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Characterization of a lytic *Lactobacillus plantarum* bacteriophage and molecular cloning of a lysin gene in *Escherichia coli*

Sung-Sik Yoon [a,*], Jae-Won Kim [a], Frederick Breidt [b], Henry P. Fleming [b]

[a] *Department of Biological Resources and Technology, College of Arts and Sciences, Yonsei University, Wonju, Kangwon-Do 220-710, South Korea*

[b] *U.S. Department of Agriculture, Agricultural Research Service, and North Carolina Agricultural Research Service, Department of Food Science, North Carolina State University, Raleigh, NC 27695-7624, USA*

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Abstract

Bacteriophage SC921, which can infect *Lactobacillus plantarum* specifically, was isolated from a fermented vegetable source, *Kimchi*. This phage is active against six of 11 strains of *L. plantarum* tested as hosts. Morphologically, it has an isometric head (60 nm in diameter) and a non-contractile tail (260 nm long and 9–11 nm wide), indicating that it belongs to Bradley's group B or the *Siphoviridae* family according to the International Committee on Taxonomy of Viruses (ICTV). The bouyant density was 1.58 g/cm^3. SDS-PAGE experimentation indicated that the phage particle contains two major structural proteins and several minor proteins. The genome was a double stranded linear DNA molecule with cohesive ends and 66.5 kb long by mapping genomic DNA digested with the restriction endonucleases: *Kpn*I, *Sma*I, and *Xba*I. The [G + C] content of the phage DNA is 39.4%. For this lysin gene study, 9.4 kb of *Kpn*I-digested DNA fragment was cloned into pUC19 and expressed in *Escherichia coli*. The *Kpn*I fragment was considered as the genetic element responsible for the lysis gene of *L. plantarum* bacteriophage. The cloned fragment in pUC19 was hybridized to a 9.4-kb fragment generated by *Kpn*I digestion of SC 921 as a probe. This confirmed that the fragment in pUC19 originated from phage DNA. The lysin gene was near the middle of the phage genome.

Keywords: *Lactobacillus plantarum*; Bacteriophage; Cloning; Heterologous expression; Lysin

1. Introduction

Lactobacilli are widely used in a variety of food fermentation processes and contribute to the flavor and texture of fermented products. The organic acids produced and the resulting low pH serve to protect these products from spoilage microorganisms (Daeschel and Fleming, 1984). The activity of phage in vegetable fermentations has received considerably less attention than lactococcal bacteriophage in dairy fermentations, presumably because starter cultures are not used in most vegetable fermentations. There are some reports concerning *Lactobacillus* phage, including: phage PL1 infecting *L. casei* (Watanabe et al., 1984), the temperate phages mv1 and mv4 (Boizet et al., 1990), ch2 (Chow et al., 1988), and LL-H (Mata et al., 1986) infecting *L. delbrueckii* subsp. *bulgaricus*, and phage fri (Trevors et al.,

* Corresponding author. Tel.: +82-33-760-2251; fax: +82-33-763-4323.

E-mail address: sungsik@dragon.yonsei.ac.kr (S.-S. Yoon).

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1983), B2 f (Nes et al., 1988) and ϕLP-1 and ϕLP-2 (Casso et al., 1995) infecting *L. plantarum*. Also, several phages infecting *L. helveticus* (Sechaud et al., 1992), ϕgle for *Lactobacillus* strain Gle (Oki et al., 1996), and ϕadh from *L. gasseri* (Henrich et al., 1995), have been reported. Early studies on phages of lactic acid bacteria (LAB) mainly provided data on morphological, serological, or physiological characteristics. Recently, extensive research has been undertaken on the molecular biology of lactic phages, including genome organization and comparative DNA studies. Morphologically, most *Lactobacillus* phages have isometric head diameters of about 50 nm and a long non-contractile or contractile tail of 170–180 nm (Jarvis, 1989).

The origin of virulent phages in LAB has been investigated. Shimizu-Kadota et al. (1983) claimed that a virulent phage (ϕFSV) of *L. casei* S-1 originated from the temperate phage (ϕFSW) in the S-1 strain, based on similarities in their morphological characteristics and genomes. *Lactococcus lactis* P335 (Moineau et al., 1986) was found to have both lytic and temperate phages, suggesting that some temperate and lytic phages may have common ancestors and that prophages may be an important source of DNA for new lytic phages (Relano et al., 1987).

Phage-encoded lysins are enzymes that hydrolyze the bacterial cell wall, such as muramidases, transglycosylases, L-alanine amidases, and endopeptidases, and are usually active against only a narrow range of organisms. After infection, phage is replicated in the host cell and cell lysis is required for release of progeny (Sable and Lortal, 1995). This cytolytic process is presumed to depend upon two phage-encoded lysis proteins, lysin and holin. As lysin lacks the secretion signal, they are accumulated in bacterial cytoplasm without the aid of holin, which inflicts lesions in the cytoplasmic membrane through which lysin can be efficiently released to the periplasm (Sable and Lortal, 1995). Over the years, several presumptive lysins and holins have been reported from phages of LAB: ϕ adh from *L. gasseri* (Hill, 1993), mv1 and mv4 from *L. bulgaricus* (Boizet et al., 1990), and ϕ LC3 (Birkeland, 1994) and Tuc 2009 (Arendt et al., 1994) from *Lac. lactis*, but molecular details about the lytic pathway of phages (Young, 1992) from LAB are still largely unknown. Lysins have the potential for use as growth and acid production inhibitors, as ripening agents to affect early starter cell lysis and intracellular enzyme release for flavor development in the dairy products (Shearman et al., 1989, 1991), and as an antimicrobial substance to prevent growth of certain LAB in the wine industry (Davis et al., 1985).

Recently, the propagation of a new virulent *L. plantarum* phage SC921 was reported (Yoon et al., 1997). Objectives of the present study were to morphologically characterize SC921 and to clone and express its lysin gene in *Escherichia coli*.

2. Materials and methods

2.1. Bacterial strains and bacteriophage

L. plantarum LA0280 was used as a standard host-indicator strain for the SC921. Short-term phage preparation mixed with two or three drops of sterile chloroform were kept at 4°C, as described by Sambrook et al. (1989). LB medium (Difco, Detroit, MI) was used for cultivating *E. coli*; 100 μg/ml ampicillin, 20 μg/ml isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma, St. Louis, MO), and 20 μg/ml 5-bromo-4 chloro-3-indolyl-β-D-galactopyranoside (X-gal, Sigma) were added as required. MRS broth (Difco) and agar plates made therefrom (1.5% agar) were used for the transfer and the growth of *L. plantarum*. Only soft agar plates (MRS broth + 0.7% agar) containing 25 mM $CaCl_2.6\ H_2O$ were used for plaque assays. MRS broth was also supplemented with 25 mM $CaCl_2$ (MRS-Ca^{2+}) for use in large-scale production of the SC921 at 37°C.

2.2. Isolation of bacteriophage

Phage was isolated from *Kimchi* as described previously (Yoon et al., 1997). The agar overlay method (Adams, 1959) was used for plaque assay in which 0.1 ml of enriched sample (Reddy, 1974) was mixed with 3 ml of MRS soft agar (pre-warmed to 50°C) containing 25 mM Ca^{2+} and 0.1 ml of freshly cultured LA0280. After brief agitation, the mixture was then poured onto previously solidified MRS hard agar plates. High titer lysate was obtained by adding a 1-ml portion of phage lysate (10^9 PFU/ml) to 100 ml of an early log-phage-culture of strain

LA0280 in MRS broth and incubating the mixture for 6 h at 37°C. The phage was stored in MRS-Ca^{2+} (final concentration 10 mM) broth at 4°C.

2.3. Host range determination

Fifteen lactic acid bacterial strains (11 *L. plantarum*, 2 *Leuconostoc mesenteroides*, 1 *L. acidophilus*, 1 *L. brevis*; Table 1) were used as indicators for the determination of phage sensitivity by an agar spotting method (2). Ten microliters of high-titre bacteriophage samples (10^9 PFU/ml) were placed on each lawn of indicator cells and left to stand for 30 min. Plaque formation was examined for lysis after 24-h incubation at 30°C and 37°C. The results were recorded as positive (+) and negative (−).

2.4. Large-scale phage purification and preparation of phage DNA

For large-scale preparation of phage, a 1-l culture of LA0280 ($A_{600} = 0.2$) was infected with a plate lysate of the SC921. Multiplicity of infection (MOI) was adjusted to be about 0.2. The cell–phage mixture was incubated statically for about 4 h. When the optical density began to decrease, chloroform and NaCl were added to a final concentration of 3% (v/v) and 0.5 M, respectively. The culture was then placed on ice for 1 h. Cell debris was removed by centrifugation (Sorvall RC-2, DuPont, Norwalk, CT) using a GSA rotor at 8500 rpm for 15 min at 4°C, followed by 0.45-μm membrane filtration (Gelman, Ann Arbor, MI). Polyethylene glycol (PEG 8,000) was added to the supernatant to a final concentration of 10% (w/v), as described by Yamamoto et al. (1970). The phage preparation was then incubated overnight at 4°C, followed by 3–4 h incubation on ice and centrifugation as described above. The precipitated fraction was dissolved in 100 mM Tris–HCl buffer (pH 8.0) containing 10 mM $MgCl_2$. The bacteriophage was purified further by CsCl-density gradient ($d = 1.70$, 1.50, 1.40) centrifugation (model L8-70, Beckman, Fullerton, CA) in a 70.1 Ti fixed-angle rotor at 30,000 rpm for 30 h at 15°C. These purified phage samples were used for electron microscopy and extraction of genomic DNA.

Table 1
Determination of host spectrum of *L. plantarum* phage SC921[a]

Strains used	Lysis[b]	Source
L. plantarum MD40	−	FFL LA0023
L. plantarum NC-8	−	FFL LA0084
L. plantarum 82	+	FFL LA0089
L. plantarum 1193	−	FFL LA0096
L. plantarum ATCC 149117	+	FFL LA0098
L. plantarum 1988	−	FFL LA0100
L. plantarum 1939	+	FFL LA0099
L. plantarum C2R	−	FFL LA0146
L. plantarum LA0280	+	KCCM11322
L. plantarum ATCC 14917	+	FFL LA0070
L. plantarum KCCM12116	+	
L. acidophilus MD30	−	FFL LA0028
L. brevis MD20	−	FFL LA0027
Leu. mesenteroides NRRL B-1145	−	FFL LA0148
Leu. mesenteroides C-33	−	FFL LA0010

[a]ATCC = American Type Culture Collection, Rockville, MD; KCCM: Korean Culture Collection of Microorganisms; FFL = Food Fermentation Lab., North Carolina State University, USA.

[b] + = Infected and plaque formed; − = not infected and no visible plaque formed.

2.5. Electron microscopy

Phage morphology of CsCl-purified preparations, negatively stained with 2% uranyl acetate, pH 4.0, were studied by electron microscopy (JEOL 100S), 80 kV. The size was determined from the average of five to seven independent measurements.

2.6. Sodium dodecyl sulfate-polyacryl amide gel for electrophoresis (SDS-PAGE)

An SDS-PAGE was performed with a Hoefer SE600 gel electrophoresis unit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for determining the size and number of the proteins of phage particles. Purified 0.1-ml samples were heated at 100°C for 5 min to ensure denaturation before loading on a gel for electrophoresis. Electrophoresis was carried out with a 4% stacking layer and 12.5% separating gel according to Laemli (1970). Standard proteins (Bio-Rad, Richmond, CA) were used as size markers with a molecular weight range of myosin 200,000; β-galactosidase 116,000; phosphorylase b 97,000; bovine serum albumin 66,000; ovalbumin 45,000; carbonic anhydrase 31,000; trypsin inhibitor 21,000; lysozyme 15,000; and aprotinin 6,500. Each well

was loaded with 10 μl of sample containing approximately 10 μg of protein. Following electrophoresis, proteins bands were stained with Coomassie blue R-250 solution (Bio-Rad), followed by destaining with 7.5% glacial acetic acid and 5% methanol, and then photographed.

2.7. Isolation of DNA and restriction enzyme analyses

The genomic DNA from the purified SC921 was extracted with a Wizard® Genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer's instructions and dissolved in 10 mM Tris–HCl (pH 8.0). Phage DNA was cut with restriction endonucleases, *Kpn*I, *Sma*I, and *Xba*I, as recommended by the manufacturer's instructions (Promega). *Exo*III and S1 nucleases were purchased from Boehringer-Mannheim (Indianapolis, IN, USA). The resulting preparations were electrophoresed on 0.7% agarose gels at 0.32–0.56 V cm^{-2}

2.8. Determination of chemical composition of genome

The G + C content of the SC921 was determined from the UV absorbance–temperature profile by the method of Mandel and Marmur (1968). The A260 was read at intervals when a 1-ml cuvette containing 25 μg of DNA in 1 × SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) buffer was slowly heated from 70°C to 90°C with an increment of 1°C in a spectrophotometer (Model 8452A, Hewlett-Packard, San Fernando, CA). Analysis of *E. coli* genomic DNA was done along with this sample as a control.

2.9. Cloning of lysin gene

All DNA manipulations were carried out according to standard methods (Sambrook et al., 1989). The phage genomic DNA and the plasmid pUC19 (Vieira and Messing, 1982) were purified by a Geneclean II kit (Bio101, La Jolla, CA). Each DNA sample was digested with *Kpn*I (Sigma) at 37°C for 1 h. A genomic library was constructed in *E. coli*. After running on low melting-temperature agarose gel (1%) electrophoresis, about 2- to 10-kb fragments were recovered from the gel and ligated to *Kpn*I-cleaved pUC19 plasmid DNA, which was treated with CIP (calf intestinal phosphatase, Sigma) to prevent self ligation. This pool of hybrid DNA molecules was used to transform *E. coli* XL1-blue ([*Sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A46 *thi*-1 *rel*A1 *lac*[F′ proAB *lac1*^{q} *lac*Z M15 *Tn*10(ter^{r})], Stratagene, La Jolla, CA). The ligation was carried out at 4°C for 12 h, and 2 μl was transformed into *E. coli* with pUC19. White transformants, presumably containing insert DNA, were selected on LB agar containing ampicillin (100 mg/ml). Recombinant plasmids were analyzed by restriction of miniprep DNA (Sambrook et al., 1989). The lysin activity was determined according to the well diffusion method of Shearman et al. (1989) as described below.

2.10. Assay of lysin activity

To screen for the phenotypic expression of the lysin gene, recombinant clones harboring the plasmids which carry the lysin gene were grown in 10 ml 2x YT medium (Difco; 16 g of Tryptone, 10 g of yeast extract, 5 g of NaCl, 1000 ml of distilled water) at 37°C. Following centrifugation at 10,000 × *g* for 10 min, cell-free supernatant was obtained by 0.45-μm filtering through membrane filter (Gelman). Pellets was suspended in 10-ml SM buffer (100 mM NaCl, 10 mM $MgSO_4.7H_2O$, 50 mM Tris–HCl, 0.01% gelatin, pH 7.5), followed by 0.1% (w/v) chloroform treatment. After 5-min incubation at room temperature for cell lysis, cell debris was removed by centrifugation at 10,000 × *g* for 15 min (cell-free extract). Each fraction was placed in wells cut into an MRS agarose plate (0.5% agarose) seeded with about 10^6 CFU/ml of *L. plantarum* LA0280 culture. A zone of lysis (plaque) developed around wells was recorded as lysin-positive after overnight incubation at 37°C.

2.11. Non-radioactive labeling and hybridization reaction

The SC921 DNA and pYJ12 were digested with *Kpn*I according to the instructions of the manufacturer (Promega). Restricted DNA samples were run on 0.7% agarose gel and visualized by ethidium bromide coloration under UV light, 254 nm. Southern blot transfer on nylon membrane (positively

charged, Boehringer-Mannheim) was done according to standard protocol (Sambrook et al., 1989). The filter was air-dried and UV cross-linked by exposure to 254-nm UV light for 3 min. The 9.4-kb fragment from pYJ12 was used as a probe. The probe was labeled and detected using a DIG high prime DNA labeling and detection kit provided by Boehringer-Mannheim, which utilizes digoxigenin-11-dUTP. The hybridization experiment and detection were done according to the manufacturer's instructions. Briefly, the membranes were washed twice for 1 min in MS buffer (0.1 M maleic acid–0.15 M sodium chloride, pH 7.5) plus 0.3% (w/v) Tween 20 and incubated for 30 min with blocking solution. Ten milliliters of dilute antidigoxigenin–AP conjugate (150 mU/ml) was added to the membrane, followed by incubation for 30 min at 68°C. After the membrane was washed twice with MS buffer (pH 7.5) and equilibrated briefly with detection buffer, the DIG-labeled DNA was visualized in situ by nitroblue tetrazolium salt/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) chromogenic substrate.

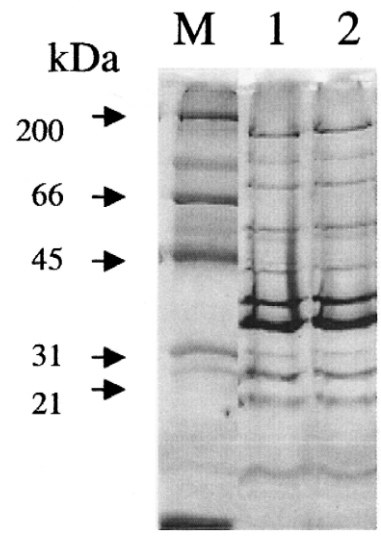

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of *L. plantarum* phage SC921 structural proteins. Lanes: M1, size standards with molecular weights of 200,000, 116,000, 66,000, 45,000, 31,000, 21,000, 15,000, 6500; 1 and 2, phage proteins stained with Coomassie blue. The size of the phage proteins is expressed in kDa.

3. Results

3.1. Host range determination

Several strains of LAB were tested for their sensitivity to the SC921 by spotting (10 μl) on the lawn of indicator cells. Phage sensitivity was shown to be different among *L. plantarum* strains. Out of the 11 *L. plantarum* strains tested, only six strains were lysed by the SC921, and the others formed no plaques at all. Other genera of lactobacilli and *Leuconostoc* were not lysed by this phage (Table 1). Among *L. plantarum* strains, strain 11322 was the most sensitive host for detection of the SC921. There was no apparent difference of plaque morphology obtained at 30°C and 37°C (data not shown).

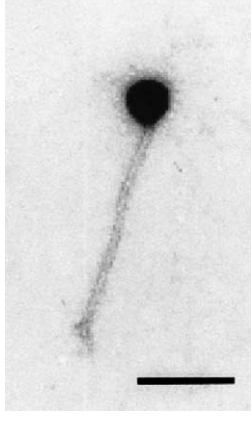

Fig. 1. Electron micrograph of *L. plantarum* phage SC921. CsCl-purified bacteriophage preparation from a lysed culture was negatively stained with 2% uranyl acetate, pH 4.0. Magnification: 85,000× (bar = 118 nm).

3.2. Morphology and protein composition

Electron microscopy of the SC921 revealed an isometric head (60 nm in diameter) and a non-contractile tail (260 nm long and 9–11 nm wide) (Fig. 1). The distal end of the tail contained some structural elements similar to the base plate, and a collar was localized faintly at the head–tail junction. Along the tail, about 40 regularly spaced rows of transversal striations were observed. The morphology of this bacteriophage thus belongs to that of Bradley's group B (Bradley, 1967), or to the *Siphoviridae* family

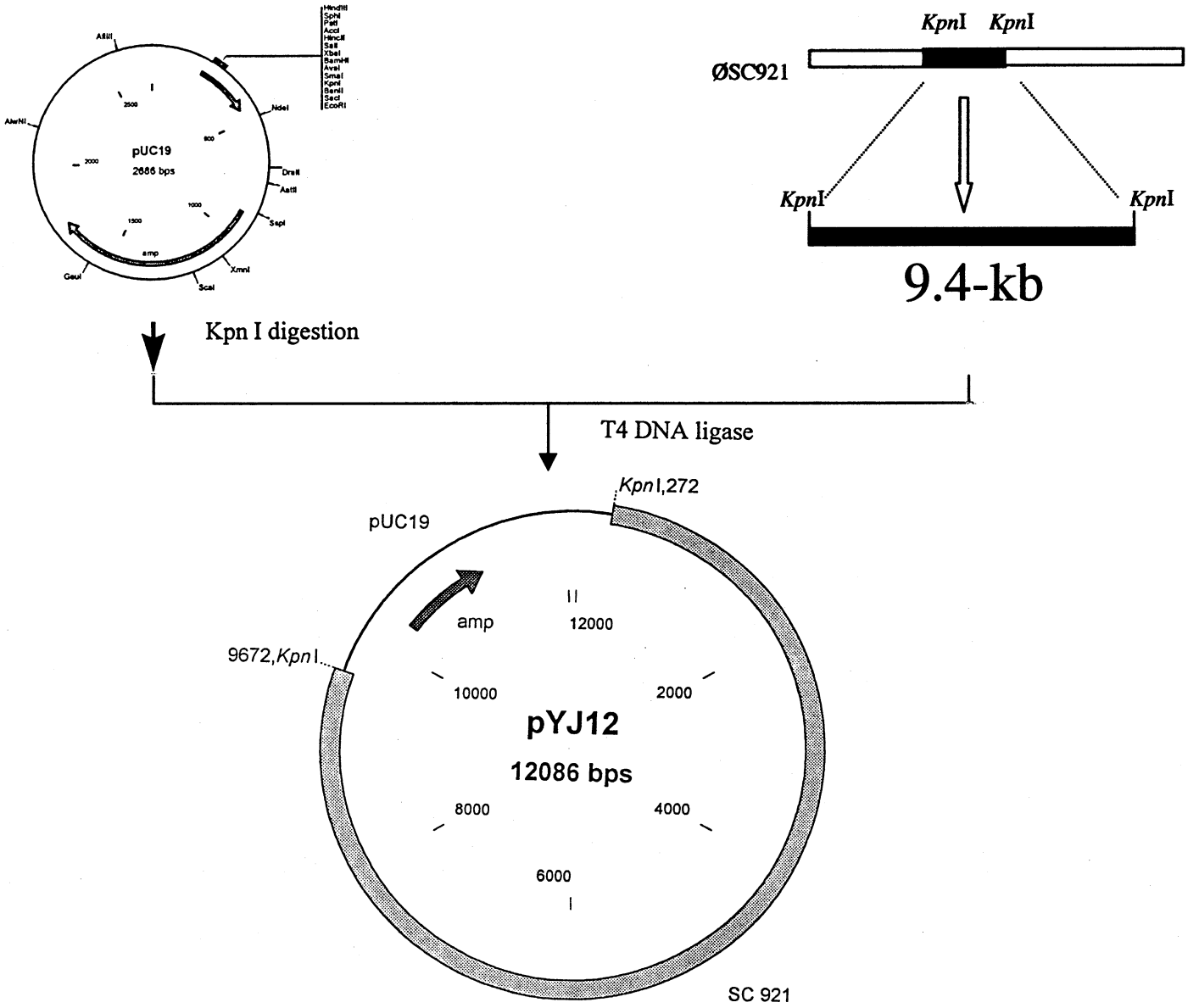

Fig. 3. Gene library was constructed into pUC19. *Kpn*I-digested 9.4-kb fragment of phage SC921 was ligated to pUC19 to construct a recombinant plasmid, pYJ12 (12 kb).

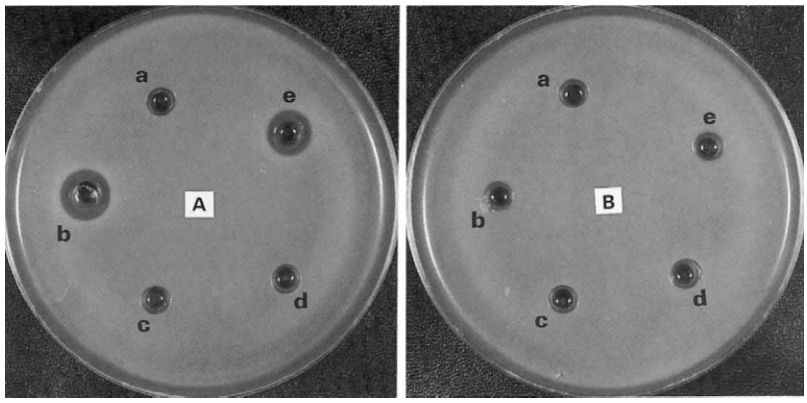

Fig. 4. Well diffusion assay for the expression of lysin gene by the cell-free extracts (A) and cell supernatants (B) of *E. coli* transformants grown in LB broth. Wells: a—*E. coli* (pYJ05); b—*E. coli* (pYJ12); c—*E. coli* (pYJ13); d—*E. coli* (pYJ14); e—*E. coli* (pYJ15).

according to classifications designated by the International Committee on Taxonomy of Viruses (ICTV) (Braun et al., 1989). The bouyant density of this phage was about 1.58 g/cm^3 by CsCl density-gradient ultracentrifugation.

SDS-PAGE showed that the virion contained two major proteins with a molecular weight of 32 and 34 kDa, and some minor proteins ranging from 13 to 112 kDa (Fig. 2). All minor proteins were barely visible after staining with Coomassie blue.

3.3. Phage genome

The G + C content of the phage DNA was estimated to be 39.4% by observation of a melting temperature of 85°C (data not shown). To determine the genomic size, the SC921 DNA was digested with three different restriction enzymes, *Sma*I, *Kpn*I, and *Xba*I. The restriction fragments were analyzed by 0.7% agarose gel electrophoresis. *Kpn*I cleaved the SC921 genome into six fragments. *Sma*I was a single site cutter, while *Xba*I had a double cut in the SC921 DNA. The length of the genome was calculated as the sum of the lengths of the restriction fragments as shown (data not shown). The genomic DNA was a double stranded linear molecule and estimated to be 66.5 kb in length.

3.4. Cloning of lysin gene in E. coli

In attempt to clone and express the lysin gene in *E. coli*, a genomic library was constructed in *E. coli*

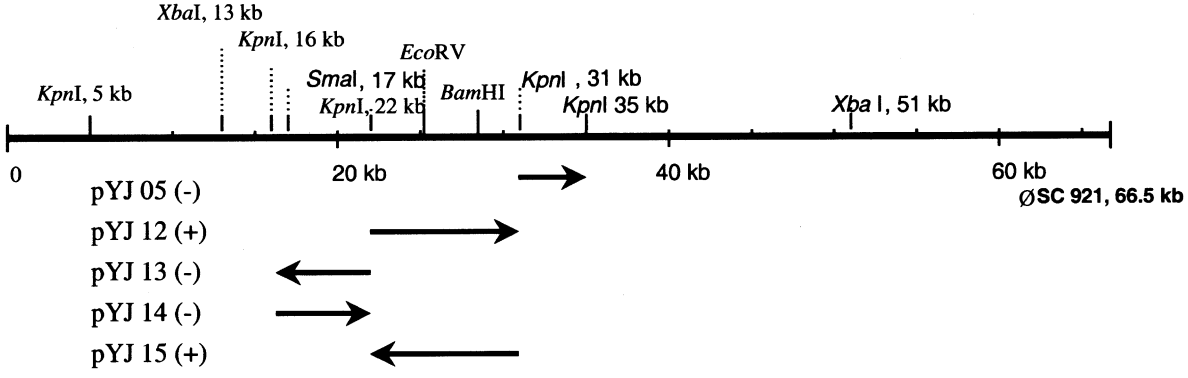

Fig. 5. Physical map of an *L. plantarum* phage SC921 genome. The arrows show the phage DNA insert cloned in pUC19. The lysin phenotype (+ or −) phenotype was assayed against *L. plantarum* LA0280 lawn as shown in Fig. 4.

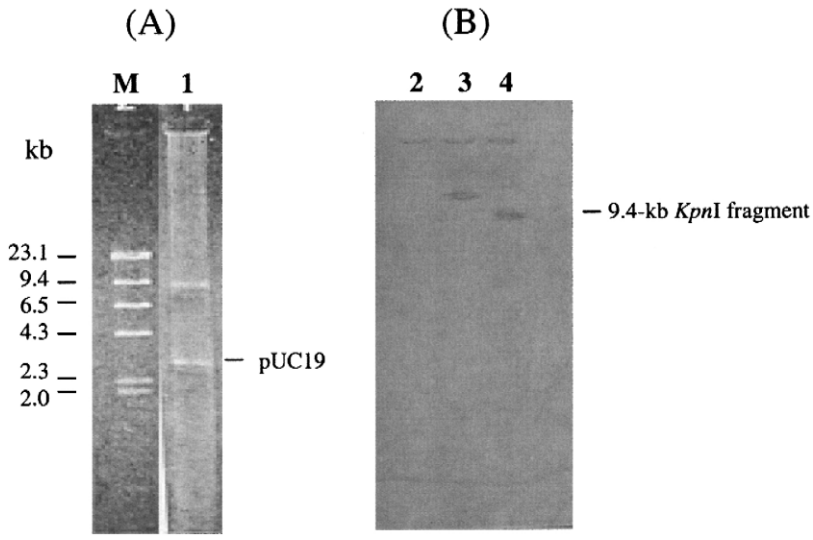

Fig. 6. Agarose gel electrophoresis of recombinant plasmid pYJ12 and *Kpn*I-generated DNA fragments of phage SC921 (A) and a Southern blots hybridization with DIG-labeled, 9.4-kb *Kpn*I fragment of SC21 DNA (B). Lanes: M, DNA/ *Hin*dIII marker; (1) fragments of pYJ12 digested with *Kpn*I; (2) pUC19; (3) pYJ12; (4) fragments of phage ϕSC921 digested with *Kpn*I.

(Fig. 3) as described above. Transformants harboring the plasmids which carry the lysin gene were selected by their ability to form plaques around colonies on the MRS soft agar plate containing indicator strain, *L. plantarum* LA0280. Out of about 3000 *E. coli* transformants subjected to the screening test for lysin activity, two plaque-forming clones were obtained. These two clones showed both Lys^+ phenotype and ampicillin resistant and two recombinant plasmids, designated pYJ12 and pYJ15, were isolated from each clone. As shown in Fig. 4, lysin activities were detected only in the cell-free extract fractions of two transformants carrying recombinant plasmids, but not in cell-free supernatants. By restriction endonuclease digestion analysis, it was confirmed that the inserted DNA into pUC19 plasmid was the same size, 9.4-kb, but placed in opposite direction (data not shown). The 9.4-kb *Kpn*I fragment was identified to be near the middle of the phage genome by restriction analysis (Fig. 5).

3.5. DNA–DNA hybridization

We were interested in confirming whether the cloned 9.4-kb *Kpn*I fragment from pYJ12 and pYJ15 originated from SC921 DNA. The 9.4-kb *Kpn*I fragment was used as the lysin gene probe for a hybridization experiment. Southern blot hybridization analysis showed this probe gave hybridization signals with both pYJ12 and a *Kpn*I-digested pYJ12 plasmid DNA fragments, but not with pUC19 as a control (Fig. 6). Thus, it was confirmed that this recombinant clone had a 9.4-kb *Kpn*I fragment of the SC921 genomic DNA.

4. Discussion

According to a recent paper (Ackermann, 1996), 96% of all phages investigated in the last 40 years have turned out to be members of either the *Siphoviridae*, *Myoviridae*, or *Podoviridae* families (Braun et al., 1989). Morphologically, the phage SC921 belongs to Bradley's group B (Bradley, 1967) or the *Siphoviridae* family, with an isometric head and a long non-contractile tail. Previously, Trevors et al. (1983) reported that *L. plantarum* bacteriophage from a meat starter culture belongs to Bradley's group A, with an isometric head and short contractile head. Bacteriophage B2 of *L. plantarum* ATCC 8014 (Nes et al., 1988) belonged to Bradley's group B, with an isometric head of 110 nm in diameter and

a long non-contractile tail (about 500 nm in length). Casso et al. (1995) also reported temperate and virulent bacteriophages of isometric heads and long non-contractile tails belong to the *Siphoviridae* family. We recently isolated *L. plantarum* bacteriophages (Fig. 7) from fermenting sauerkraut of which morphological characteristics are very close to the *Podoviridae* family (Braun et al., 1989). Thus, there has been so far no consistent data on the morphology of *L. plantarum* bacteriophage. For a short-tailed phage group, they had isometric heads 50–54 nm in diameter and rigid tails 160 nm long with contractile sheaths 17×150 nm (Sechaud et al., 1992). The sheath contained about 40 rows of transverse striations, indicative of a helical organization of protein subunits. On the other hand, long-tailed phages had isometric heads 50–60 nm in diameter and slightly flexible 260 nm long tails with non-contractile sheaths 17 by 250 nm. The sheath of the SC921 had a cris-cross appearance that was distinct from the striated sheaths of the short-tailed phages.

Since many phages require divalent cations, particularly calcium for promoting the infection, the efficiency of plating (EOP) was tested with varying concentration of $CaCl_2$ (0, 10, 25, 50, 100 mM tested) in MRS broth. The result (Fig. 8) showed that calcium concentrations profoundly affected plaque formation with the overlay method. Highest EOP was obtained at 25 mM $CaCl_2$. The EOP increased with the $CaCl_2$ concentration up to 25 mM, but decreased at the higher levels.

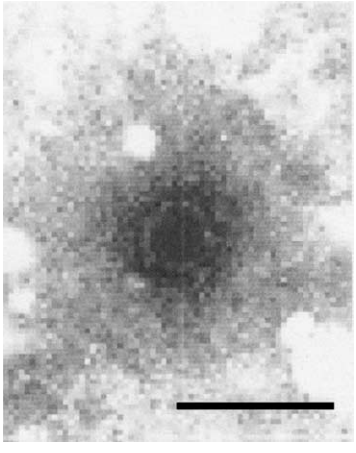

Fig. 7. Electron micrograph of *L. plantarum* phage from sauerkraut. Magnification, 85,000× (bar = 100 nm).

It is now generally accepted that organization of phage genome consists of a *cos*-type (cohesive ends) and a *pac*-type (packaging sites). A *cos*-type phage always contains the precise genome length of concatameric phage DNA packaged in the procapsid. The phage packaging sequence starts at a *cos* site and continues to the next *cos* site, where the DNA is nicked by a specific phage gene-encoded nuclease called terminase (Black, 1988). For *pac*-type phages, packaging of DNA starts at a *pac* site and continues until the empty phage capsid is filled (headful mechanism). This mechanism requires that procapsid package more than one genome length of phage DNA molecules that are terminally redundant and circularly permuted (Black, 1988; Forsman and Alatossava, 1991). In order to confirm the genomic organization of the SC921, both *Exo*III enzyme and S1 nuclease were added separately to the DNA sample. The *Exo*III sequentially degrades the DNA molecule from 3′- to 5′-end, but S1 nuclease is active only on single-stranded DNA (Sambrook et al., 1989). As shown in Fig. 9, the size of phage genomic DNA was reduced after treatment with both enzymes. Because no subfragments (Forsman and Alatossava, 1991) were detected after treating the restriction digests of DNA at 70°C for 10 min, the phage genome was considered as a linear double-stranded molecule with cohesive ends. Similar results have been found for several virulent lactococcal phages (Powell et al., 1989). As is often the case, all lactococcal phage genomes, which have been investigated, contain double-stranded linear DNA (Jarvis et al., 1991).

Lysins belong to a group of murein-degrading enzymes (muramidase enzymes) and have been divided into several subgroups: lysozyme, transglycosidase, L-alanine amidase, or endopeptidase (Sable and Lortal, 1995). The *lytA* gene encoding the lysin A of bacteriophage US3 (Platteeuw and de Vos, 1992) was cloned and sequenced to 774 bp in genome size, corresponding to a protein of 258 amino acids with deduced molecular mass of 29 kDa protein. Interestingly, the amino acid sequence of *LytA* was similar to that of the autolysin of *Streptococcus pneumoniae*, suggesting that the lysin could be an amidase. Recently, the bacteriophage c2 lysin was

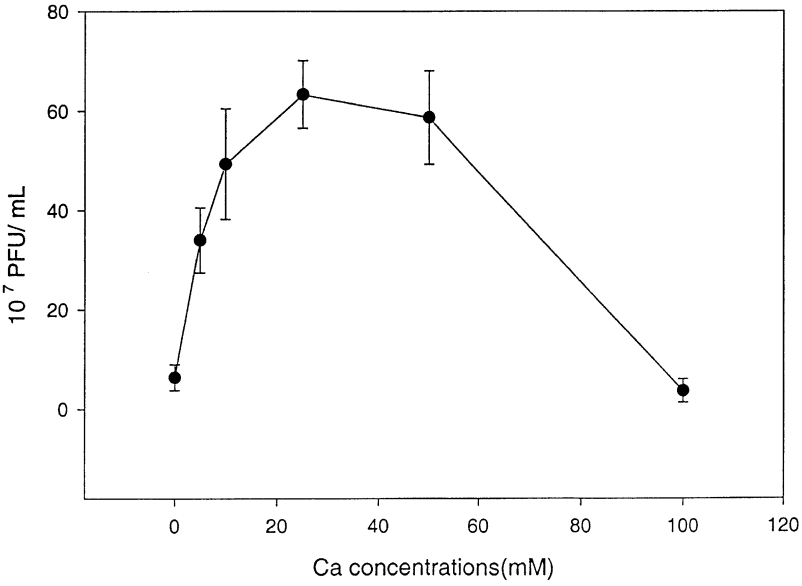

Fig. 8. Effect of $CaCl_2$ concentration on the plaque formation by *L. plantarum* phage SC921.2 M $CaCl_2$ stock solution pre-warmed to 50°C was adjusted to each concentration in 0.7% MRS soft agar containing indicator strain and overlaid on MRS hard agar (1.5%), followed by 24-h incubation at 37°C.

expressed in *E. coli* and sequenced (Ward et al., 1993). This sequence showed a high degree of similarity with the ML3 lysin gene. In the case of the dairy lactobacilli, the lysin of the phage PL-1 infecting *L. casei* seems to be specific to the host strain. In 1986, the genome of the virulent phage, LL-H infecting *Lac. lactis* was characterized and the gene coding for the phage lysin cloned in *E. coli* (Trautwetter et al., 1986). The *lysA* gene encoding the lysin of temperate bacteriophage mv1 infecting *L. delbruechii* subsp. *bulgaricus* was also cloned in *E. coli* (Boizet et al., 1990). Moreover, a protein produced heterologously by *E. coli* was active on some strains of *L. delbrueckii* subsp. *bulgaricus* and *L. helveticus* and *S. salivarius* subsp. *thermophilus*, but not for *Lactococcus* strains. Heterologous expression and activity of the lysin enzyme from bacteriophages in *E. coli* would require that the protein be secreted through the cell membrane by aid of the holin gene product. There were no attempts to confirm the expression of the holin gene, which precedes the lysin gene, by this experiment. For the positive results of recombinant clones with lysin activity, the effect of *E. coli* autolysin was excluded in the present study because the cell-free extract of *E. coli* (control) did not show lysin activity against *L. plantarum* LA0280 (Fig. 4). The physical location of the

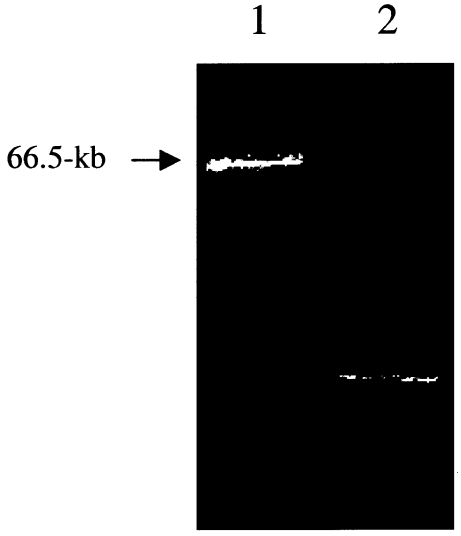

Fig. 9. Analysis of the double-stranded phage DNA terminus. The DNA of *L. plantarum* phage SC921 was digested with both *Exo*III enzyme and S1 nuclease, and electrophoresed on a 0.9% agarose gel. Lanes: (1) intact DNA of phage SC921; (2) DNA treated with both *Exo*III and S1 nuclease.

SC921 lysin gene (Fig. 5) in the double stranded DNA is consistent with other work (Ward et al., 1993), in which it has been located in the middle of the genome.

In considering that a cloned lysin gene(s) has formed the basis of a novel strategy for generating autolytic strains of lactococci, the autolytic strains have potential application in cheese-making to improve flavors and reduce the ripening time (Jarvis, 1989). Likewise, the lysin gene product could be applied to delay over-ripening problems in vegetable fermentations, which is known to be caused mainly by acid-tolerant LAB, including *L. plantarum*. Subcloning and analysis of genetic make-up of the holin or possibly the holin–lysin gene cassette is being studied further.

5. Conclusion

A lytic bacteriophage active against *L. plantarum* is of interest from the industrial point of view because it can be applied to vegetable fermentation for delaying over-acidification. One *L. plantarum* phage species was isolated from the fermented vegetable, *Kimchi*. This phage is able to infect 6 of 11 strains of *L. plantarum* tested. Morphologically, it belongs to Bradley's group B or the *Siphoviridae* family according to International Committee on Taxonomy of Viruses. The phage particle contains two major structural proteins and several minor proteins. The organization of the genome was a double stranded linear DNA molecule with cohesive ends and the length of the genome was estimated to be 66.5 kb. The [G + C] content of the phage DNA is about 39.4%. For this lysin gene study, 9.4 kb of *Kpn*I-digested DNA fragment was cloned into pUC19 and successfully expressed in *E. coli*, indicating this fragment contains genes responsible for the lysis of *L. plantarum*. The cloned fragment in pUC19 was hybridized to a 9.4-kb fragment generated by *Kpn*I digestion of the SC921 as a probe and locates near the middle of the linear phage genome. As suggested by some workers previously, lysins have the potential for use as growth and acid production inhibitors in vegetable fermentations and in cheese-making to improve flavors and reduce the ripening time.

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