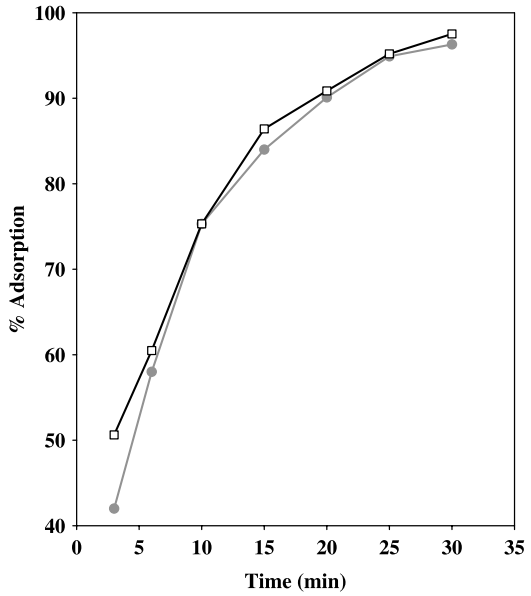

Fig. 2. Adsorption curves of *L. plantarum* phage ΦJL-1 in MRS medium without calcium supplement (●) or with 20 mM calcium supplement (□).

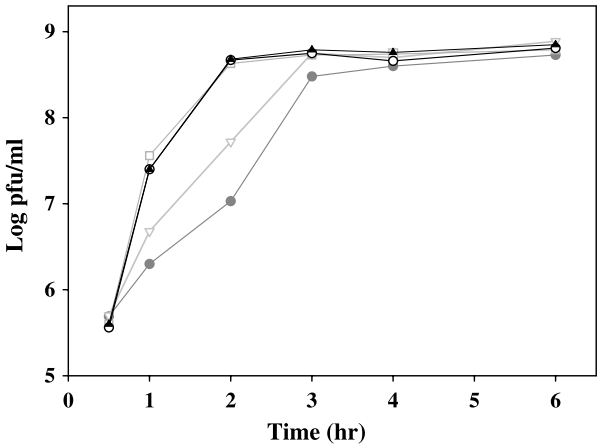

Fig. 3. The effect of calcium on phage ΦJL-1 propagation in MRS media at 30 °C without calcium supplement (●), and with 1 (▽), 10 (▲), 20 (□), or 30 mM (○) mM calcium supplement.

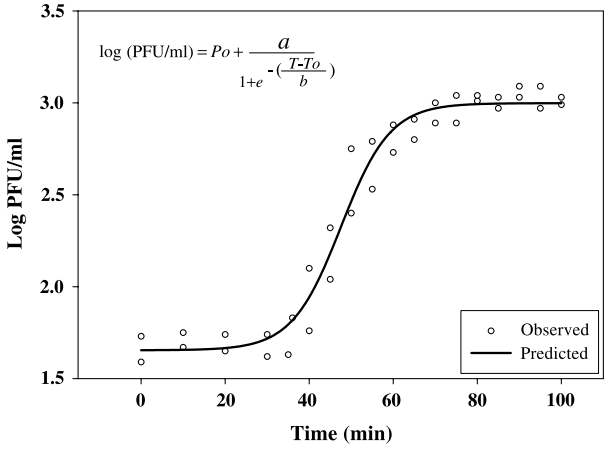

Fig. 4. One-step growth curve of *L. plantarum* phage ΦJL-1 in MRS broth at 30 °C. Three replicates of the growth curve were performed. The root mean square is 0.106 with R^2 value of 0.998.

After heating at intervals between 15 s to 3 min, the tube was placed in an ice-water bath. Samples were assayed to determine surviving pfu. *D* values were calculated as the time required for one log reduction in pfu/ml.

3. Results

3.1. Phage isolation and characterization

Brine samples from the first week of fermentation were screened for the presence of phage active against *L. plantarum* MU45. Phage from the positive plate (containing clear zones) of the spot test underwent plaque purification. The phage ΦJL-1 formed small, clear, round plaques (about 1.7 mm in diameter) on the MU45 lawn. High-titer phage stock contained 10^9 pfu/ml. The ultrastructure of the phage was examined by electron microscopy, as seen in Fig. 1. The phage has an isometric head of 59 nm in diameter and a long, flexible, non-contractile, and regularly striated tail (182 nm long and 11 nm wide). A complex base plate (approximately 25 nm in diameter) on the tail was also present. The structural features of this phage were consistent with morphotype B1 according to Ackermann (1996), and classify it into the family Siphoviridae, according to the International Commit-

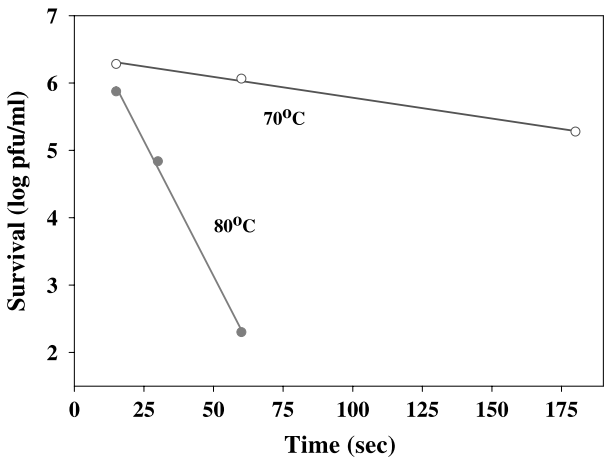

Fig. 5. Phage ΦJL-1 population over time at 70 and 80 °C in water. The initial pH was 6.5.

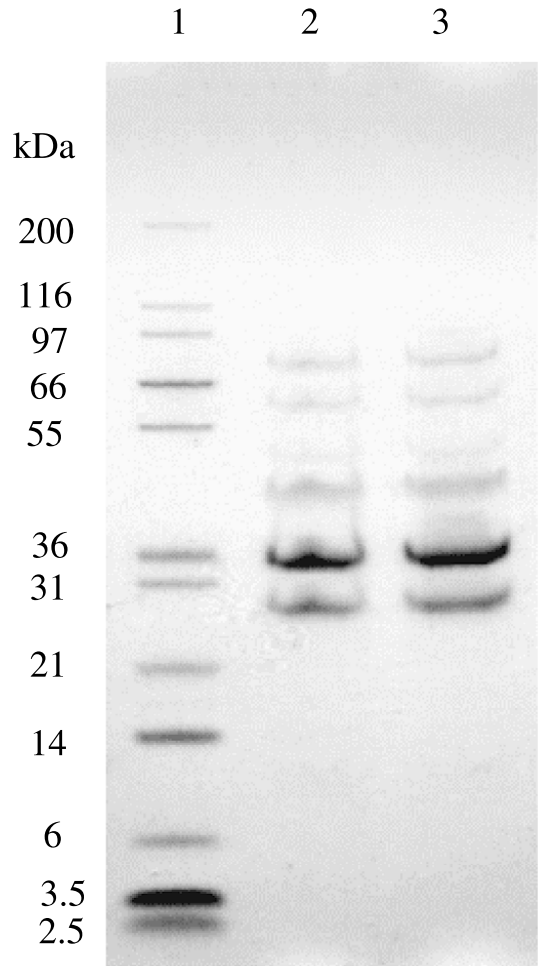

Fig. 6. SDS-PAGE of *L. plantarum* phage ΦJL-1 structural proteins (lanes 2 and 3) and molecular mass markers (lane 1). Mark 12 unstained standard was used as molecular weight standard.

tee on Taxonomy of Viruses (Mathews, 1982). The optimal MOI of ΦJL-1 was determined to be 0.01–0.03 (data not shown).

3.2. Calcium effects on phage adsorption and propagation

The adsorption rates of ΦJL-1 in MRS broth with 0 and 20 mM $CaCl_2$ supplementation are shown in Fig. 2. In MRS broth without $CaCl_2$ supplementation, about 75% of ΦJL-1 phage particles adsorbed to host cells within 10 min, 90% in 20 min, and 96% in 30 min. Similar results were obtained in MRS media supplemented with 5, 10, 15, 25, and 30 mM of calcium chloride. The adsorption of ΦJL-1 in calcium-free BES buffer was then investigated, and the percent adsorptions were similar to those in MRS media with or without calcium supplementation. However, phage growth rates were faster in calcium-supplemented media at an MOI of 0.02 (Fig. 3). Cell lysis was observed in MRS media containing 10, 20, or 30 mM added $CaCl_2$ within 2 h; while clearance required 3 or 4 h in the media containing 1 or 0 mM supplemented $CaCl_2$. There was no difference in the phage propagation rates with MRS media supplemented with $CaCl_2$ concentrations in the range of 10–30 mM. The final phage titer in all media, with or without calcium supplementation, reached the same level in 4 h.

3.3. Host range, kinetics, and thermal lability

The host range of ΦJL-1 was determined with 32 selected strains from 4 genera of LAB. ΦJL-1 was lytic against both *L. plantarum* B17 and its mutant MU45, which is deficient in malolactate fermenting ability. Of the 31 remaining LAB strains tested, none was sensitive to ΦJL-1 (Table 1). A one step growth curve for ΦJL-1 was determined in MRS broth at 30 °C. Fig. 4 shows that the latent period was about 35 min, the rise period was 40 min, and the average burst size was 22 pfu/cell. Thermal lability of ΦJL-1 was investigated by thermal treatments at 70, 80, 90, and 100 °C. Survivor curves of the phage at 70 and 80 °C are shown in Fig. 5. The *D* values were calculated as 2.7 min at 70 °C and 0.2 min at 80 °C. Phage titers decreased below the detection limit (20 pfu/ml) after heating for longer than 60 s at 80 °C, or 15 s at 90 or 100 °C.

3.4. Protein and sequence analysis

The structural proteins of phage ΦJL-1 were analyzed by SDS-PAGE (Fig. 6). Six structural proteins

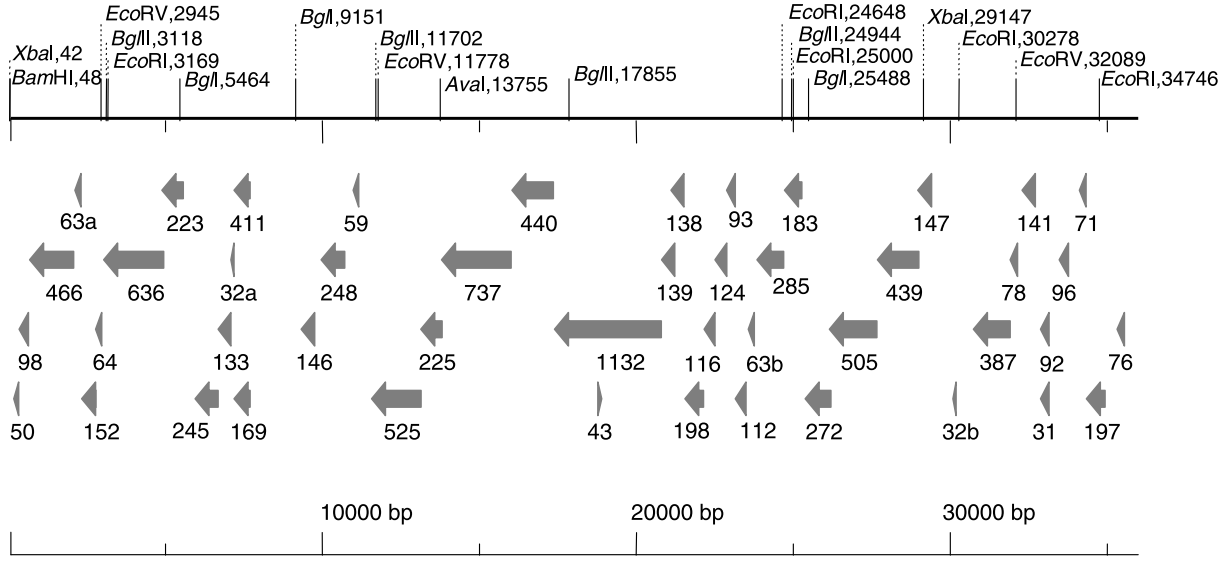

Fig. 7. Restriction and ORF map of contig 2 of phage ΦJL-1 DNA. Locations (in bp) of the restriction endonuclease (*Ava*I, *Bam*HI, *Bgl*I, *Bgl*II, *Eco*RI, *Eco*RV, and *Xba*I) sites are as indicated. The orientations of the ORFs are indicated by arrows. The number below each arrow represents the number of amino acids encoded by each ORF.

were revealed with molecular masses estimated at 76, 61, 50, 45, 34, and 28 kDa. Chromosomal DNA was submitted to the Department of Energy Joint Genome Institute (Walnut Creek, CA). Preliminary sequence data (http://www.jgi.doe.gov) showed two major contiguous sequences, Contigs 1 and 2. Contig 2 was constructed from 1216 reads and consists of 35,701 bps with 46 possible open reading frames (ORFs, Fig. 7). Contig 1 consisted of 831 bps. A restriction map of contig 2 of ΦJL-1 (Fig. 7) was constructed based on this primary sequencing data and confirmed by our restriction analyses (data not shown).

4. Discussion

Adsorption of phage particles to bacterial cells is the initial step of phage infection. While 75% adsorption occurred in 10 min, 96% adsorption occurred in 30 min. Caso et al. (1995) reported that 92% of phage ΦLP1-A was absorbed onto *L. plantarum* ATCC 8014 in 45 min. Adsorption is not only dependent on the presence of specific receptors on the cell surface (Topley and Wilson, 1990), but can also depend on the presence of certain cations in the media. Bacteriophages usually require higher concentrations of divalent cations, such as calcium or magnesium, at some stage of their infection cycle than the concentration required for the growth of host cells (Watanabe and Takesue, 1972). In this study, excess of Ca^{2+} (5–30 mM) in MRS media did not affect the adsorption rate in the first 30 min, but did promote rapid phage propagation and cell lysis. These results suggested that the levels of calcium and/or other cations in MRS media were sufficient for the initial infection steps but not for subsequent cycles. Watanabe and Takesue (1972) reported that calcium ions were required for the penetration of phage genomes into the host cells of *Lactobacillus casei*. Calcium appears to be required for other ΦJL-1 processes. The percent adsorption in calcium-free BES buffer was almost the same as that in calcium-containing BES buffer or in MRS media, suggesting that calcium is not required for phage adsorption. This was perhaps because sodium ions were present in calcium-free BES buffer and these monovalent cations were as effective as Ca^{2+} in facilitating phage adsorption. It was reported that in pure distilled water or at low concentration of monovalent ions (≤ 0.01 mM), most phages do not adsorb to bacteria (Luria et al., 1978). The sodium concentration in 50 mM BES buffer (pH 7.2) used in this study was much higher than 0.01 mM and may have been sufficient to facilitate phage adsorption. Watanabe and Takesue (1972) studied the adsorption of phage PL-1 to *L. casei* in Tris–maleate buffer in the presence or absence of calcium. They concluded that calcium was not required in Tris–maleate buffer for the phage adsorption. This is not known what Ca^{2+}-independent factors are involved in adsorption of these phages.

The morphology of ΦJL-1 was similar to most other *Lactobacillus* phage (about 50 nm in diameter, 170–180 nm in length; Jarvis, 1989). ΦJL-1 had a burst size of 22 pfu/cell, which was larger than that for *L. plantarum* 8014 phage B2 (12–14 pfu/cell; Nes et al., 1988), but almost 10 times smaller than that for *L. plantarum* phage fri (200 pfu/cell; Trevors et al., 1983). Phage B2 had a larger head (110 nm in diameter) and a longer tail (500 nm) than ΦJL-1 (59 and 182 nm, respectively). Both phage B2 and fri had a much longer latent period (75 min) than ΦJL-1 (35 min). ΦJL-1 was lytic only against two closely related strains of *L. plantarum*, BI7 and an isogenic mutant MU45. ΦJL-1 was distinct from phage SC921 isolated from Kimchi (Yoon et al., 2001) because ΦJL-1 was not lytic for the SC921 host, *L. plantarum* ATCC 14917. High strain specificity was also observed in other *L. plantarum* phage such as *L. plantarum* phage B2 and fri (Douglas and Wolin, 1971; Trevors et al., 1983).

Like most tailed phage, ΦJL-1 had a genome consisting of a linear, double-stranded DNA. The estimated genome size of ΦJL-1 (36.7 kbp) was smaller than those of other known *L. plantarum* phage (Table 2), including: ΦLP2 (47 kbp), SC921 (66.5 kbp), B2 (73 kbp), ΦLP1 (80 kbp), and fri (133 kbp). The ΦJL-1 genome size was similar to those of *Lactobacillus sake* phage PWH2 (35 kbp), *Lactobacillus bulgaricus* phage ch2 (35 kbp), and *L. casei* phage J-1 (37 kb), but smaller than that of *Lactobacillus gasseri* phage phi adh (43.8 kbp). The restriction digestion fragment sizes reported for these phages showed no similarity with ΦJL-1. Based on the primary sequencing data, 46 ORFs were identified. Additional ORFs may be identified after the genome sequencing is completed. Six structural proteins with

Table 2
Genome sizes of several *Lactobacillus* bacteriophages

Phage	DNA size (kbp)	Family	Host	Reference
ΦJL-1	36.7	Siphoviridae	*L. plantarum* MU45	This study
ΦLP2	47	Siphoviridae	*L. plantarum*	Caso et al., 1995
SC921	66.5	Siphoviridae	*L. plantarum* 0280	Yoon et al., 2001
B2	73	Siphoviridae	*L. plantarun* ATCC 801	Nes et al., 1988
ΦLP1	80	Siphoviridae	*L. plantarum*	Caso et al., 1995
fri	133	Myoviridae	*L. plantarum* A	Caso et al., 1995
phi adh	43.8	Siphoviridae	*L. gasseri*	Altermann et al., 1999
PWH2	35	Siphoviridae	*L. sake* Ls2	Leuschner et al., 1993
ch2	35	Siphoviridae	*L. bulgaricus* CH2	Chow et al., 1988
J-1	37	Siphoviridae	*L. casei* S-1	Khosaka, 1977

molecular weights ranging from 28 to 76 kDa were identified by SDS-PAGE. A detailed sequence analysis and identification of the ORFs corresponding to the observed structural proteins will be the subject of future research.

ΦJL-1 was rapidly inactivated at temperatures above 70 °C. This heat sensitivity may be exploited in designing heat processes to prepare vegetables prior to starter culture inoculation. Breidt et al. (2000) reported that blanching whole pickling cucumbers for 15 s (0.25 min) at 80 °C reduced microbial cell counts by 2–3 log cycles from an initial population of typically 10^6 cfu/g. This blanching treatment was adequate to eliminate 1 log cycle of ΦJL-1 from fresh cucumbers and, consequently, would be predicted to decrease the risk of potential phage infection problem with the starter culture MU45 for controlled low-salt cucumber fermentation.

The results from this study revealed that ΦJL-1 was active against the potential starter culture (MU45) for commercial cucumber fermentation. Phage infection could adversely affect the fermentation process by delaying acidification of the brine, thereby allowing spoilage or pathogenic organisms to grow and affecting the quality or safety of the fermented product. A study with phage B2 and its host *L. plantarum* ATCC 8014 as a meat starter culture showed that the phage infection significantly delayed (8–10 days) lactic acid production and concomitant pH drop during the production of salami dry sausage (Nes and Sorheim, 1984). Phage infection could also destroy biocontrol organisms, giving false safety assurance.

ΦJL-1 is the first reported *L. plantarum* phage isolated from cucumber fermentation. The discovery of this phage provides valuable information that must be considered during development of any procedures for controlled cucumber fermentation or biocontrol systems using *L. plantarum* MU45. Further research is needed to evaluate the impact of ΦJL-1 on these systems.

Acknowledgements

This work was supported in part by a research grant from Pickle Packers International, St. Charles, IL.

We thank Valerie M. Knowlton for the electron microscopy analysis, Dr. Paul Predki and colleagues at the Department of Energy Joint Genome Institute for the generous aid with DNA sequencing. In addition, the authors thank Dr. Jason A. Osborne for statistical consultation, Mr. Rodolphe Barrangou-Poueys and Ms. Evelyn Durmaz for helpful discussions, and Ms. D.D. Toler for excellent secretarial assistance.

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