ABSTRACT

LU, ZHONGJING. The ecology and genetics of bacteriophages in commercial vegetable fermentations. (Under the direction of Drs. Henry P. Fleming and Fred Breidt, Jr.)

The objectives of this study were to investigate the ecology of bacteriophages (phages) infecting lactic acid bacteria (LAB) in commercial sauerkraut fermentations, to explore the possible role phages may play in microbial succession during the fermentations, to characterize predominant phages in commercial sauerkraut fermentations, to isolate a phage infecting a starter culture, *Lactobacillus plantarum* MU45, from a commercial cucumber fermentation, and to determine and analyze the complete genome sequence of the *L. plantarum* phage.

A total of 171 independent phage isolates, including at least 26 distinct phages, were obtained from four commercial sauerkraut fermentations (90-ton fermentation tanks) in 2000 and 2001. Host range and the temporal sequence of occurrence of these phages were determined. Twenty-eight distinct bacterial hosts, including *Leuconostoc*, *Lactobacillus*, and *Weissella* species, were identified by ITS restriction and 16S rDNA sequence analyses. It was found that there were two phage-host systems in the fermentations, with the dividing line occurring between day 3 and day 7 after the start of the fermentations, corresponding to the population shift from heterofermentative to homofermentative LAB. The data strongly suggested that phages may play an important role in the microbial ecology and the succession of LAB species in vegetable fermentations. Eight phage isolates which were independently obtained two or more times were further characterized. They belonged to the *Myoviridae* or *Siphoviridae* family, and showed distinct host ranges and DNA fingerprints. These results

demonstrated for the first time the complex phage ecology present in commercial sauerkraut fermentations, providing new insights into the bioprocess of vegetable fermentations. More research is needed to evaluate the impact of these phages on vegetable fermentations.

Additionally, a virulent phage ΦJL-1, active against *L. plantarum* starter cultures, was isolated from a commercial cucumber fermentation. The phage has an isometric head and a long non-contractile tail, and belongs to morphotype B1 within the *Siphoviridae* family. The host range of phage ΦJL-1 was limited to two related strains of *L. plantarum*, MU45 and BI7. Using *L. plantarum* MU45 as a host, the phage had an average burst size of 22 and a latent period of 35 min. The phage possesses a linear double-stranded DNA genome consisting of 36,674 bp with a G+C content of 39.4%. Forty-six possible open reading frames (ORFs) were identified. According to the N-terminal amino acid sequencing and bioinformatic analyses, proven or putative functions were assigned to 17 ORFs (39%), including 6 structural protein genes. It was found that functionally related genes were clustered together in the ΦJL-1 genome, resulting in a modular genome structure: DNA packaging, head and tail morphogenesis, lysis, DNA replication, and transcriptional regulation modules. This genomic organization was similar to several other phages infecting lactic acid bacteria. An attempt was made to experimentally determine the anti-receptors of phage ΦJL-1 via phage display technology.

The results from this study indicate that phage infection is common in vegetable fermentations, suggesting that a phage-control strategy will be needed in any vegetable fermentations relying on use of starter cultures. The genetic information obtained from phage ΦJL-1 and other phages is fundamental to the understanding of the phage-host interaction in

vegetable fermentations and to the development of phage-control strategies for high and consistent quality of fermented vegetable products.

THE ECOLOGY AND GENETICS OF BACTERIOPHAGES IN COMMERCIAL VEGETABLE FERMENTATIONS

by

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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

FOOD SCIENCE

Raleigh

2002

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BIOGRAPHY

Zhongjing (Jean) Lu is originally from China. She holds a B.S. degree in Chemical Engineering and had several years' college teaching/research experience before coming to the United States as a visiting scientist at Purdue University in 1991. A few years later, she decided to pursue an advanced degree in Food Science. She received her B.S. in Food Science from Louisiana State University in 1996, and a M.S. degree in Food Science from North Carolina State University in 1999. Thereafter, she began her Ph.D. program in Food Science at North Carolina State University under the direction of Drs. Henry P. Fleming and Fred Breidt, Jr.

ACKNOWLEDGMENTS

I would like to express deepest appreciation to Dr. Henry P. Fleming for giving me the opportunity to complete my studies under his direction. He has been not only my advisor and mentor, but also a great friend. His wisdom, guidance, encouragement, understanding, and patience made my study and research enjoyable and memorable.

I wish to thank Dr. Fred Breidt for his insight, guidance, and encouragement throughout the course of this study. Without his guidance and strong support, my graduate studies would not have been so rewarding.

I also wish thank Dr. Todd R. Klaenhammer for his insight, inspiration, advice, and support. It is he who makes me so interested in bacteriophages and phage display technology. He is one of the greatest teachers I have ever had. I enjoy his lectures and challenging questions.

A special thank goes to Dr. Francis G. Giesbrecht for his advice, understanding, and a long-term support. He has served on both my Master and Ph.D. committees.

I greatly appreciate Dr. Eric Altermann for his great help with sequence analysis, Dr. Paul Predki for his generous help with bacteriophage genome sequencing, Vethachai (Eddie) Plengvidhya for assistance in host identification, and Bush Brothers & Company (Shiocton, Wis) for collecting cabbage and brine samples. I would like to thank Dr. Sung-Sik Yoon for introducing me to bacteriophage research, and Dr. Roger F. McFeeters for his continuous support. I am grateful to the following individuals for their help and friendship: Rodolphe Barrangou-Poueys, Evelyn Durmaz, Dean Toler, Roger Thompson, Laura Reina, Janet Hayes, Suzanne Johanningsmeier, Debra Clare, and Sabine Morris.

Finally, I wish to thank my parents, husband, daughter, sister, and friends for their love, understanding, encouragement, and patience throughout all these years.

The next morning [after the experiment], on opening the incubator, I experienced one of those rare moments of intense emotion which reward the research worker for all his pains: at the first glance I saw that the culture which the night before had been very turbid, was perfectly clear: all the bacteria had vanished, they had dissolved away like sugar in water. As for the agar spread, it was devoid of all growth and what caused my emotion was that in a flash I had understood: what caused my clear spots was in fact an invisible microbe, a filterable virus, but a virus which is parasitic on bacteria.

Felix d'Herelle, French Bacteriologist.
On his discovery of bacteriophages in 1916.

(Felix d'Herelle. 1949. The Bacteriophage. Science News **14**: 44-59.)

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SAUERKRAUT FERMENTATION

Introduction

Fermentation is a very ancient method of preserving vegetables and other foods. Most vegetable fermentations are a spontaneous fermentation by a group of organisms, lactic acid bacteria (LAB) naturally associated with the plants. The primary function of the LAB is the production of lactic acid and other substances from sugars present in the raw materials. These substances, mainly acids, inhibit the growth of the various undesirable spoilage organisms, thereby converting the food from a microbiologically unstable to a stable product. Many vegetables such as cabbage, cucumbers, olives, peppers, cauliflower, carrots, radishes, and beans are home-fermented or commercially fermented in many countries. In the United States, sauerkraut, cucumbers, and olives are of significant commercial importance.

Sauerkraut is fermented, shredded cabbage. The German word, sauerkraut, literally translated means acid (sour/sauer) cabbage (kraut). It is believed that initial styles of sauerkraut were made by dressing cabbage leaves with sour wine or vinegar (Vaughn, 1985). Later, cabbage was broken or cut into pieces, packed into containers and covered with acidic 'verjuice' from green (unripe) grapes or other sour fruit. The time of transition from acidic liquids to salt brine is unknown. Vaughn (1985) speculated that the method used today developed during the period AD 1550 to 1750. The basic method has not changed greatly over centuries. The preservation relies on the salt added, exclusion of oxygen, lactic acid produced, and the removal of fermentable sugars. Sauerkraut fermentation remains an important means of preservation today because of the unique flavors produced and low energy requirements for storage and processing (Fleming et al., 1988).

Sauerkraut Processing

A variety of cabbage can be used for the commercial production of sauerkraut. Generally, cabbage leaves contain approximately 5% fermentable sugars including glucose and fructose (about 2.5% each), and sucrose (0.1-0.4%). In cabbage core, sucrose

constitutes a much greater fraction of the fermentable sugars. Malic acid concentration is higher in the leaves than the core. Overall, the core accounted for 23% and the leaves 77% of the cabbage weight (Fleming et al., 1988). An optimal cabbage for sauerkraut making contains at least 3.5% sugar within its tissue. Some cabbage contains antibacterial compounds such as methyl methanethiosulfinate and glucosinolate hydrolysis products including isothiocyanates, thiocyanates, and nitriles (Kyung and Fleming, 1994a, 1994b; Shofran, et al., 1998).

Sauerkraut is produced in the United States in bulk tanks raging in capacity from 45 to 150 tons (Fleming et al., 1988). Mature heads of cabbage are trimmed to remove the outer green leaves (up to 30% of the total weight). The cores are cut by a reversing corer that leaves the cut core in the head (Fig. 1). The head of cabbage is shredded as fine as 0.08 to 0.16 cm. Shredded cabbage is dry-salted and conveyed into the tanks. The tanks are usually covered with plastic sheeting, which is then weighted down with water or weak brine to minimize the contact of shredded cabbage with air, thereby inhibiting the growth of yeasts and molds. Brine begins to form as soon as the cabbage is salted due to osmotic extraction of water from the cut cabbage tissue. Anaerobic condition within the tank is quickly established due to the depletion of oxygen and production of CO_2 by the growing certain LAB strains. This favors the growth of other LAB.

In the sauerkraut-producing areas of the United States, an average temperature of about 18°C and 2.25% NaCl (by weight) are considered ideal for making top quality sauerkraut (Pederson and Albury, 1969; VanGarde and Woodburn, 1994). Higher temperature and/or higher salt concentration inhibit *Leuconostoc mesenteroides*, resulting in less complex flavor or unacceptable harsh flavor (Pederson and Albury, 1969; VanGarde and Woodburn, 1994). However, commercial fermentation occurs at ambient temperatures, which can vary from 10 to 30°C, depending on time of year and weather conditions. Dry salting practices can lead to variations in salt concentration within and among fermentation tanks (Pederson and Albury, 1969). Fermentation is allowed to proceed for as little as a few weeks to as long as a year before processing. Sauerkraut is often stored in the fermentation tanks and canned as needed to fill customer orders. This is an economical way to store sauerkraut but results in a variability in the amount of acid

in end-product (Fleming et al., 1988). Excessive acidity in stored sauerkraut may require a dilution of the brine. This dilution not only reduces desirable flavor and nutrients such as vitamin C, but also causes waste problems. Sauerkraut may be packaged in cans, glass jars, or plastic bags (Fleming et al., 1995). Sauerkraut can be made shelf stable by pasteurization at 74-82°C for about 3 min. Refrigerated sauerkraut is made from an unpasteurized product to which sodium benzoate (0.1% w/w) and potassium metabisulphite are added as preservatives.

Fermentation microbiology

Although sauerkraut recipes are very simple: shredded cabbage and salt, the fermentation of sauerkraut is a complex process involving the sequential growth of various microorganism populations (Pederson and Albury, 1969; VanGarde and Woodburn, 1994). A wide variety of microorganisms are present on fresh cabbage. Gram-negative aerobic bacteria are typically predominant. Lactic acid bacteria only count for a very small portion of the initial population (Mundt et al., 1967; Mundt and Hammer, 1968; Mundt, 1970). However, the majority of the natural flora are suppressed early in the fermentation by the salt added, which gives LAB a competitive advantage. It is believed that the fermentation is initiated by *Leuconostoc mesenteroides*, and succeeded in sequence by *Lactobacillus brevis*, and *Lactobacillus plantarum* (Fig. 2). The correct sequential growth of various LAB is critical in making a stable product with the typical flavor and aroma of sauerkraut. Sequential growth of specific lactic acid bacteria depends on the biological (their initial abundance on cabbage, growth rate, salt- and acid-tolerance), the chemical (sugar and salt concentrations, acids, and pH) and physical (temperature, anaerobiosis) conditions (Daeschel et al., 1987). As these conditions change during the fermentation, so do the dominant LAB species (Pederson and Albury, 1969).

Sauerkraut fermentation can be divided into two stages: heterofermentative or gaseous stage, and homofermentative or non-gaseous stage (Fleming et al., 1988). Heterofermentative *Leuconostoc mesenteroides* initiates and dominate the gaseous stage because it is present at an initially higher number (Mundt et al., 1967; Mundt and Hammer, 1968; Mundt, 1970), has a shorter generation time (Stamer et al., 1971), and

grows more rapidly at lower temperature than other LAB found on plants (Pederson and Albury, 1969). This species converts sugars to lactic acid, acetic acid, ethanol, carbon dioxide, mannitol, and dextran (Fig. 3). Other chemical substances are also produced in variable amounts (Pederson and Albury, 1969). The quality characteristics of sauerkraut are largely dependent upon the growth of this species (Pederson and Albury, 1969). As the fermentation continues, LAB cells are exposed to increasingly lower pH values through their own metabolic activity. When pH quickly reaches 4.5 (approx. 1% lactic acid). *L. mesenteroides* rapidly dies off due to its relatively low acid tolerance (McDonald et al., 1990). The gaseous stage establishes an anaerobic environment for continuing lactic acid fermentation. Homofermentative *Lactobacillus brevis*, *Pediococcus pentosaceus* (formerly *cerivisiae*), and *Lactobacillus plantarum* take over and predominate during the non-gaseous stage. These LAB utilize the remaining sugars to produce large amount of acid (primarily lactic acid) (Fig. 3), and further lower the pH. They also remove mannitol, a bitter-flavor compound produced by *Leuconostocs* (VanGarde and Woodburn, 1994). *Lactobacillus plantarum* terminates the lactic acid fermentation with the final pH of about 3.5 and total acidity of 1.7 to 2.3%, expressed as lactic acid (Pederson and Albury, 1969; Vaughn, 1985). As a general rule, one-half as much acid is produced as there is sugar in the cabbage (VanGarde and Woodburn, 1994). A variety of products give a complex flavor which distinguishes fermented products from acid-added products.

Currently, commercial sauerkraut fermentations in the United States are natural fermentations. Top quality sauerkraut is not produced in every batch. The products can be highly variable due to the variations in the microorganisms on cabbage and environmental conditions. Many attempts have been made to apply starter cultures to sauerkraut fermentation for consistent and high quality of products (Pederson and Albury, 1969; Narbors and Salunkhi, 1969). However, development of a starter culture is difficult given the normal and desirable succession of microorganisms in a spontaneous fermentation and the inability to pasteurize the raw material without detrimental textural problems (Harris, 1998). It is difficult to duplicate the complex changes produced during natural fermentation and resulting characteristics of sauerkraut. In most cases, inoculation with single or mixed starter cultures of *L. mesenteroides*, *Lb. brevis*, and *Lb. plantarum*

resulted in little, if any, improvement in product quality over uninoculated controls (Keipper et al., 1932; Lopez et al., 1954; Narbors and Salunkhi. 1969; Yago et al., 1985). Stamer (1968) evaluated the flavor of cabbage juice fermented with pure single strain cultures of lactic acid bacteria. Juice fermented with *L. mesenteroides* was mild and pleasantly aromatic, while that fermented by *Lb. brevis* was harsh and vinegar-like. *Lb. plantarum* and *P. pentosaceus* produced products were dull in taste, lacked body, and were judged to be unacceptable. More research is needed for the development of controlled fermentation to achieve consistent and high quality sauerkraut products.

Defects of sauerkraut and their control

A variety of defects is known to affect sauerkraut quality. Softening is a common texture defect in sauerkraut. It is caused by enzymes from the plant or from yeasts and molds. Salt and acid inhibit enzymes from the plant, while anaerobic conditions prevent the growth of yeasts and molds, and thus the production of softening enzymes from these microorganisms. Poor flavor kraut lacks the proper combination of acids and other products of fermentation. Certain organisms need to be present in a specific sequence for the finished fermentation to have optimum flavor. These microbial populations can be controlled by selecting fresh, sweet cabbage, by adding 2.25-2.5% salt, by storing the fermentation container at temperature below 27°C, and by stopping the fermentation when the quality is optimum (VanGarde and Woodburn, 1994). Sauerkraut may darken at the surface due to oxidation. Enzymes from yeasts and molds growing on the surface are the major cause of the oxidation. Uneven salting and a high temperature increase the rate of color change. The discoloration problem can be minimized by weighting shredded cabbage down below the surface to prevent the surface growth of yeasts and molds, by proper salting and temperature control. Pink sauerkraut is another problem in sauerkraut, but not very common. Cabbage contains a plant pigment (anthocyanin) that can exist in different color forms depending on pH. It is blue near neutral pH, colorless in slightly acid conditions, pink or red at low pH. The pigment is harmless to consume, as is pink sauerkraut (VanGarde and Woodburn, 1994). Pink kraut can also result from yeast growth, especially if the salt was unevenly distributed and the temperature is high. This also is not harmful. Slimy or ropy kraut has been recognized as a defect. It is thought to

be the result of dextran produced by *L. mesenteroides*. Given the low concentration of sucrose in cabbage, this is less likely than some other type of extracellular polysaccharide, possibly produced by an organism other than *L. mesenteroides* (Harris, 1998).

BACTERIOPHAGES

Introduction

Bacteriophages (phages) are viruses that infect bacteria. It was over a century ago when Hankin (1896) reported the bacteriocidal activity of filtered water collected from the Ganges and Jumma rivers on *Vibrio cholera*. He speculated that drinking the river water was effective in preventing the spread of cholera. Twenty years later, Frederick Twort (1915) in the United Kingdom and Felix d'Herelle (1917) in France independently identified bacteriophages as filterable, transmissible agents of bacterial lysis (Ackermann and DuBow, 1987a). d'Herelle called bacteriophages “eaters of bacteria.” However, the submicroscopic "sperm-like" morphology of phages remained undetected until the first electron microscopes became available during the 1940s.

Phages are the largest of all virus groups. They have been found in over 140 bacterial genera. Phages have been found in every conceivable habitat in nature, and may be found there in enormous numbers (Ackermann, 2001). Many phages occur as prophages in lysogenic hosts rather than free in the environment.

Phages are classified by morphotype and host genus. Phages may presently be divided into 21 morphotypes (Fig. 4), corresponding to 13 phage families (Table 1). Phages are tailed, cubic, filamentous, or pleomorphic. Over 5100 phages have been examined in the electron microscope since 1959. About 96% of these phages are tailed phages (Ackermann, 2001). Tailed phages constitute the order *Caudovirales* (Ackermann, 1999), and are distributed unevenly into three families: *Siphoviridae* (61%), *Myoviridae* (25%) and *Podoviridae* (14%) (Ackermann, 2001). The tail of tailed phages is unique or rare in viruses (Fig. 5). Generally, it is a hollow proteinic tube of fixed length and width (with respect to species), built of a number of stacked rows of subunits, and provided with conspicuous transverse striations in the *Myoviridae* and *Siphoviridae*

families. Most tailed phages have isometric heads, and only some 15% of tailed phages have elongated heads. Tailed phages are virtually ubiquitous and occur in all parts of the bacterial world, including lactic acid bacteria (LAB) of industrial importance, such as lactococci, lactobacilli, and pediococci (Jarvis, 1989).

Like other viruses, phages are small particles, usually about 20-200 nm long, consisting of genetic material (DNA or RNA, single- or double-stranded) surrounded by a layer of proteins, which form a shell or "capsid" that protects the genomic material. Viruses are sub-microscopic, obligate intracellular parasites. No known virus has the biochemical or genetic potential to generate the energy necessary for driving all biological processes, e.g. macromolecular synthesis. They are therefore metabolically inert in their extracellular form, a "virion." They replicate within living host cells by using cellular machinery and resources, such as ribosomes, ATP, nucleotides, tRNAs, and amino acids. Most of the known virus types code for their own capsid proteins. But satellite viruses obtain capsid proteins from a helper virus that coinfects the same host cell (Hurst and Lindquist, 2000). Virus particles are produced from the assembly of pre-formed components.

Phages have some unique features, which set them apart from other virus groups. Phages are relatively small, in the < 0.45 μm size range. They have a simple structure and genetics - genome encapsulated by capsid protein. The majority do not have envelopes. The typical phage replication cycle is very short and often in the order of 20 minutes. When a phage infects a cell, only the phage genome, not the whole virion, enters the cell. The empty capsid remains outside. Phage infection generally leads to lysis of the host.

Genetics of bacteriophages

Phage genomes typically consist of either single or double stranded DNA or RNA, or segmented dsRNA (Table 1). Phage genome may vary in size from 4 kb to 725 kb (Ackermann, 1999) and may be circular or linear. In the case of phage ϕCH 1 from *Natronobacterium magadii* the phage head contains both DNA and RNA (Whitte et al., 1997). Generally, ssDNA molecules are circular, whereas all other viral nucleic acids are linear (Birge, 2000a). The circularity can be attributed to the difficulty of protecting

linear single-strand DNA from exonucleolytic attack. Temperate phages are generally dsDNA > 20 kb.

The genome of tailed phages is usually a single molecule of linear dsDNA. Typically, tailed phages consist of DNA and protein only, devoid of lipids and carbohydrates. Tailed phages contain about 50% DNA, the highest DNA content of all viruses (Ackermann, 1999). The reported highest DNA contents elsewhere in virology are 20% for adenoviruses and 14-15% for tectiviruses, respectively (Murphy et al., 1995). The high DNA content of tailed phages evidently reflects the high buoyant density of phage particles. The molecular weight of tailed phage DNA varies between 17 and 725 kb. Its distribution shows a sharp peak at 50 kb (Ackermann and DuBow, 1987b). *Bacillus megaterium* phage G has the largest known genome (725 kb) in the viral world. As far as known, large parts of tailed phage genomes are nonessential. Phage P22 can lose 40% of its genome without affecting lytic growth or lysogeny. This leaves many opportunities for the acquisition of foreign genes. Guanine-cytosine (GC) contents of tailed phages usually parallel those of their hosts.

Tailed phage DNAs have numerous particulars of genome anatomy or composition, some of which are rare or absent in other viruses, such as *pac* and *cos* sites. A *pac* site is the site on the DNA molecule where the first cut occurs for initiation of DNA packaging. *Cos* sites or cohesive ends are single-stranded "sticky" DNA overhangs of 7 to 21 nucleotides that enable phage genomes to circularize after infection and are also involved in DNA packaging. Phages with *pac* sites may be virulent or temperate. Phages with *cos* sites are generally temperate. *Pac* and *cos* sites have been found in many tailed phages and are mutually exclusive, so that tailed phages can be divided into *pac* and *cos* types. The basic difference between *pac*- and *cos*-type phages is reflected in their genomic maps. *Pac*-type phages, with their circularly permutated genomes, generally have circular maps whereas *cos*-type phages, with nonpermutated genomes, have linear maps.

Many new insights were obtained by a detailed analysis of new data from lambdoid phages, especially of head and lysis genes. These include: genes with related functions clustered together; genes of different phages could be homologous (related) or analogous (encoding different proteins with identical functions); gene orders were more

conserved than nucleotide sequences; tailed phages could form "quasi-species" with little sequence similarity, but the same gene orders and transcription patterns; and tailed phages routinely acquired genes from their hosts, phages, or other sources (Ackermann, 1999). The present survey of the available functional maps confirms or shows that:

1. Genes with related functions cluster together

2. Roughly 50% of genes are morphopoietic.

3. Morphopoietic genes are generally located at the left end or the center of the genome.

4. Head genes usually precede tail genes.

5. Genes for lysis, integration-excision, or DNA replication, *pac* sites and origins of replication occupy variable positions with respect to morphopoietic genes. No pattern is apparent and no hypothetical "ur-genome" can be constructed with these elements.

6. DNA polymerases may or may not be present and are not a universal feature of tailed phages.

No genomes of other dsDNA viruses are remotely comparable to those of tailed phages. At least in lambdoid phages, functional clustering of genes provides finer levels of regulation because genes whose products interact with each other occupy adjacent positions. This would constitute a powerful mechanism to ensure evolutionary stability.

There is ample evidence for horizontal gene transfer: not only between phages, but also between phages, plasmids, transposons, other viruses, bacteria, and eukaryotes. Some phage proteins can even be traced to humans (Ackermann, 1999). Amino acid sequence alignments (notably of DNA polymerases, integrases, and peptidoglycan hydrolases) indicate frequent events of horizontal gene transfer in tailed phages. Common capsid and tail proteins have not been detected. Tailed phages often have unusual bases. These modified bases may replace normal bases completely or in part. These unusual bases protect phage DNA against degradation by bacterial restriction endonucleases (Ackermann, 1999).

Lifestyles of bacteriophages

Phage infections begin with adsorption of viral particles to specific receptor sites on the host cell surface. All tailed phages adsorb to bacteria by their tails. Most adsorb to

the cell wall. Some tailed phages adsorb to cell pili, flagellae, or capsules, but all reach eventually the cell wall by pilus retraction, sliding along the flagellae, or digestion of the capsule (Ackermann, 1999). Once adsorbed, tailed phages digest the cell wall using specialized enzymes located at the tail tip and inject their DNA through the cytoplasmic membrane. The empty capsid remains outside. After entering the cell, the phage genome may cyclize by joining cohesive ends (λ, P2) or by site-specific recombination of terminally redundant ends (P1, P22), or remains linear (T4, T7). Cyclization offers protection against host-coded exonucleases.

Tailed phages may have two possible lifecycles: lytic and lysogenic cycles. In the lytic cycle, the phage becomes metabolically active. It takes over host metabolism, shuts off host syntheses, and redirects the host metabolic machinery to begin viral replication and transcription. In tailed phages, phage DNA is characteristically transcribed in three partially overlapping stages: early, middle, and late (Ackermann, 1999). Early genes prepare the host for the phage, initiate DNA replication, and induce the synthesis of regulatory proteins. Typically, lytic phages use host RNA polymerases for transcription of their early genes. Middle genes for DNA synthesis start to operate 3 to 8 minutes after infection and continue to function during the late phase. Middle genes are under phage control to the extent that phages code for DNA polymerases. Finally, late genes, starting as early as 8 minutes after infection, code for structural and lysis proteins and DNA packaging. There is no obvious pattern in the direction of transcription.

DNA replication is as varied as the phages themselves. Rolling-circle replication is a basic mechanism used by the *cos*-type phages. Bidirectional replication is used by T4 and other phages that remain linear genomes. Most tailed phages produce DNA concatemers (a DNA molecule containing several complete phage genomes); those which do not are exceptional. Terminases (phage-coded enzymes) bind to and cut DNA concatemers to size, generally at *pac* or *cos* sites. Assembly of phage particles is a highly ordered, sequential process. Packaging of DNA into preformed capsids (a headful mechanism) seems to be a common feature of most dsDNA viruses with cubic symmetry (Ackermann, 1999). Heads and tails are assembled separately and joined later.

All tailed phages are released by a single event called lysis, in which the cell bursts suddenly and is destroyed. There is no gradual release by transport vesicles,

budding, or extrusion. Lysis is mediated by two types of enzymes: endolysins and holins, which cluster together in a "lysis cassette". Endolysins are peptidoglycan hydrolases, attacking the murein layer of the bacterial cell wall. Holins are small proteins that cause nonspecific lesions in the plasma membrane, allowing endolysins to reach the cell wall. The molecular mechanisms of releasing other phages or eukaryotic viruses are different from those of tailed phages. Filamentous phages such as M13 do not cause cell lysis or death. The progeny is secreted into the medium as the culture grows (Felici et al., 1995). Eukaryotic viruses do not need endolysins because their hosts do not have peptidoglycan. It is noteworthy that cell lysis may not be always accompanied by release of progeny phage particles. An example is "lysis from without", which occurs when infecting a culture with such a high multiplicity of phage particles that all cells lyse as a result of numerous attempts to introduce viral DNA into their cytoplasm.

In the lysogenic cycle, phage DNA is integrated into the host chromosome, resides there, and replicates as a part of the chromosome. Integration is mediated by specific enzymes called integrases. The integrated phage genome is called a prophage. Phages that can establish the prophage state are called temperate phages. A bacterium that carries a temperate phage is referred to as a lysogen. Prophages maintain themselves in a quiescent state by producing a protein repressor that prevents the expression of lytic genes. The lysogenic state (lysogeny) protects the phage from environmental conditions that might damage the capsid or viral nucleic acids (Suttle, 2000). It also confers immunity to the host cell from superinfection (lytic infections by the same phages). The superinfection immunity is due to the presence of repressors that bind to newly arrived phage DNA and turn off lytic functions (Birge, 2000b). Lysogenic infection may give competitive advantages to the host via phage genes, such as restriction modification systems, antibiotic resistance and others properties. Moreover, the phage can serve as a vector of genetic information, carrying host DNA from one cell to another. Lytic extinction of many virulent phage-host systems may force the phage to enter the lysogenic pathway (Paul and Kellogg, 2000). The lysogenic state is not always maintained in all cells of a culture. A prophage can reactivate spontaneously or in response to external stimuli, exit from the host chromosome, and shift to lytic growth. The excision of prophage may require special enzymes excisionases. The sites for

integrase (*int*), prophage attachment (*att*), and excisionase (*xis*) cluster together to form a "lysogeny cassette." The entrance and exit of phage DNA from the host chromosome may or may not be site-specific.

Some phages may be only lytic and some only temperate; some, however, may be either one, depending on growth conditions. Moebus (1983) showed that 10% of 300 marine phage strains examined were temperate. Ackermann and DuBow (1987) indicated that, of 1200 strains of diverse bacteria, an average of 47% contained inducible prophage. Nearly 100% of naturally occurring *Pseudomonas* trains were lysogenic for some temperate phages (Levin and Lenski, 1983). On infection with a temperate phage, a lysogenic decision is made whether to enter a lytic or lysogenic lifestyle. This molecular decision depends on multiplicity of infection (MOI, or the attack ratio of infectious phage particles per host cell; high MOIs favor lysogeny to ensure survival of the phage), nutrient levels (low nutrient concentrations favor lysogeny), physiological status of the host cell, temperature, and levels of toxic agents in their environment (Paul and Kellogg, 2000; Suttle, 2000). Some phages such as P1 do not insert into the host chromosome but, rather, exist as a plasmid within the cytoplasm. Such a state is called pseudolysogeny (Paul and Kellogg, 2000). Pseudolysogeny is an unstable state that may occur under extreme starvation conditions, where there is not enough energy to make the "lysogenic decision."

Nutrients, many agents, and environmental signals can trigger or induce reversion to the lytic pathway, liberating phages. The signal may indicate that there are ample nutrients in the environment, the host is doing well, or it is time to leave. Most inducing agents act on DNA replication (Ackermann and DuBow, 1987a). UV light and mitomycin C are the most widely used inducing agents in laboratory study. However, these two agents may not induce the same prophages. Other agents include radiation, chemical carcinogens, mutagens, base analogues, antibiotics, pressure, and temperature. In λ phage, the induction involves the activation of the recA gene, a part of the SOS response (multiple gene activation) in *E. coli* cell that helps the bacterium survive UV radiation. The repressed phage "senses" that its host has been damaged. RecA protein, which ordinarily facilitates recombination DNA molecules, becomes a special protease: it

cleaves the λ repressor, thereby triggering induction. The phage quickly begins lytic growth (Ptashne, 1986). Induction of only a portion of the indigenous bacterial population could result in a doubling of the phage population.

It is noteworthy that not all phages have lytic or lysogenic lifecycle. Some filamentous phages such as M13 and M13-like phages replicate without causing cell lysis or death. The progeny is secreted into the medium as the culture grows. Such infection only slows down the growth rate of host cells (Felici et al., 1995).

Phage genomics

The first phage genome (coliphage ϕX174) was published in 1977 (Sanger et al., 1977). Currently, there are about 120 complete phage genome sequences in the database. The best characterized of these are the *E. coli* phages λ (Sanger et al., 1982) and T7 (Dunn and studier, 1983), and the *Bacillus subtilis* phage Φ29 (Paces et al., 1985). To date, a total of about 23 lactic acid bacterial phage genome sequences have now become available in databases. These include 14 lactococcal phages TP901-1 (Brøndsted et al., 2001.), Tuc2009 (Arendt et al., 1994), bIL170 (Crutz-Le Coq et al., 2002), bIL285 (Chopin et al., 2001), bIL286 (Chopin et al., 2001), bIL309 (Chopin et al., 2001), bIL310 (Chopin et al., 2001), bIL311 (Chopin et al., 2001), bIL312 (Chopin et al., 2001), sk1 (Chandry et al., 1997), BK5-T (Boyce et al., 1995), c2 (Lubbers et al. 1995), r1t (van Sinderen et al., 1996), and ul36 (Labrie and Moineau, 2002); 5 streptococcal phages 7210 (Le Marrec et al., 1997), DT1 (Tremblay and Moineau, 1999), Sfi11 (Lucchini et al., 1998), Sfi19 (Desiere et al., 1998), and Sfi21 (Desiere et al., 1998); 3 lactobacillus phages ϕadh (Fremaux et al., 1993), ϕg1e (Kodaira et al., 1997), and LL-H (Mikkonen et al., 1996); and 1 oenococcal phage L5 (Olwage et al., 1993). Owing to their economical importance, dairy phages (phages isolated from dairy products) became the best-investigated phage group in the database (Brüssow, 2001).

These available phage sequences have been marvelously informative for the biology of the individual phages. Many new insights have been obtained from detailed analysis of these sequences. Comparative phage genomics has provided substantial knowledge on phage evolutions, genetic diversity, horizontal/vertical gene transfers, module similarity, and lytic/lysogenic cycles.

Sequence data on a variety of phage genomes suggest that phages have evolved by exchange of functional modules, individual genes, or gene segments by various genetic recombination events (Botstein, 1980; Lucchini et al., 1999). Phages are a major source of horizontally transferred DNA in bacteria and prophage DNA accounts in many bacteria for important interstrain genetic variability (Brüssow and Hendrix, 2002). It has been found that multiple phages contribute to bacterial pathogenicity. A number of famous bacterial toxins, such as botulinus toxin, Shiga toxin, and cholera toxin, are phage encoded (Brüssow and Hendrix, 2002; Miyamoto et al., 1999).

Comparative genomic analysis shows that genes are organized in functional modules which carry out particular biological functions. Gene orders in tailed phage genome are more conserved than nucleotide sequences. The modules can be entire sets of genes or single genes, or gene segments encoding distinct protein domains like those in streptococcal phages (Neve et al., 1998). Many *Siphoviridae* LAB phages shared a comparable structural gene cluster and an identical modular genome organization: DNA packaging, head, tail, tail fiber, lysis, lysogen, DNA replication, transcriptional regulation (?) modules (Brüssow and Desiere, 2001; Venema et al., 1999; Desiere et al., 1999; Desiere et al., 2001; Luccini et al., 1998; 1999).

Comparative genomics allowed the establishment of associations between phenotype and genotype and thus the prediction of gene functions (Desiere et al., 1999). The genetic information obtained from phage sequence analysis has found many practical applications, such as induced lysis of LAB to enhance cheese ripening (de Ruyter et al., 1997), or to eliminate undesirable bacteria during fermentations (Payne et al., 1996), re-routing of carbon fluxes for the production of a specific amino acid enantiomer, and construction of genetic traps for phages. Phage-resistant LAB starters containing the cloned phage origin of replication on a plasmid have been designed. When the cell is infected, the phage drives the replication of the plasmid, and no longer drives its own DNA replication (Brüssow, 2001).

Compared with other organisms, the total number of phage sequences in the database is small. More phage genome sequences from a diverse array of phages and comparative sequence analysis are needed to elaborate upon a sequence-based theory and to improve our understanding of these viruses and their interaction with their hosts.

BACTERIOPHAGE ECOLOGY IN NATURE

Introduction

Ecology is the study of the relationship between organisms and their surroundings (Hurst and Lindquist, 2000). Therefore, phage ecology is the study of the relationship between phages, other organisms, and their environments. There are an estimated $>10^{30}$ phages on the planet, outnumbering all other forms of life combined (Brüssow and Hendrix, 2002). Ecologically, phages are as varied and as versatile as their hosts with some able to survive extremes of temperature (up to 95°C) and extremes of pH as low as pH 1 (Sharp, 2001**)**. Phages can be isolated from most environments populated by bacteria. Phage particles typically outnumber prokaryotic cells by about 10-fold in environmental samples (Brüssow and Hendrix, 2002). Thus, phage numbers generally reflect that of the bacterial population (Sharp, 2001). Phage is an important factor in regulating the abundance and distribution of the bacterial population in nature (Suttle, 2000).

The ecological role of phages in the environment has been a subject of intense investigation over the past several years. The development of techniques to study natural viral populations *in situ* has progressed tremendously. Various aspects of phage ecology in nature, including abundance, diversity, role in microbial mortality and water column trophodynamics, viral decay rates, repair mechanisms, and lysogeny, are becoming understood (Paul and Kellogg. 2000). Most of these studies have been performed in aquatic environments.

Phage ecology in aquatic environments

Bacteriophages are known to be the most numerically abundant form of life in the surface waters of this planet. Direct counting techniques including epifluorescence microscopy and TEM have brought this fact to light. A major question posed to microbial ecologists concerning the tremendous number of viruses in aquatic environments is: So what? Are viruses causing significant bacterial mortality, short-circuiting the Microbial Loop, and causing dissolved organic matter release?

It is estimated that in normal aquatic environments, viral lysis accounts for approximately 25% of total bacterial mortality, roughly equivalent to that caused by flagellate grazing (Paul and Kellogg. 2000; Suttle, 1994). However, this percentage can shift under extreme conditions (high salinity or anoxia) to nearly 100% of the bacterial mortality (Paul and Kellogg. 2000). Thus, phages can have major ecological effects on environments. Viral lysis also has important implications for our understanding of nutrient and energy cycling on a global scale (Suttle, 2000). Lysis results in release of cellular material into the surrounding water, which supplies nutrients to other organisms. This can place phages in a key position in nutrient cycling in aquatic communities (Suttle, 2000). Viruses themselves may be a source of carbon for higher tropic levels.

Viral infection can play an important regulatory role in microbial ecosystems through selective predation of host strains which are numerically abundant. Based on the findings of chemostat studies, it is likely that fast-growing, dominant hosts are either phage-susceptible or lysogens. In either case, it is easily anticipated that natural selective events, such as nutrient input, stimulate rapid growth of a specific bacterial strain. Once this subpopulation achieves a critical density or growth rate, an epidemic of phage infection will occur, selectively limiting the abundance of the fast-growing host and preventing the excessive dominance of a single strain within the community (Wommack and Colwell, 2000.). Thus, phages may ensure the coexistence of competing bacteria by infecting only the most abundant hosts or "killing the winner".

It is estimated that phage decay rates average between 0.2 and 1.0 day^{-1}. UV damage by sunlight may be the greatest factor contributing to phage mortality, while photoreactivation plays a major role in phage repair. Lysogeny is a common occurrence among marine bacteria. On average, 10% of the population contains inducible prophage (Paul and Kellogg, 2000). This may be important in maintaining the phage population.

The genetic diversity of phages in environments (soil, freshwater, or the ocean) is becoming known. Often differences in phage morphology are reflected in differences in DNA hybridization or restriction patterns for phages sharing a common host (Paul and Kellogg, 2000). There is also conservation of blocks of viral DNA ("modular cassettes") in morphologically variable phage isolated on the same host. In fact, evidence for gene transfer of viral genes is based on blocks of common or similar genes in widely differing

phages. Transduction of host genes has been observed in isolates in culture, in isolates added to microcosms, and in natural population. Although the frequency seems low, when extended across an entire estuary, transduction could result in 10^{14} gene transfer events annually, which could have given rise to the great diversity of bacterial strains (Paul and Kellogg, 2000).

Phage diversity can be considered to be the number of morphologically distinct groups of viruses that infect bacteria. More relevant from an ecological perspective is the diversity of phages that infect a given host or group of hosts. One approach for examining diversity that has direct ecological implications is to examine the host range of viral isolates. Phages that have broad host ranges are less likely to be dependent on a specific host for replication, and their effect on community structure may be less than phages with very narrow host ranges. In general, phages have relatively narrow host ranges and only infect members of a single species, and are often restricted to strains within a species. However, a study shows that the host range of cyanophages can be extremely complex. Some cyanophage isolates have very restricted host ranges and only infect a single strain of *Synechococcus*, while other isolates appear to have broad host ranges that overlap with those of other cyanophages (Suttle, 2000).

Phenotypic (e.g. major capsid proteins or morphological criteria) or genetic (e.g. restriction fragment length polymorphism, hybridization or nucleic acid sequence analysis) approaches can also be used for examining phage diversity in natural viral communities (Suttle, 2000). An even more powerful approach for examining the diversity is to couple PCR amplification of a region of viral specific genes with denaturing gradient gel electrophoresis (DGGE) (Suttle, 2000). DGGE can be used to resolve DNA fragments of the same size that differ in as little as a single base pair**.** As well, DGGE eliminates the need to isolate the phages or to clone PCR products, and offers a rapid means of detecting predominant populations. DGGE has been used to genetically fingerprint marine virus communities (Short and Suttel, 2002**).**

Bacteriophages in food fermentation systems

Most food fermentations occur under non-sterile conditions. Phages can be an important component of microbial flora present on raw materials or in fermentation

environments. Therefore, bacteria that drive fermentations can be infected by these phages (Boucher and Moineau, 2001). Phages active against lactic acid bacteria have been isolated from various fermentation environments: dairy (Everson, 1991; Peitersen, 1991), meat (Trevors et al., 1983), sourdough (Foschino et al., 2001), and silage (Caso et al., 1995). Phages in vegetable fermentation were first reported by Faville and Fabian (1949) following observation that *Lactobacillus plantarum* cultures were lysed by phages isolated from the soil where cucumbers had grown. This report went largely unnoticed. Not until recently was it shown that phages were present in sauerkraut fermentation (Yoon et al., 2001; 2002). Although it is well-known that vegetable fermentations, particularly sauerkraut fermentations, depend on the sequential growth of a variety of LAB, virtually nothing is known about the role phages may play in the succession. Little research has been done on abundance, diversity, and genetics of phages, and the ecological role phages play in vegetable fermentations.

Molecular methods for specific phage detection

Several molecular methods have been developed for rapid detection of some *Lactococcus* or *Streptococcus* phages in dairy fermentations. An enzyme linked immunosorbent assay (ELISA) using monoclonal antibodies against a major capsid protein of *Lactococcus* phage ul36 has been used for detecting lactococal phages in whey and milk (Moineau et al., 1993). DNA probes have been used for detection of lactococcal phages in cheese whey (Moineau et al., 1992). PCR-based methods targeting genes (e.g. *int*) or conserved regions in phage genome have been used for the detection of *Streptococcus thermophilus* phage in cheese and yogurt fermentations (Brüssow et al., 1998; O'Sullivan et al., 2000).

PHAGE DISPLAY TECHNOLOGY

Introduction

Most biological processes depend on molecular binding events (Felici et al., 1995). Identification and isolation of the binding partners to molecular targets such as

proteins, carbohydrates, nucleic acids, lipids or whole cells are fundamental to the understanding of biological processes and to the search for novel molecules with desired chemical, physical, or biological properties (binding affinity, specificity, kinetics, and stability).

Phage display is a powerful new technology for selecting and engineering proteins or peptides that bind a target molecule of interest. The technology was first developed with the *E. coli* specific phage M13 as a means of selecting cloned peptides that bind to molecular targets (Smith, 1985). The success of M13 phage display has prompted the development of numerous alternative display systems such as λ, T4, T7, and phagemid systems.

Phage display offers several unique advantages over hybridoma and other methodologies: simple, efficient, and inexpensive. Phage display has been used in a wide range of applications.

Principles of phage display

With phage display technology, a foreign DNA is genetically fused to a coat protein gene in a phage, resulting in the presentation of a foreign protein on the phage surface, while the DNA encoding the fusion resides within the virion. This physical link between phenotype (the displayed peptide) and genotype (the encoding DNA) in one genetic package allows a direct selection and enrichment of both displayed protein and its coding sequence at the same time (Kristensen et al., 2000; Rodi and Makowski, 1999). An enormous ($>10^7$) collection of phages (known as phage display library) can be constructed. The library can be highly diverse with respect to insert size, sequence, conformation, flexibility, and distribution of hydrophobic or charged regions, with each phage displaying only one kind of peptide (Collins and Röttgen, 1997). The library members with desired binding characteristics can be isolated by a simple *in vitro* affinity selection procedure called "biopanning," in which the phage library is incubated with an immobilized target of interest, and non-binding phage particles are washed away. The bound phage is eluted and amplified by re-infection in bacteria. Such amplified phages are then used in the next selection round. After a few rounds of biopanning, the phage population is enriched for high affinity binders. The technology provides an easy and

efficient means to find “a needle in a vast molecular haystack” (Rodi and Makowski, 1999). Vast numbers ($>10^8$) of different peptides or proteins and their coding sequences can be simultaneously screened. The amino acid sequence of displayed proteins can be deduced by nucleotide sequencing of the inserted DNA (Rodi and Makowski, 1999; Sidhu, 2000).

Applications of phage display

Phage display technology can be used to isolate proteins or peptides that bind with high specificity and affinity to virtually any target of interest. The technology of varying formats has been applied successfully in a large number of studies to identify molecules with desired binding properties for research, medical, and industrial application. Large collections of antibody fragments have been displayed on phage surfaces and screened with different antigens or with complex protein mixtures such as sera for diseased individuals (Felici et al., 1995). Phage displayed antigens have been found immunogenic in rabbits and/or mice (Wan et al., 2001; de la Cruz et al., 1988; Willis et al., 1993; Minenkova et al., 1993). Structure-function relationship of proteins, protein-protein interaction, and enzyme-substrate specificity have been studied using the phage display system (Forrer et al., 1999).

Tremendous selection power offered by the phage display system can now be used in conjunction with controlled random mutagenesis, leading to exciting protein engineering opportunities to improve or alter the binding properties of displayed proteins, or to produce novel proteins. These mutant proteins or protein mimicry with desired phenotype can be used as reagents to understand molecular recognition, as minimized mimics for receptors, or as lead structures for the development of new drugs and vaccines (Sidhu, 2000; Griffiths and Duncan, 1998; Felici et al., 1995). A wide variety of such proteins or protein domains have been functionally displayed on phage surfaces, such as hormones (Lowman and Wells. 1993), antibodies (Jones, 1998; Clackson et al., 1991), growth factors (Ballinger et al., 1998), enzymes (Soumillion et al., 1994), and antigens (Felici et al., 1995). Several groups have used phage display to engineer zinc-finger domains with designed DNA-binding specificities that can be used to control gene expression (Wolfe et al., 1999). Phage displayed proteins can act as organ- or cell-

specific binders for developing targeted drug-delivery systems (Pasqualini and Ruoslahti., 1996), as specific inhibitors to enzymes or receptors (Ley et al., 1996), and as 'molecular sponges' for protein purification or toxic waste remover (Smith et al., 1998; Petrenko et al., 1996). Phage display technology has become an invaluable component of biotechnology. New applications are continually arising.

Limitations of phage display

Despite the great potential of phage display technology to probe protein-ligand interactions, this technology is only in its infancy. Inherent limitations imposed by the display mechanism and by a biological system limit the scope of proteins that can be displayed. Only a subset of the proteins or peptides consisting of the 20 natural amino acids can be displayed and only a fraction of these has been successfully displayed with any given system (Sidhu, 2000). For studying the biological activity of natural protein or peptide, the technology is useful only if the displayed protein is folded properly, and no additional subunits or post-translational modifications are required (Jacobsson and Frykberg, 1996). The capacity to construct large libraries in any cloning system depends on the overall efficiency of cloning and packaging fusion protein into the capsid (phage) or transformation (plasmids). In addition, the system may not display all fusion proteins equally well. So far, no single display format has proven universally applicable. The technology needs to be developed further and fine tuned for the selection of any desired selectable property of a protein.

REFERENCES

Ackermann, H-W., and DuBow, M.S. 1987a. General properties of bacteriophages. In: Viruses of prokaryotes, Vol. 1, pp. 1-11. CRA Press, Boca Raton, FL.

Ackermann, H-W., and DuBow, M.S. 1987b. Natural groups of bacteriophages. In: Viruses of prokaryotes. Vol. 2, pp. 1-161, 171-218. CRC Press, Boca Raton, FL.

Ackermann, H-W. 1999. Tailed bacteriophages: the order Caudovirales. In: Advances in virus research. Vol. 51. pp. 135-201. K. Maramorosch, F.A. Murphy, and A.J. Shatkin, eds., Academic Press, New York.

Ackermann, H-W. 2001. Frequency of morphological phage descriptions in the year 2000. Brief Review. Arch. Virol. **146**:843-857.

Arendt, E.K., Daly, C., Fitzgerald, G.F., and van de Guchte, M. 1994. Molecular characterization of lactococcal bacteriophage Tuc2009 and identification and analysis of genes encoding lysin, a putative holin, and two structural proteins. Appl. Environ. Microbiol. **60** (6):1875-1883.

Ballinger, M.D., Jones, J.T., Lofgren, J.A., Fairbrother, W.J., Akita, R.W., Sliwkowsky, M.X., and Wells, J.A. 1998. Selection of heregulin variants having higher affinity for the ErbB3 receptor by monovalent phage display. J. Biol. Chem. **273**:11675-11684.

Birge, E.A. 2000a. Genetics of other intemperate bacteriophages. In: Bacterial and bacteriophage genetics. 4th ed. pp. 215-251. Springer, New York.

Birge, E.A. 2000b. Genetics of temperate bacteriophages. In: Bacterial and bacteriophage genetics. 4th ed. pp. 253-292. Springer, New York.

Botstein, D. 1980. A theory of modular evolution for bacteriophages. Ann. N.Y. Acad. Sci. **354**:484-491.

Boucher, I. and Moineau, S. 2001. Phages of *Lactococcus lactis*: an ecological and economical equilibrium. Recent Research Developments in Virology. **3**:243-256.

Boyce, J.D., Davidson, B.E., and Hillier, A.J. 1995. Sequence analysis of the *Lactococcus lactis* temperate bacteriophage BK5-T and demonstration that the phage DNA has cohesive ends. Appl. Environ. Microbiol. **61** (11):4089-4098.

Brøndsted, L., Ostergaard, S., Pedersen, M. Hammer, K., and Vogensen, F. 2001. Analysis of the complete DNA sequence of the temperate bacteriophage TP901-1: Evolution, structure, and genome organization of lactococcal bacteriophages. Virology. **241**:93-109.

Brüssow, H. 2001. Phages of dairy bacteria. Annu. Rev. Microbiol. **55**:283-303.

Brüssow, H. and Hendrix, R.W. 2002. Phage genomics: small is beautiful. Cell. **108**:13-16.

Brüssow, H. and Desiere, F. 2001. Comparative phage genomics and the evolution of *Siphoviridae*: insights from dairy phages. Mol. Microbiol. **39**(2):213-222.

Brüssow, H., Bruttin, A., Desiere, F., Lucchini, S., and Foley, S. 1998. Molecular ecology and evolution of *Streptococcus thermophilus* bacteriophages – a review. Virus Genes. **16:**95-109.

Caso, J. L., de los Reyes-Gavilán, C. G., Herrero, M., Montilla, A., Rodriguez, A., and Suarez, J. E. 1995. Isolation and characterization of temperate and virulent bacteriophages of *Lactobacillus plantarum*. J. Dairy Sci. **78**:741-750.

Chandry, P.S., Moore, S.c., Boyce, J.D., Davidson, B.E., and Hillier, A.J. 1997. Analysis of the DNA sequence, gene expression, origin of replication and modular structure of the *Lactococcus lactis* lytic bacteriophage sk1. Mol. Microbiol. **26**:49-64.

Chopin, A., Bolotin, A., Sorokin, A., Ehrlich, S.D. and Chopin, M. 2001. Analysis of six prophages in Lactococcus lactis IL1403: different genetic structure of temperate and virulent phage populations. Nucleic Acids Res. **29** (3):644-651.

Clackson, T., Hoogenboom, H.R., Griffiths, A.D., and Winter, G. 1991. Making antibody fragments using phage display libraries. Nature. **352**:624-628.

Collins, J. and Röttgen, P. 1997. Evolutive phage-display - a method as basis for a company. http://bib.gbf.de/ergebnisbericht/1997/englisch/section_b/b-collins-eng.html

Crutz-Le Coq, A.M., Cesselin, B., Commissaire, J., and Anba, J. 2002. Sequence analysis of the lactococcal bacteriophage bIL170: insights into structural proteins and HNH endonucleases in dairy phages. Microbiology. **148**:985-1001.

Daeschel, M.A., Andersson, R.E., and Fleming, H.P. 1987. Microbial ecology of fermenting plant materials. FEMS Microbiology Reviews. **46**:357-367.

de la Cruz, V.F., Lal, A.A., and McCutchan, T.F. 1988. Immunogenicity and epitope mapping of foreign sequences via genetically engineered filamentous phage. J. Biol. Chem. **263**:4318-4322.

de Ruyter, P.G.G.A., Kuipers, O.P., Meyer, W.C., and de Vos, W.M. 1997. Food-grade controlled lysis of *Lactococcus lactis* for accelerated cheese ripening. Nature Biotechn. **15**:976-979.

Desiere, F., Lucchini, S. and Brüssow, H. 1998. Evolution of *Streptococcus thermophilus* bacteriophage genomes by modular exchanges followed by point mutations and small deletions and insertions. Virology. **241** (2), 345-356.

Desiere, F., Lucchini, S., and Brüssow, H. 1999. Comparative sequence analysis of the DNA packaging, head, and tail morphogenesis modules in the temperate cos-site *Streptococcus thermophilus* bacteriophge Sfi21. Virology. **260**:244-253.

Desiere, F., Mahanivong, C., Hillier, A.J., Chandry, P.S., Davidson, B.E., and Brüssow, H. 2001. Comparative genomics of lactococcal phages: insight from the complete genome sequence of *Lactococcus lactis* phage BK5-T. Virology. **283**:240-252.

Dunn, J.J. and Studier, F.W. 1983. Complete nucleotide sequence of bacteriophage T7 DNA and the locations of T7 genetic elements. J. Mol. Biol. **166** (4):477-535.

d'Herelle, F. 1917. Sur un microbe invisible antagoniste des bacilles dysentériques. CR Acad Sci Ser C. **165**:373-375.

Everson, T.C. 1991. Control of phage in the dairy plant. Bull. Int. Dairy Fed. **263**:24-28.

Faville, L. W., and F. W. Fabian. 1949. The influence of bacteriophage, antibiotics, and Eh on the lactic fermentation of cucumbers. Michigan State College Agricultural Experiment Station Technical Bulletin 217. Michigan State Agricultural Experiment Station

Felici, F., Luzzage, A., Monaci, P., Nicosia, A., Sollazzo, M., and Traboni, C. 1995. Peptide and protein display on the surface of filamentous bacteriophage. Biotechnol, Annu. Rev., **1**:149-183.

Fleming, H.P., McFeeters, R.F., and Humphries, E.G. 1988. A fermentor for study of sauerkraut fermentation. Biotechnology and Bioengineering. **31**:189-197.

Fleming, H.P., Kyung, K.H., and Breidt, F. 1995. Vegetable fermentations. In: Biotechnology. 2nd ed., Vol. 9, pp 629-661, H.J. Rehm and G. Reed, eds. VCH, New York.

Forrer, P., Jung, S., and Plückthun. 1999. Beyond binding: using phage display to select for structure, folding and enzymatic activity in proteins. Curr. Opin. Struct. Biol. **9**:514-520.

Foschino, R., Perrone, F. and Galli, A. 1995. Characterization of two virulent *Lactobacillus fermentum* bacteriophages isolated from sour dough. J. Appl. Bacteriol. **79**:677-683.

Fremaux, C., De Antoni, G.L., Raya, R.R., and Klaenhammer, T.R. 1993. Genetic organization and sequence of the region encoding integrative functions from

Lactobacillus gasseri temperate bacteriophage phi adh. Gene **126**(1):61-66.

Griffiths, A.D and Duncan, A.R. 1998. Strategies for selection of antibodies by phage display. Curr. Opin. Biotechnol. **9**:102-108.

Hankin, E.H. 1896. Ann. De l'Inst Pasteur. **10**:511.

Harris, L.J. 1998. The microbiology of vegetable fermentations. In: Microbiology of Fermented Foods. 2nd ed., Vol. 1. pp. 45-72, B.J.B. Wood, ed. Blackie Academic and Professional, New York.

Hurst, C.J. and Lindquist, H.D.A. 2000. Defining the ecology of viruses. In: Viral ecology. pp. 3-40. C.J. Hurst, ed., Academic Press, New York.

Jarvis, A.W. 1989. Bacteriophages of lactic acid bacteria. J. Dairy Sci. **72**:3406-3428.

Jacobsson, K. and Frykberg, L. 1996. Phage display shotgun cloning of ligand-binding domains of prokaryotic receptors approaches 100% correct clones. Biotechniques **20**:1070-1076.

Jones, P. 1998. Display of antibody chains on filamentous bacteriophage. Methods Mol. Biol. **80**:449-459.

Keipper, C.H., Peterson, W.H., Fred, E.B., and Vaughn, W.E. 1932. Sauerkraut from pretreated cabbage. Industrial and Engineering Chem. **24**:884-898.

Kodaira, K.I., Oki, M., Kakikawa, M., Watanabe, N., Hirakawa, M., Yamada, K., and Taketo, A. 1997. Genome structure of the *Lactobacillus* temperate phage ϕgle: the whole genome sequence and the putative promoter/repressor system. Gene. **187**:45-53.

Kristensen, P., Ravn, P., Jensen, K.B., and Jensen, K. 2000. Applying phage display technology in agind research. Biogerontology. **1**:67-78.

Kyung, K.H. and Fleming, H.P. 1994a. Antibacterial activity of cabbage juice against lactic acid bacteria. J. Food Sci. **59**:125-129.

Kyung, K.H. and Fleming, H.P. 1994b. S-mehyl-L-cysteine sulfoxide s the precursor of methyl methanethiolsufinate, the principal antibacterial compound in cabbage. J. Food Sci. **59**:350-355.

Labrie, S. and Moineau, S. 2002. Complete genomic sequence of bacteriophage ul36 : Demonstration of phage heterogeneity within the P335 quasi-species of lactococcal phages. Virology. **296** :308-320.

Le Marrec, C., van Sinderen, D., Walsh, L., Stanley, E., Vlegels, E., Moineau, S., Heinze, P., Fitzgerald, G., and Fayard, B. 1997. Two groups of bacteriophages infecting *Streptococcus thermophilus* can be distinguished on the basis of mode of packaging and genetic determinants for major structural proteins. Appl. Environ. Microbiol. **63** (8):3246-3253.

Levin, B.P. and Lenski, R.E. 1983. Coevolution in bacteria and their viruses and plasmids. In: Coevolution. pp. 99-127. D.J. Futuyma, and M. Slaktin, eds., Sinauer Associates, Sunderland, MA.

Ley, A.C., Markland, W., and Ladner, R.C. 1996. Obtaining a family of high-affinity, high-specificity protein inhibitors of plasmin and plasma kallikrein. Mol. Diversity. **2**:119-127.

Lopez, a., Schwartz, M.G., Pratt, D.E., and Powers, J.J. 1954. Nutritional supplementation of lactic acid flora of sauerkraut. Food Res. **19**:564-574.

Lowman, H.B. and Wells, J.A. 1993. Affinity maturation of human growth hormone by monovalent phage display. J. Mol. Biol. 234:564-578.

Lubbers, M.W., Waterfield, N.R., Beresford, T.P., Le Page, R.W., and Jarvis, A.W. 1995. Sequencing and analysis of the prolate-headed lactococcal bacteriophage c2 genome and identification of the structural genes. Appl. Environ. Microbiol. **61**(12):4348-4356.

Lucchini, S., Desiere, F. and Brüssow, H. 1998. The structural gene module in *Streptococcus thermophilus* bacteriophage phi Sfi11 shows a hierarchy of relatedness to *Siphoviridae* from a wide range of bacterial hosts. Virology **246** (1), 63-73.

Lucchini, S., Desiere, F., and Brüssow, H. 1999. Comparative genomics of *Steptococcus thermophilus* phage species supports a modular evolution theory. J. Virol. **73**:8647-8656.

McDonald, L.C., Fleming, H.P., and Hasan, H.M. 1990. Acid tolerance of *Leuconostoc mesenteroides,* and *Lactobacillus plantarum*. Appl. Environ. Microbiol. **56**:2120-2124.

Mikkonen, M., Räisänen, L., and Alatossava, T. 1996. The early gene region completes the nucleotide sequence of *Lactobacillus delbrueckii* subsp. *lactis* phage LL-H. Gene. **175**:49-57.

Minenkova, O.O., Ilyichev, A.A., Kishchenko, G.P., and Petrenko,V.A. 1993. Design of specific immunogens using filamentous phage as the carrier. Gene. **128**:85-88.

Miyamoto, H., Nakai, W., Yajima, N., Fujibayashi, A., Higuchi, T., Sato, K., and Matsushiro, A. 1999. Sequence analysis of Stx2-converting phage VT2-Sa shows a

great divergence in early regulation and replication regions. DNA Res. **31**:6(4):235-240.

Moebus, K. 1983. Lytic and inhibition responses to bacteriophages among marine bacteria, with special reference to the origin of phage-host systems. Helgland, Meeres. **36**:375-391.

Moineau, S., Fortier, J., and Pandian, S. 1992. Direct detection of lactococcal bacteriophages in cheese whey using DNA probes. FEMS Microbiol. Lett. **92:**169-174.

Moineau, S., Bernier, D., Jobin, M., Hebert, J., Klaenhammer, T. R., and Pandian, S. 1993. Production of monoclonal antibodies against the major capsid protein of the *Lactococcus* bacteriophage ul36 and development of an enzyme-linked immunosorbent assay for direct phage detection in whey and milk. Appl. Environ. Microbiol. **59:**2034-2040.

Mundt, J.O. 1970. Lactic acid bacteria associated with raw plant food material. J. Milk & Food Technol. **33**:550-553.

Mundt, J.O. and Hammer, J.L. 1968. *Lactobacilli* on plants. Appl. Microbiol. **16**:1326-1330.

Mundt, J.O., Graham, W.F., and McCarty, I.E. 1967. Spherical lactic acid-producing bacteria of southern-grown raw and processed vegetables. Appl. Microbiol. **15**:1303-1308.

Murphy, F.A., Fauquet, C.M., Bishop, D.H.L., Ghabrial, S.A., Jarvis, A.W., Martelli, G.P., Mayo, M.A., and Summers, M.D., eds. 1995. Virus taxonomy. Sixth report of the international committee on taxonomy of viruses. Arch. Virol. (Suppl. 10), Springer, Vienna.

Narbors, W.T. and Salunkhi, D.K. 1969. Pre-fermentation inoculations with *Leuconostoc mesenteroides* and *Lactobacillus plantarum* on physico-chemical properties of fresh and dehydrated sauerkraut. Food Technology. **23**:67-71.

Neve, H., Zenz, K.I., Desiere, F., Koch, A., Heller, K.J., and Brüssow, H. 1998. Comparison of the lysogeny modules from the temperate *Streptococcus thermophilus* bacteriophages TP-J34 and Sfi21: implications for the modular theory of phage evolution. Virology. **241**:61-72.

Olwage, M.M., Dorrington, R.A., and Dicks, L.M.T. 1993. Characterization of the genome of *Leuconostoc oenos* phage L5. Unpublished.

O'Sullivan D., Ross, R. P., Fitzegerald, G. F., and Coffey, A. 2000. Investigation of the relationship between lysogeny and lysis of *Lactococcus lactis* in cheese using

prophage-targeted PCR. Appl. Environ. Microbiol. **66:**2192-2198.

Paces,V., Vlcek, C., Urbanek, P., and Hostomsky, Z. 1985. Nucleotide sequence of the major early region of Bacillus subtilis phage PZA, a close relative of phi 29. Gene. **38**(1-3), 45-56.

Paul, J.H. and Kellogg, C.A. 2000. Ecology of bacteriophages in nature. In: Viral ecology. pp. 211-246. C.J. Hurst, ed., Academic Press, New York.

Pasqualini, R. and Ruoslahti, E. 1996. Organ targeting *in vivo* using phage display peptide libraries. Nature. **380**:364-366.

Payne, J. MacCormic, C.A., Griffin, H. G., and Gasson, M.J. 1996. Exploitation of a chromosomally integrated lactose operon for controlled gene expression in *Lactococcus lactis*. FEMS Microbiol. Lett. **136**:19-24.

Pederson, C.S. and Albury, M.N. 1969. The sauerkraut fermentation. New Your State Agricultural Experiment Station Technical Bulletin 824, Geneva, Cornell University, New York.

Peitersen, N. 1991. Practical phage control. Bull. In. Dairy Fed. **263**:1-43.

Petrenko, V.A., Smith, G.P., Gong, X., and Quinn, T. 1996. A library of organic landscapes on filamentous phage. Protein Eng. **9**:797-801.

Ptashne, M. 1986. A genetic switch gene control and phage λ. Cell Press & Blackwell Scientific Publications.

Rodi, D.J. and Makowski, L. 1999. Phage display technology - finding a needle in a vast molecular haystack. Curr. Opin. Biotechnol. **10**:87-93.

Sanger, F., Air, G.M., Barrell, B.G., Brown, N.L., Coulson, A.R., Fiddes, C.A., Hutchison, C.A., Slocombe, P.M., and Smith, M. 1977. Nucleotide sequence of bacteriophage phi X174 DNA. Nature. **265** (5596):687-695.

Sanger, F., Coulson, A.R., Hong, G.F., Hill, D.F. and Petersen, G.B. 1982. Nucleotide sequence of bacteriophage lambda DNA. J. Mol. Biol. **162** (4): 729-773.

Sharp, R. 2001. Bacteriophages: biology and history. J. Chem. Technol. Biotechnol. **76**:667-672.

Shofran, B.G., Purrington, S.T., Breidt, F., and Fleming, H.P. 1998. Antimicrobial properties of sinigrin and its hydrolysis products. J. Food Sci. **63**:621-624.

Short, S.M. and Suttle, C.A. 2002. Sequence analysis of marine virus communities reveals that groups of related algal viruses are widely distributed in nature. Appl.

Environ. Microbiol. **68**:1290-1296.

Sidhu, S.S. 2000. Phage display in pharmaceutical biotechnology. Curr. Opin. Biotechnol. **11**:610-616.

Smith, G.P. 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science. **228**:1315-317.

Smith, G.P., Petrenko, V.A., Matthews, L.J. 1998. Cross-linked filamentous phage as an affinity matrix. J. Immunol. Methods. **215**:151-161.

Soumillion, P. Jespers, L., Bauchet, M., Marchard-Brynaert, J., Sartiaux, P., and Fastrez, J. 1994. Phage display of enzymes and in vitro selection for catalytic activity. Appl. Biochem, Biotechnol. **47**:175-190

Stamer, J.R. 1968. Fermentation of vegetables by lactic acid bacteria. In: Proceedings, Frontiers in food research. New York State Agricultural Experiment Station, Cornell University, Geneva, New York, pp. 46-52.

Stamer, J.R., Stoyla, B.O., and Dunckel, B.A. 1971. Growth rates and fermentation patterns of lactic acid bacteria associated with the sauerkraut fermentation. J. Milk & Food Technol. **34**:521-525.

Suttle, C.A. 1994. The significance of viruses to mortality in aquatic microbial communities. Microb. Ecol. **28**:237-243.

Suttle, C.A. 2000. Ecological, evolutionary, and geochemical consequences of viral infection of cyanobacteria and eukaryotic algae. In: Viral ecology. pp. 247-296. C.J. Hurst, ed., Academic Press, New York.

Tremblay, D.M. and Moineau, S. 1999. Complete genomic sequence of the lytic bacteriophage DT1 of *Streptococcus thermophilus*. Virology **255** (1):63-76.

Trevors, K. E., Holley, R. A., and Kempton, A. G. 1983. Isolation and characterization of a *Lactobacillus plantarum* bacteriophage isolated from a meat starter culture. J. Appl. Bacteriol. **54**:281-288.

Twort, F.W. 1915. An investigation on the nature of the ultramicroscopic viruese. Lancet. **2**:1241-1243.

VanGarde, S.J. and Woodburn, M. 1994. Fermented pickles. In: Food preservation and safety. Book I, Principles, pp 60-73. Iowa State University Press, Ames, Iowa.

van Sinderen, D., Karsens, H., Kok, J., Terpstra, P., Ruiters, M.H., Venema, G., and Nauta, A. 1996. Sequence analysis and molecular characterization of the temperate lactococcal bacteriophage r1t. Mol. Microbiol. **19**:1343-1355.

Vaughn, R.H. 1985. The microbiology of vegetable fermentations. In: Microbiology of Fermented Foods. Vol. 1. pp. 49-109. B.J.B. Wood, ed., Elsevier, New York.

Venema, G., Kok, J. and Sinderen, D.V. 1999. From DNA sequence to application: possibilities and complications. Antonie van Leeuwnehoek. **76**:3-23.

Wan, Y., Wu, Y., Bian, J., Wang, X.Z., Zhou, W., Jia, Z.C., Tan, Y., and Zhou, L. 2001. Induction of hepatitis B virus-specific cytotoxic T lymphocytes response *in vivo* by filamentous phage display vaccine. Vaccine. **19**:2918-2923.

Whitte, A., Baranyi, U., Klein, R., Sulzner, M., Luo, C., Wanner, G., Kruger, D.V., and Lubitz, W. 1997. Characterization of *Natronobacterium magadii* phage Ch 1, a unique archaeal phage containing DNA and RNA. Mol, Microbiol. **23**:603-616.

Willis, A.E., Perham, R.N., and Wraith, D. 1993. Immunological properties of foreign peptides in multiple display on a filamentous bacteriophage. Gene. **128**:79-83.

Wolfe, S.A., Greisman, H.A., Ramm, E.I., and Pabo, C.O. 1999. Analysis of zinc fingers optimized via phage display: evaluating the utility of a recognition code. J. Mol. Biol. **285**:1917-1934

Wommack, K.E. and Colwell, R.R. 2000. Virioplankton: viruses in aquatic ecosystems. Microbiol. Mol. Biol. Rev. **64**(1):69-114.

Yago, M.R. Vila, Feria, A., and Rodrigo, M. 1985. Nota. Influencia de la temperatura y de la inoculacion con *Lactobacillus plantarum* sobre el processo de fermentacion para obtencion de col acida. Revista de Agroquimica y Tecnologia de Alimentos. **25**:295-304.

Yoon, S.S., Kim, J-W., Breidt, F., and Fleming, H.P. 2001. Characterization of a lytic *Lactobacillus plantarum* bacteriophage and molecular cloning of a lysin gene in *Escherichia coli*. Int. J. Food Microbiol. **65**:63-74.

Yoon, S.S., Barrangou-Poueys, R., Breidt, F., Klaenhammer, T.R., and Fleming, H.P. 2002. Isolation and characterization of bacteriophages from fermenting sauerkraut. Appl. Environ. Microbiol., **68**:973-976.

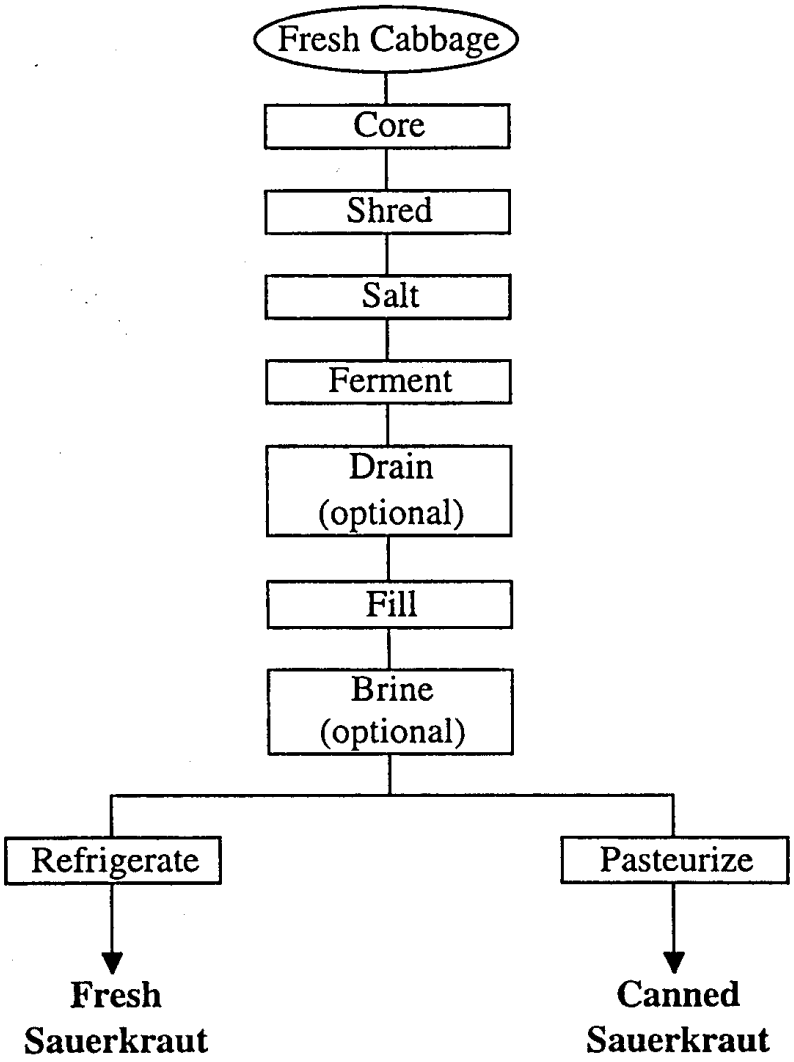

Figure 1. Flow diagram for sauerkraut processing. (Adapted from Harris, 1998.)

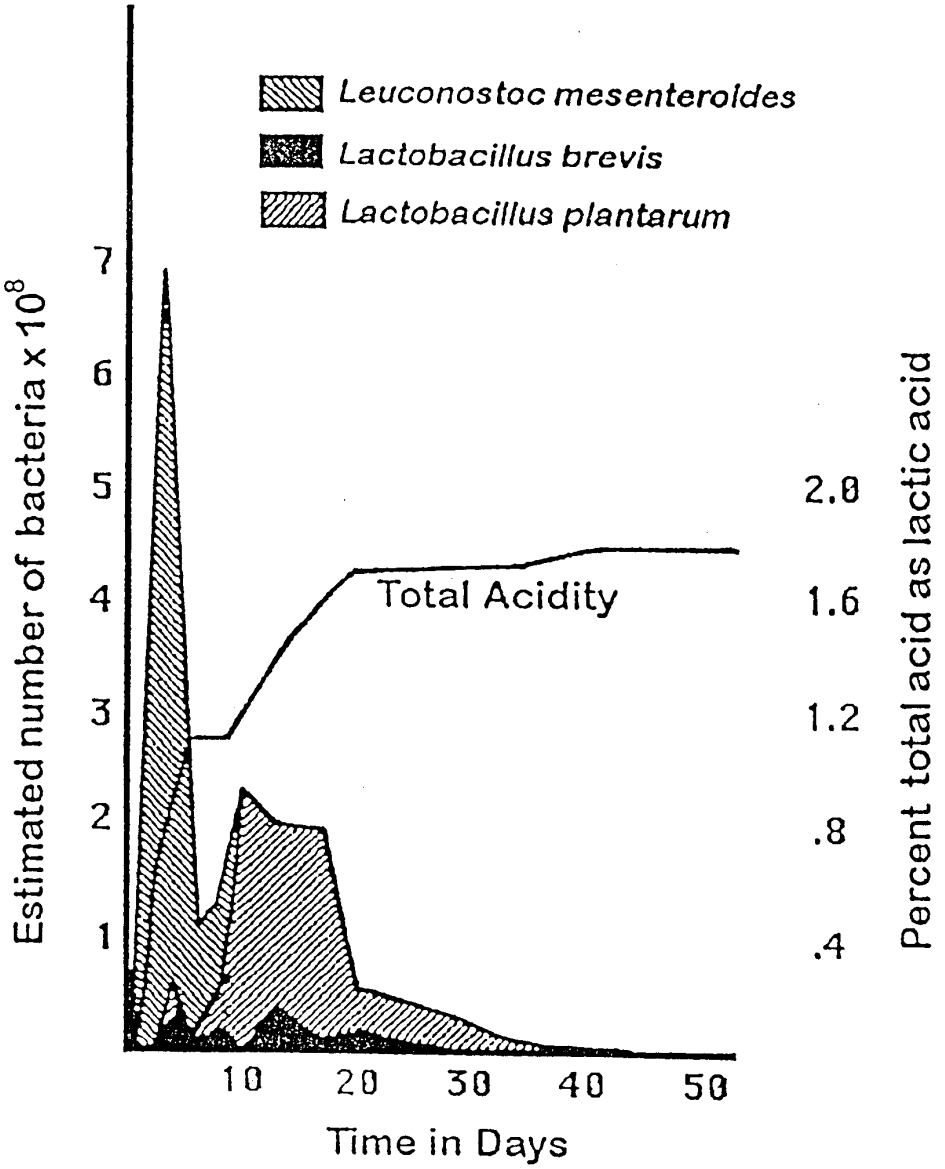

Figure 2. Sequential growth of lactic acid bacteria and acid production during sauerkraut fermentation at 2.25% NaCl and 18°C. (Adapted from Pederson and Albury, 1969.)

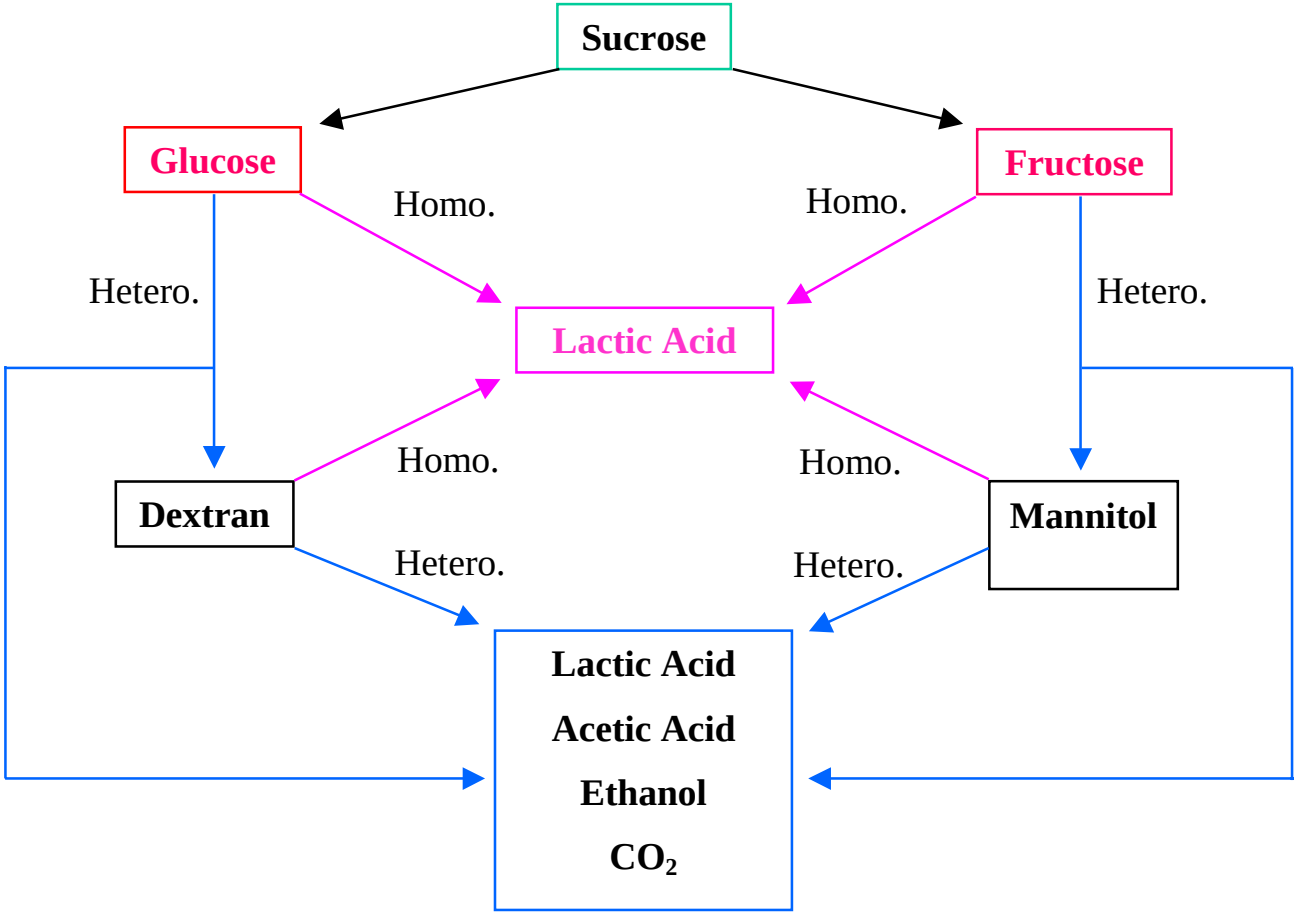

Figure 3. Major substrates and products in sauerkraut fermentations by lactic acid bacteria. (Modified from Pederson and Albury, 1969.) Homo. and Hetero. stand for homofernentation and heterofermentation, respectively.

Table 1. Morphotypes and basic properties of phage families[a]

Morphotype	Shape	Nucleic acid	Family	Particulars
A1 to A3	Tailed	DNA, 2, L	*Myoviridae*	Tail contractile
B1 to B3			*Siphoviridae*	Tail long, noncontractile
C1 to C3			*Podoviridae*	Tail short
D1	Polyhedral	DNA, 1, C	*Microviridae*	Conspicuous capsomers
D3		2, C, S	*Corticoviridae*	Complex capsid, lipids
D4		2, L	*Tectiviridae*	Lipid vesicle, pseudotail
E1		RNA, 1, L	*Leviviridae*	
E2		2, L, seg.	*Cystoviridae*	Envelope, lipids
F1	Filamentous	DNA, 1, C	*Inoviridae*	a. Long filaments
F2				b. Short rods
F3		2, L	*Lipothrixviridae*	Envelope, lipids
F4		2, L	*Rudiviridae*	TMV-like
G1	Pleomorphic	DNA, 2, C, S	*Plasmaviridae*	Envelope, lipids, no capsid
G2		2, C, S	*Fuselloviridae*	Same, lemon-shaped

C Circular; *L* linear; *S* superhelical; *seg.* segmented; *1* single-stranded; *2* double-stranded

[a]Adapted from Ackermann, 2001.

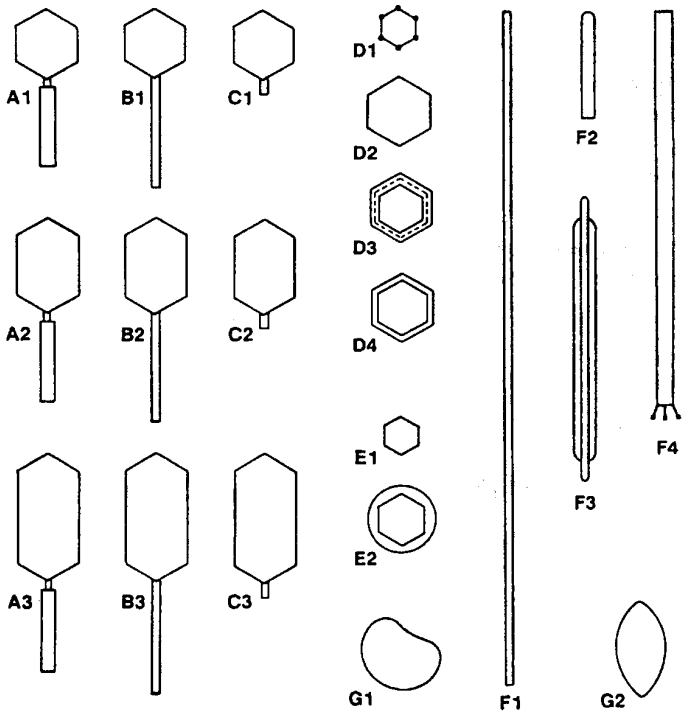

Figure 4. Morphotypes of bacteriophages. See Table 1 for explanation. (Adapted from Ackermann, 2001).

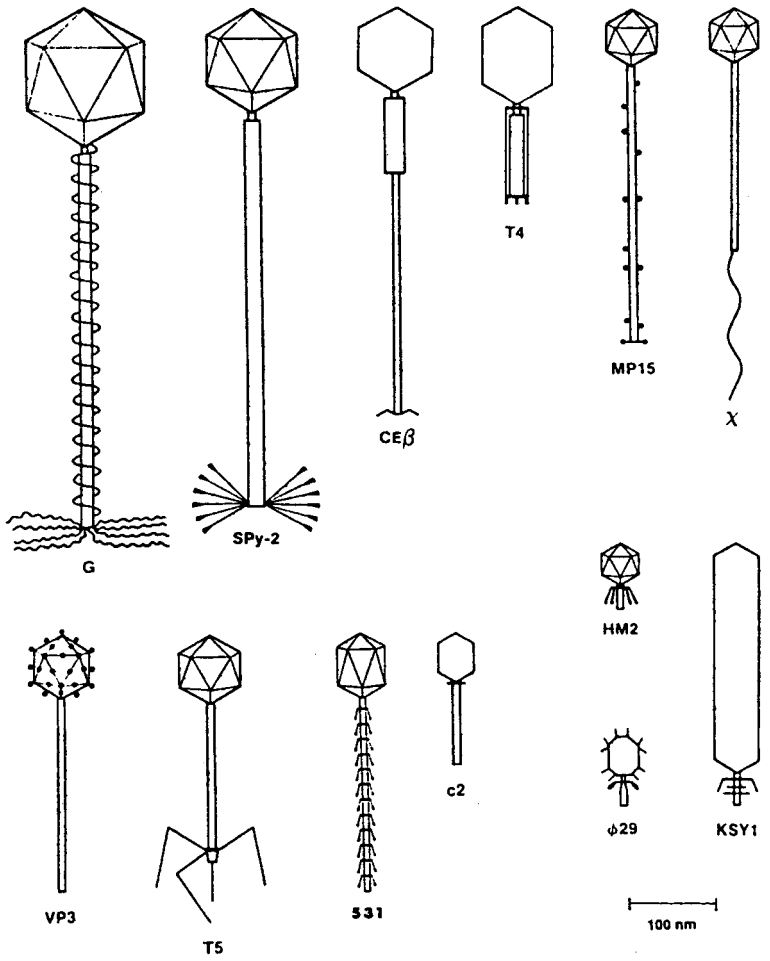

Figure 5. Morphology of selected tailed phages. (Adapted from Ackermann, 1999).

Chapter 2

Bacteriophage Ecology in Commercial Sauerkraut Fermentations

ABSTRACT

The ecology of bacteriophages infecting lactic acid bacteria (LAB) in commercial sauerkraut fermentations was investigated. Brine samples over a 60- or 100-day period were taken from four commercial sauerkraut fermentation tanks in 2000 and 2001. A total of 171 independent phage isolates, including at least 26 distinct phages, were obtained. In addition, 28 distinct host strains were isolated and identified as lactic acid bacteria by ITS restriction and 16S rRNA sequence analyses. These host strains included *Leuconostoc*, *Weissella*, and *Lactobacillus* species. It was found that there were two phage-host systems in the fermentations, with the dividing line occurring between day 3 and day 7 after the start of the fermentations, corresponding to the population shift from heterofermentative to homofermentative LAB. The data strongly suggested that phages may play an important role in the microbial ecology and the succession of lactic acid bacterial species in vegetable fermentations. It was found that two phage isolates were capable of infecting two lactobacillus species. Twenty-eight phage isolates were obtained from samples taken on day 60, when the brine pH was ≤ 3.5. Eight phage isolates which were independently obtained two or more times were further characterized. They belonged to the *Myoviridae* or *Siphoviridae* family, and showed distinct host ranges and DNA fingerprints. These results demonstrated for the first time the complex phage ecology present in commercial sauerkraut fermentations, providing new insights into the bioprocess of vegetable fermentations. Knowledge of phage ecology in vegetable fermentations is essential for developing phage-control strategies for high and consistent quality of fermented vegetable products.

Keywords: bacteriophage, bacteriophage ecology, lactic acid bacteria, sauerkraut fermentation, vegetable fermentation

INTRODUCTION

Like most vegetable fermentations, sauerkraut fermentation is spontaneous and relies on a very small population of lactic acid bacteria (LAB) which are naturally present on the fresh vegetables for preservation. It is known that a succession of various LAB species and their metabolic activities are responsible for the quality and safety of these products (Pederson and Albury, 1969). The process is characterized by an initial heterofermentative stage, followed by a homofermentative stage. Heterofermentative *Leuconostoc mesenteroides* initiates the fermentation and quickly predominates the early stage of the fermentation because it is present at an initially higher number (Mundt et al., 1967; Mundt and Hammer, 1968; Mundt, 1970), has a shorter generation time (Stamer et al., 1971), and grows more rapidly at lower temperature than most other LAB found on plants (Pederson and Albury, 1969). The quality characteristics of sauerkraut are largely dependent upon the growth of this species (Pederson and Albury, 1969). As the fermentation proceeds, pH drops quickly to 4.5 (approx. 1% lactic acid). *L. mesenteroides* rapidly dies off due to its relatively low acid tolerance (McDonald et al., 1990). *Lactobacillus brevis* and homofermentative *Lactobacillus plantarum* take over. They continue fermenting the remaining cabbage sugars to produce primarily lactic acid and further lower the pH. *Lactobacillus plantarum* completes the fermentation with the final pH around 3.5 (Pederson and Albury, 1969; Vaughn, 1985). The correct sequence of LAB species is essential in achieving a stable product with the typical flavor and aroma of sauerkraut. It is generally thought that microbial succession is largely due to the initial microbial load on cabbage, the growth rates and salt- and acid-tolerances of LAB, salt and acid concentrations, pH, and temperature (Pederson and Albury, 1969; Fleming, 1982; Daeschel et al., 1987).

Bacteriophages (phages) are ubiquitous in nature. They can be an important component of microflora on vegetables. An initial studies in our laboratory showed the presence of phages active against LAB in sauerkraut fermentation (Yoon et al., 2002). As phages are obligate intracellular parasites that typically infect bacteria and cause cell lysis, they can potentially contribute to bacterial mortality, and change the microbial community, thereby influencing microbial succession in the fermentations. However, no

research has been done on the phage ecology in vegetable fermentation and the role phages play in microbial succession.

The objectives of this study were to investigate the diversity and ecology of phages active against LAB in commercial sauerkraut fermentations, to explore the possible role of phages in microbial succession in the fermentation, and to characterize predominant LAB phages isolated from the commercial fermentations. Knowledge of the diversity and ecological role of phages is important for better understanding of the complex bioprocess. This information should facilitate the development of controlled vegetable fermentation strategies whereby starter cultures could be used.

MATERIALS AND METHODS

Commercial sauerkraut fermentation and sample collection

Four commercial sauerkraut fermentation tanks (one in 2000, three in 2001) were examined in this study. Each tank had a 90-ton capacity. Fresh cabbage was trimmed of outer leaves. The trimmed cabbage was then shredded, dry salted, and conveyed into tanks. Natural fermentations were carried out with 2.3% NaCl (after equilibration with the shredded cabbage) and an average temperature of 18°C. The fermentation procedure did not change over the 2-year study period.

Fermentation samples were obtained between October 2000 and December 2001. On 2 October 2000 (day zero), a commercial fermentation tank was filled with salted shredded cabbage. Fresh shredded cabbage samples (500 g) were collected in sterile plastic bags prior to salting. From the tank into which the cabbage was conveyed, brine samples (100 ml each) were taken on days 1, 3, 7, 9, 14, 22, 30, 60, and 100 after the start of fermentation. A stainless steel tube (1 cm in diameter) was rinsed with tap water and used to take brine samples from a depth of about 60 cm from the top of the fermentation tank (about 60 cm from the edge of the tank). Each brine sample was transferred into two separate 50-ml sterile plastic tubes with screw caps (Corning Costar Corp., Cambridge, Mass.) and immediately placed in insulated boxes. The collected samples (cabbage or brine) were shipped on ice to our laboratory through overnight mail, and processed immediately. On 24 September 2001 (day zero), three tanks were packed. Brine samples

(100 ml each) from each of the three tanks were taken on days 1, 3, 7, 9, 14, 22, and 60 following the same procedure as described above, and shipped to our laboratory under refrigeration.

Preparation of shredded cabbage samples for microbiological and chemical analyses

For microbiological analysis, 30 g shredded cabbage sample was homogenized in a stomacher with 270 g saline (0.85% NaCl) for 3 min at the maximal speed (Stomacher 400, Tekmar, Cincinnati, OH). The cabbage extract (1 ml) was obtained from the stomacher bag for microbiological analysis. For chemical analysis, 100 g shredded cabbage sample were blended with 200 g water for 3 min in a Waring blender (Dynamic Products Corp., New Hartford, CT) at the maximal speed. The resulting cabbage slurry was frozen overnight at -20°C, thawed next day, and homogenized in a stomacher at the normal speed setting for 1 min. The cabbage extract (30 ml) was transferred from the filter side of the stomacher bag to a 50-ml sterile plastic tube and frozen at - 20°C for later analysis.

The treatment of brine samples for host and phage isolations

Each brine sample was divided into several portions for microbiological and chemical analyses, and for isolation of phages and their hosts (Fig. 1). One ml brine was saved for immediate microbiological analysis and host isolation. The remaining brine sample was centrifuged aseptically at 10,000 x g (GSA rotor, Sorvall Centrifuges, Newtown, CT) and 4°C for 10 min to remove solid particles. The supernatant was filtered (Whatman filter paper, No. 4, W & R Balston Limited, England). A portion (30 ml) of the filtrate was stored at -20°C for later chemical analysis. The pH of the remaining filtrate was measured by a pH meter (Model 825 MP; Fisher Scientific Co., Pittsburgh, PA), and then adjusted to approximately 6.3 with 3.0 N NaOH. After filter-sterilizing (0.45 μm-pore-size filters), the brine was stored at 4°C for later use as a potential phage source.

Microbiological analysis

After serial dilution, samples of cabbage extract or of brine were plated on PCA (Difco Laboratories, Detroit, MI), VRBG (VRB agar, Difco, supplemented with 1% glucose, Sigma Chemical Co, St. Louis, MO), MRS agar (Difco), MMRS (MRS agar, Difco, supplemented with 0.02% sodium azide, Fisher Scientific Co., Fairlawn, N.J., to prevent the growth of yeasts and molds), and YM agar (Difco) by using a spiral plater (Autoplate 4000; Spiral Biotech, Inc., Bethesda, MD). The plates were incubated at 30°C for 1 day (PCA and VRBG) or 2 days (MRS, MMRS), or at room temperature for 4 days (YM). The colonies on the plates were enumerated with an automated colony counter (Protos Plus, Bioscience International, Rockville, MD) to determine the total aerobic (PCA), *Enterobacteriaceae* (VRBG), LAB (MRS and MMRS), and yeast and mold (YM) counts.

Chemical analyses and sensory evaluation

The salt (NaCl) content in brine was determined by titration with standard $AgNO_3$ using dichlorofluorescein as an indicator (Fleming and others 1992). Sugars, alcohols, and organic acids were determined by high-performance liquid chromatography (HPLC). Sugars and mannitol were separated by a Carbopac PA1 column (Dionex Corp., Sunnyvale, CA) with a 0.8 ml/min flow rate of 0.12 N NaOH at room temperature, and detected by a pulsed amperometric detector (model PAD-2; Dionex). Cellobiose was used as an internal standard. Organic acids and ethanol were analyzed using a cation-exchange column (Aminex HPX-87H, Bio-Rad Laboratories, Richmond, CA) with a 0.8 ml/min flow rate of 0.03 N H_2SO_4 at 75°C. A UV detector (UV-6000, Thermo Separation Products Inc., San Jose, CA) and a differential refractometer (Waters 410, Warters, Milford, MA) were connected in series for detection of organic acids (at 210 nm) and ethanol, respectively. Isobutyric acid was used as an internal standard. The quality of the sauerkraut products from two of the four tanks was evaluated and compared with similar products from local grocery stores by a sensory panel.

Isolation of phages and their hosts

In 2000, each brine sample was diluted and plated on MRS agar plates (Fig. 1). The plates were incubated at 30°C for 2 days. From these plates, 96 isolated colonies were randomly picked and used for inoculating 96 wells in a microplate (microplate I, Corning Costar Corp., NY). Each well in microplate I contained 200 µl MRS broth and cells was overlayed with 75 µl mineral oil after inoculation. The microplate was then incubated overnight at 30°C. Ten µl of each of the 96 overnight cultures in microplate I were transferred into two new microplates (IIA and IIB, Fig. 1). These cultures would serve as potential hosts for phage enrichment and isolation. Each well in microplate IIA or IIB contained 200 µl MRS broth. Microplate IIA was incubated at 30°C for 6 h, whereas microplate IIB was temporarily stored at 4°C for later use. A filter-sterilized brine sample (50 µl) was added to each well in microplate IIA to enrich the phages in the brine. The incubation of microplate IIA was continued overnight. Meanwhile, microplate IIB was removed from 4°C and incubated at 30°C to provide fresh cultures for spot tests. After several hour's incubation, microplate IIA was placed in microplate carriers (SH-3000 Swinging Bucket Rotor, Sorvall), and centrifuged at 3,300 x g and 4°C for 10 min. Ninety-six individual spot tests were performed by spotting 10 µl supernatant from a well in Plate IIA onto the corresponding bacterial lawn that resulted from 100 µl of overnight culture from the corresponding well in Plate IIB. The 96 plates were incubated overnight at 30°C. Primary phage-host relationships were indicated by positive spot-test plates (showing a clear or hazy zone, or plaques in the bacterial lawn) and confirmed by plaque assay after the host was colony-purified. Each host isolate was assigned an identification (ID) with a numeric label indicating the day when it was isolated, followed by the alpha-numeric identification of the well on microplate IIB in which the host was grown. The corresponding phage isolate was assigned the same ID with a prefix 'Φ'. For example, Φ1-A3 indicated that the phage was isolated on day 1 and propagated in well A3 of microplate IIA, and its principal host was 1-A3. Theoretically, the detection limit of phages in 2000 was 20 pfu/ml brine sample because 50 µl of the brine sample was used for phage enrichment. All of the resulting phage isolates were purified by one round of single-plaque isolation according to the method described in Chapter 2. Additional spot

tests were performed to determine host range and phage typing, and thereby identifying distinct phages and hosts.

In 2001, the hosts isolated in 2000 were used to propagate phages in brine samples from three commercial fermentation tanks. With a few exceptions, hosts isolated from a specific day in 2000 were used mainly for phage isolation from the brine samples of the same day of the fermentation in 2001. For example, the hosts isolated from day 3 in 2000 were used to isolate phages from only day-3 brine sample in 2001. Fresh host cultures were prepared in 1.5-ml microfuge tubes instead of microplates. A half ml MRS broth supplemented with 10 mM of $CaCl_2$, 0.5 ml filter-sterilized brine, and 20 μl of host culture (in log-phase) was added to a 1.5-ml microfuge tube, which was then incubated for 10-12 hr at 30°C and then micro-centrifuged (Model 16KM, Fisher Scientific) at 8,850 x g for 1 min. A half ml supernatant was transferred to a new microfuge tube containing 1 ml MRS/10 mM $CaCl_2$ and 0.1 ml fresh host culture. After overnight incubation at 30°C and micro-centrifugation at 8,850 x g for 1 min, the phage lysate (in supernatant) was used for spot test against the host used for the phage propagation. The theoretical detection limit of phage in 2001 was 2 pfu/ml of brine sample because a larger volume (500 μl) of brine sample was used for phage enrichment compared with that in 2000.

Maintenance of Bacterial and phage isolates

All bacterial isolates were temporarily maintained on MRS agar at 4°C. For long-term storage, host glycerol stocks were made with MRS broth supplemented with 16% glycerol, and kept at -84°C. All phage lysates were filter-sterilized and maintained at 4°C. Phage glycerol stocks (in MRS broth supplemented with 16% glycerol) were kept at -84°C.

Host identification

All host isolates were subjected to biochemical tests for gas (CO_2) production from glucose with a Durham tube in MRS broth, malolactic enzyme activity in MD medium (Daeschel et al., 1984), dextran (slime) production on 5% sucrose agar (Atlas,

1993), and catalase activity using 5% H_2O_2. *Lactobacillus plantarum* and *Listeria monocytogenes* strains (from our culture collection) were used as negative and positive controls, respectively. Each individual host isolate obtained in 2000 was tested against every single phage isolate obtained in 2000 with spot tests to determine phage typing of the host. Based on phage typing, distinct hosts were determined. Host strains were identified with restriction analysis of the Intergenic transcribed spacer (ITS) regions between the 16S and 23S rDNA genes. Genomic DNA from each host was extracted from an overnight culture using a Wizard Genomic DNA purification kit according to the protocol for genomic DNA isolation provided by Promega Cooperation (Madison, WI). ITS-PCR was performed according to the method described by (Breidt and Fleming, 1996). The forward (5'-GAAGTCGTAACAAGG-3') and reverse (5'-GGGTTTCCCCATTCGGA-3') primers (Genosys Biotechnologies Inc., The Woodlands, TX) were used to amplify the ITS regions of rRNA operons. The amplification was performed with a thermal cycler (GTC-2 Genetic, Precision Scientific Inc., Chicago, IL) in a total volume of 100-µl. Each reaction mixture contained 70 µl water, 4 µl template DNA (10 to 100 ng), 10 µl 10X PCR buffer, 10 µl 25 mM $MgCl_2$, 2 µl of each primer, 1 µl of dNTP mixture (25 mM each dNTP), and 1 µl *Tag* DNA polymerase (5 Unit/µl, Promega). Samples were overlaid with 75 µl of mineral oil and subjected to an initial denaturation step (94°C for 5 min), followed by 25 cycles of denaturation (94°C for 1 min), annealing (55°C for 5 min), and extension (72°C for 2 min), and finished with a final extension step at 72°C for 5 min. After cooling to 4°C, 20 µl of each PCR product was mixed with 1µl *Rsa*I endonuclease (16 U/µl, Stratagen, Woodinville, WA), and then incubated at 37°C for 1 hr. A 10 µl sample from the restriction digest was mixed with 5 µl bromphenol blue tracking dye solution, loaded onto 5% polyacrylamide gels using a vertical gel electrophoresis box with the glass plates (BRL Model V16, Life Technologies, Inc., Gaithersburg, MD). Electrophoresis was carried out in a 1X TBE buffer at a constant 75 V for 5 to 6 hr. A 100 bp DNA ladder-size standard (Invitrogen, Carlsbad, CA) was used as a standard. The gels were stained with ethidium bromide (0.5 µg/ml, in water, Sigma) and visualized under transmitted UV light (UV Transilluminator, model 2040EV, Stratagen). The restriction patterns were compared with our database to

determine the genus and species of the hosts, which were confirmed later by 16S rDNA sequence analysis. Chromosomal DNA (4 µl) from each host was subjected to PCR amplification in a total volume of 100 µl containing 1 µl *Tag* polymerase (Promega). The thermal cycler (RoboCycler Gradient 96, Stratagen) was programmed for an initial denaturation at 94°C for 10 min, 25 cycles of 1 min at 94°C, 2 min at 61°C, and 2 min at 72°C, followed by a final 5-min extension at 72°C. Forward (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (5'-GTCTCAGTCCCAATGTGGCC-3') primers specific for LAB were used to amplify the region (about 350 bp) with a hypervariable nucleotide base sequence of the 16S rRNA gene. The PCR products were purified by using the Qiaquick PCR Purification Kit (Qiagen) in accordance with the manufacturer's protocol and examined by 1% agarose gel electrophoresis before shipped to University of California at Davis (Davis, CA) for sequencing. A search of the GenBank DNA database was conducted by using the BLAST algorithm. A similarity of >98% to the partial 16S rDNA sequences of type strains was used as the criterion for identification.

Characterization of phages

Each phage isolate obtained in 2000 was tested against all host isolates obtained from the same tank to determine the host range by spot test on MRS agar media. Based on host ranges, distinct phages were determined. Eight frequently occurring phages were selected for further characterization by their morphology, major structural protein profiles, and restriction endonuclease digestion patterns using the methods described in Chapter 2. Briefly, phages were concentrated from large-scale phage lysate by PEG precipitation and purified by ultra-centrifugation in CsCl step gradient (Sambrook et al., 1989). Electron micrographs of phages were taken by transmission electron microscopy. Structural proteins of phages were analyzed by SDS-PAGE. Phage DNA was phenol- and chloroform-extracted, and then digested with *Hind* III restriction endonuclease 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.

RESULTS AND DISCUSSION

Microbiological analysis

Figure 2 showed the cell count profiles in the commercial sauerkraut fermentation tank studied in 2000. The initial PCA count on day 0 was 4 x 10^6 cfu/g. VRBG count for *Enterobacteriaceae* was 2 x 10^6 cfu/g and LAB counts were about 1 x 10^4 cfu/g. Yeast and mold counts were below the detection limit (10^2 cfu/ml). Once the fermentation started, *Enterobacteriaceae* quickly died off due to the combined inhibitory effect from salt added and the acid produced by LAB, which became predominant. Meanwhile YM count increased because yeasts and molds were salt- and acid-tolerant. As the fermentation continued, LAB, yeast and mold counts gradually decreased, largely due to fewer nutrients available, increasing concentration of organic acids, and lowering pH. On day 30, PCA, MRS, or MMRS counts were about the same (close to 1 x 10^7 cfu/ml), whereas YM count was 1 log cycle lower (1 x 10^6 cfu/ml). From day 1 to day 60, MRS count was close to MMRS count, indicating that the majority of colonies on MRS were LAB. However, on day 100, MMRS count was under the detection limit, whereas MRS and YM counts were close to or above 10^6 cfu/ml, respectively, indicating the majority of colonies on MRS plate were not LAB, but yeast and mold. The overall cell count profiles over the 60-day period in three other tanks (data not shown) were similar to those described above.

Chemical analyses and sensory evaluation

The fermentable substrate composition of shredded cabbage used for the fermentations was shown in Table 1. The cabbage used in 2000 contained 119.2 mM (2.15%, by wt.) glucose, 90.8 mM (1.64%) fructose, 4.8 mM (0.17%) sucrose, and 5.6 mM (0.07%) malic acid, which were lower than those reported by Fleming et al. (1988). The cabbage used in 2001 contained much lower glucose (81.7 to 92.4 mM), fructose (73.8 to 83.5 mM), and malic acid (3.5 to 4.7 mM), compared with that in 2000. No sucrose was detected in the cabbage used in 2001. The variation in overall fermentable

substrate composition of cabbage can result from the difference in varieties or maturation stages of cabbage used.

The substrate consumption, acid production, and pH reduction were rapid during the first two weeks of the fermentation in the tank studied in 2000 (in Fig. 3). Mannitol was produced during the first week of the fermentation (Fig. 3a). This commonly occurs in the heterofermentative stage when fructose is used as an electron acceptor by such species as *Leuconostocs mesenteroides* and *Lactobacillus brevis* (Daeschel et al., 1987). When cabbage sugars were exhausted, mannitol concentration started to decrease (Fig. 3a), as mannitol then serves as a substrate for homofermentative species (Daeschel et al., 1987). This results in additional acid production and a further pH decrease (Fig. 3b). Small amounts of residual sugars (2.2 mM glucose and 3.8 mM fructose) were present at the end of fermentation. Similar sugar and acid profiles were obtained in the three tanks studied in 2001 (data not shown) except that no residual sugars were observed. The difference in residual sugar might have resulted from the difference in initial sugar content of the cabbage used in fermentation. As mentioned earlier, the cabbage used in 2000 had a much higher sugar content than those used in 2001. LAB could be inhibited by low pH or other factors before they could utilize all natural sugars present in vegetables. Incomplete fermentation due to high initial sugar content was also observed in cucumber fermentation (Lu et al., 2001; 2002a; 2002b).

The overall pH profiles (Fig. 4) were similar in all four tanks. Brine pH decreased rapidly in the first week of fermentation, slowly in the second and third weeks, and remained almost unchanged thereafter. The pH on day 60 was in the range of 3.4 to 3.5 in all four tanks. It was noticed that the pH on day 1 in the tank studied in 2000 was lower than the three tanks studied in 2001 (Fig. 4). This may be due to the variation of cabbage used or other unknown factors. Malic acid was exhausted in all four tanks (data not shown), indicating the activity of malolactic enzyme from certain LAB strains, especially some *Lb. plantarum* strains. The salt content in brines from the four tanks was in the range of 2.2-2.3% (data not shown). The quality of the sauerkraut products from two of the four tanks was evaluated by a sensory panel. The color, flavor, and texture of the final product from either tank were very similar to those of the references obtained from local

grocery stores (data not shown), indicating that all four tanks underwent normal fermentations.

Isolation of phages and their hosts

In 2000, 864 randomly picked bacterial isolates from MRS plates were obtained from 9 brine samples taken from a commercial sauerkraut fermentation tank over a 100-day period. These isolates were tested as potential hosts for phage enrichment and isolation. About 35 of them were unable to grow in the 96-well microplate and thus abandoned. Based on spot tests, 61 phage isolates along with 61 hosts were obtained. After purification of individual host and phage isolates, 46 phage-host relationships were confirmed by plaque assays. Additionally, 2116 (46 X 46) spot tests were performed to determine host range and phage typing. Table 2 shows 44 phage isolates (on the top row) and 46 host isolates (in the first column). Two phage isolates (Φ1-F9 and Φ14-A4) lost their infectivity for unknown reasons, and were not included in Table 2. Among the 44 phage isolates, 12 were obtained from day 1, 11 from day 3, 2 from day 7, 1 from day 9, 9 from day 14, 5 from day 22, and 4 from day 60. No phage was isolated on day 30 and day 100. Probably the hosts or phages, or both, were under the detection limit.

A complex pattern of host ranges and phage typing is shown in Table 2. Multiple phage isolates could attack the same host strain, while multiple hosts were susceptible to the same phage. A striking observation, however, was that phages isolated from day 1 or day 3 only infected hosts isolated from day 1 and/or day 3, not from other days, whereas phages isolated after day 3 only attacked hosts isolated from day 7 or thereafter. The restricted infectivity of phages to hosts from specific days implied that there were at least two groups of phage-host systems in the fermentation, with the dividing line occurring between day 3 and day 7. Based on the time of their isolation or hosts, the 44 phage isolates in Table 2 could be divided into two large groups: phage group I, including 23 phage isolates obtained on days 1 and 3, and phage group II, including the rest 21 phages isolates obtained after day 3. Accordingly, hosts could also be divided into two groups based on the time of their isolation: group I isolated from days 1 and 3, group II isolated after day 3. Within the same phage group, multiple phage isolates could attack the same host strain. Within the same host group, multiple hosts could be susceptible to the same

phage. The results clearly indicated that microbial populations shifted between day 3 and day 7, which were consistent with well-established observations (Pederson and Albury, 1969). The results also indicated that the appearance of a new group of phages was closely correlated to the bacterial succession. A parallel bacterial ecology study confirmed the transition from hetero- to homo-fermentaiton occurred between day 3 and day 7 (unpublished data).

Table 2 also shows that a number of phage isolates (such as Φ1-A4, Φ1-D6, and Φ1-G7) have the same host range, and thus are possibly one single phage strain. Based on host range analysis, 26 distinct phages were determined (Table 3). It is noteworthy that 9 phages were independently isolated more than once on the same or different days in 2000. Phage Φ7-E1 was isolated six times (once on day 7 or day 22, and four times on day 14), whereas Φ14-C8 appeared among phage isolates four times (twice on day 14, two times on day 60), suggesting that these phages were predominant on day 14 and/or day 60. Four phage isolates were obtained from day 60, when the brine pH was very low (3.5). Host range is an important aspect of diversity that has ecological repercussions. Many phages were capable of infecting two or more bacterial strains (Table 3). Phages Φ14-C8, Φ14-E10, and Φ22-A2 had very broad host ranges, capable of infecting five or six strains. The broad host range phenomenon has been observed for a number of phages infecting *E. coli*. The LamB protein, which was initially named the λ receptor (Werts et al., 1994), also serves as a specific cell surface receptor for a series of other phages (Charbit and Hofnung, 1985). It was reported that some complex phages used more than one receptor and, therefore, had alternative routes of uptake into cells (Werts et al., 1994). Broad-host-range phages may promote genetic diversity and genetic exchange in microbial communities (Jensen et al., 1998). Table 3 also shows that a number of phages were capable of cross-infecting each other's host. In contrast, several phages had a very restricted host range and only infected a single strain. They could have a significant effect on community structure. The large variety of phage types shown in Table 3 indicate that there is substantial diversity among the phages in commercial sauerkraut fermentation system.

Host isolates (such as 1-A4, 1-D6, 1-F9, and 1-D10) having the same phage typing were considered as phage-related host strains. Based on phage typing analysis, 28 distinct phage typing hosts were determined (Table 4). Based on ITS restriction digestion pattern and 16S rRNA gene sequence analysis (sequence data not shown), these hosts were identified as 9 *L. mesenteroides*, 1 *Leuconostoc pseudomesenteroides*, 1 *Leuconostoc citreum*, 3 *Leuconostoc fallax*, 1 *Weissella kimchii*, 10 *L. plantarum*, 2 *Lactobacillus paraplantarum*, and 1 *Lactobacillus brevis* strains (Table 4). No *Pediococcus pentosaceus* was found in the host list. *Leuconostoc fallax* and *W. kimchii* were newly identified LAB in sauerkraut fermentation with *L. fallax* only recently been reported in sauerkraut fermentations (Barrangou et al., 2002). The ITS-PCR products and their restriction profiles obtained from 10 phage hosts are representatively shown in Figure 5. The four *L. mesenteroides* strains (1-A4, 1-F8, 3-A4, and 3-B1) shared an identical ITS or restriction profile, which were different from those of *L. pseudomesenteroides* (3-B11), *Lb. brevis* (7-E1), *L. fallax* (3-G10), and *W. kimchii* (3-H2). The two *Lb. plantarum* strains (14-C8 and 22-D10) shared a unique ITS profile.

Biochemical tests showed that all hosts were catalase-negative (Table 4), a common characteristic of LAB. All *Leuconostocs* and *Weissella* strains produced gas from glucose, a heterofermentative feature. In addition, these *Leuconostocs* and *Weissella* strains also produced a characteristic slime of dextran from sucrose, a distinguishing characteristic of most *Leuconostocs* and some *Weissella* strains. None of the *Lb. plantarum* and *paraplantarum* strains produced gas, which was consistent with their homofermentative nature. The *Lb. brevis* strain (7-E1) produced gas because it was heterofermentative. Neither the *Lb. plantarum* nor *Lb. brevis* strain produced slime colonies on sucrose agar, which clearly differentiated them from the *leuconostocs*. Most *leuconostocs* did not have malolactic activity. In contrast, most *lactobacilli* showed malolactic activity, which can be used by some LAB to protect them from acidic environments because decarboxylation of malic acid consumes an intracellular proton, resulting in an increase in a cytoplasmic pH (McFeeters et al., 1982; Gottschalk, 1986).

It was found that hosts isolated from days 1 and 3 (group I hosts) all belong to the genus *Leuconostoc* or *Weissella*, and hosts isolated after day 3 (group II hosts) were all *Lactobacillus*. This was consistent with the observations that group I phages infected

group I hosts and did not infect group II hosts. Generally, phages only replicate within one genus and species, but can typically replicate over a number of strains. Because the two groups of hosts were from different genera, it was concluded that they were susceptible to different groups of phages. In host group I, a number of strains, including three *L. fallax* strains and one *W. kimchii* strain, were only sensitive to a single phage, while other strains were susceptible to more than one phage. In host group II, most strains were *Lb. plantarum* strains and sensitive to two or more phages. There was, with one exception, no overlapping phage sensitivity, either between *Leuconostoc* species or between *Lactobacillus* species. The exception was the *Lb. brevis* strain 7-E1 which was susceptible to one *Lb. brevis* phage (Φ7-E1) and two *Lb. plantarum* phages (Φ14-E10 and Φ22-A2). It is unknown at the point why the two *Lb. plantarum* phages were able to infect two different bacterial species. While different in fermentation ability, *Lb. brevis* and *Lb. plantarum* usually occupy the same ecological niche. They may exhibit similar receptors on cell surfaces. Phages may to extend their host range by acquiring pieces of a tail fiber from unrelated phages (Haggard-Ljungquist et al., 1992). Host 22-D10 was susceptible to five distinct *Lb. plantarum* phages isolated on days 7, 14, 22, and 60.

Sauerkraut fermentation is a dynamic biochemical system. The chemical composition and microbial ecology of the system are continuously changing. Because phage infection is both density-dependent and species-specific (or nearly so), the numbers and types of phages appearing may reflect the activity of both phages and their hosts. It is noteworthy that all of the 23 *Leuconostoc* and *Weissella* phage isolates were obtained from day 1 and day 3 (Table 2) and no *Leuconostoc* and *Weissella* phages were isolated after day 3. This suggests that *Leuconostoc* and *Weissella* strains were only active during the first 3 days, but died off thereafter. The mortality of *Leuconostoc* and *Weissella* could be due to a combination of factors, including organic acids, low pH, and phages. It has been suggested that viral infection can contribute significantly (22%, range: 4.5 to 45%) to overall bacterial mortality in many marine and freshwater environments (Binder, 1999). Results from this study showed that an average of 12% (12 of the 96) of the randomly picked LAB isolates (mainly *Leuconostoc*) from day 1 or day 3 were susceptible to phage infection. Such a significant phage infection could contribute

greatly to the overall mortality in the *Leuconostoc* population, thereby influencing the dynamics of LAB populations in the fermentation. Even if these phages caused only a small proportion of the mortality of leuconostocs, they still could have a profound effect on the relative proportions of different species or strains in the fermentation. In contrast, no *Lactobacillus* phages were isolated until day 7, indicating that the *Lactobacillus* population was very low at the early stage of fermentation. As fermentation proceeded, *Lactobacillus* population increased, providing hosts for propagation of *Lactobacillus* phages. The number of isolated *Lactobacillus*-specific phages increased from two on day 7 to nine on day 14, and then decreased to four on day 60 (Table 2), indicating the changes in the populations of lactobacilli. The correlation between the appearance of new groups of phages and the bacterial succession strongly suggests that phages play a significant role in the succession, rising with the population and eventually causing its elimination.

In 2001, a total of 111 phage isolates were obtained from the three tanks by using the 28 distinct host strains isolated in 2000 as the principal hosts (Table 5). Twenty-four (86%) of the 28 distinct hosts were found susceptible to the phages present in two or three tanks at selected time points. The results indicated that the phages isolated in 2001 were the same as, or very similar to, those isolated in 2001 because they attacked the same set of LAB hosts. The large number and variety of phage isolates affirmed a complex phage ecology in sauerkraut fermentation, with some phages re-occurring. This result was consistent with that from the previous year. The temporal relationship between phages and their hosts suggested that phages could influence the numbers, types, and sequences of microbial populations in the fermentation. Phages Φ1-F8, Φ7-E1, Φ14-C8, and Φ22-D10 were frequently isolated in 2000 (Table 3). Their hosts were used at multiple time points in 2001 to investigate the presence and persistence of these phages in three different fermentation tanks. Table 5 shows that all of these four hosts were susceptible to phages from any of the three tanks at one or more time points. Phages attacking 7-E1, 14-C8, and 22-D10 were found in all three tanks from day 14 or 22, to day 60, supporting the suggestion made earlier that these frequently-appearing phages were predominant on day 14 and thereafter. In addition to distinct hosts, several other

host isolates, which were considered the same as those distinct ones based on phage typing, were also included for testing phages to see if they gave the same results. For instance, 7-E1 and 14-A2 were identical or related strains, based on phage typing (Table 2). Both isolates were used for testing day-14 brines from three tanks. They gave the same results (Table 5), suggesting that the two isolates were the same. With the same method, 14-C8 and 22-F3 gave identical results, and so did 22-D10 and 60-D8. An exception was 1-F8 and 3-B5. 1-F8 was found to be susceptible to day-3 samples from any of the three tanks, whereas 3-B5 was only susceptible to two of the three samples. This does not, however, prove that the two isolates were different. It was possible that the phage active against a particular host strain was under detection limit in a particular volume of the sample. This was supported by the observations shown in Table 5. Host 7-E1 was found to be susceptible to only one of the three day-7 samples. However, it showed susceptibility to two day-9 samples, then to all three day-14 samples, and remained such thereafter, indicating the phage titer in these tanks increased from day 7 to day 22 and maintained at the level above the detection limit (2 pfu/ml) thereafter. A total of 24 phage isolates were obtained from the three different tanks on day 60 (pH <3.5) by using eight bacterial isolates, including three redundant host isolates (14-C8 and 22-F3 were considered the same LAB strain as 60-H11, and 22-D10 was the same as 60-D8). The results were consistent with those obtained in the previous year. Although some phages were frequently isolated, no evidence showed that a single phage dominated the fermentation system. Each type of microorganism in one way or another is involved in the microbial succession and directly or indirectly contributes to the final characteristic properties of the products.

Phage characterization

Eight of the 26 distinct phages isolated in 2000 (Φ1-A4, Φ1-F8, Φ3-A4, Φ3-B1, Φ3-B11, Φ7-E1, Φ14-C8, and Φ22-D10) were selected for further characterization. These phages appeared two or more times during phage isolation process (Tables 2 and 3), representing frequently occurring or predominant phages. The electron micrographs (Fig. 6) showed that the eight phages all had tails, and belonged to two morphological

families. Φ3-B1, Φ7-E1, and Φ14-C8 belonged to the *Myoviridae* family, having a contractile tail. Phges Φ1-A4, Φ1-F8, Φ3-A4, Φ3-B11, and Φ22-D10 had a long noncontractile tail, and belonged to the *Siphoviridae* family. Each of these phages revealed unique morphological features (Table 6). These phages differed in their host ranges (Table 3). The principal hosts of these phages included four *L. mesenteroides* strains (1-A4, 1-F8, 3-A4, and 3-B1), one *L. pseudomesenteroides* strain 3-B11), two *Lb. plantarum* strains (14-C8 and 22-D10), and one *Lb. brevis* strain (7-E1; Table 4). The major structural proteins of the eight phages were analyzed by SDS-PAGE. Six different structural protein profiles were observed (Fig. 7). Phages Φ1-A4, Φ3-B1, Φ7-E1, Φ14-C8, and Φ22-D10 presented distinct ones. The remaining phages (Φ1-F8, Φ3-A4, and Φ3-B11) showed similar protein profiles. *Hind* III restriction digestion analysis of the eight phage DNAs showed eight unique restriction banding patterns, indicating that these phages were genetically distinct (Fig. 8). The data from the eight selected phages provided a glimpse of the genetic diversity of LAB phage population in commercial sauerkraut fermentations. Detailed sequence analysis is needed to investigate the extent of the diversity, to identify LAB-phage-specific gene sequences that could be used for rapid molecular methods to quantify the predominant phage and host populations in vegetable fermentations, without the need for cultural methods.

Knowledge of the diversity, abundance, and properties of phages in vegetable fermentations is essential for developing phage-control strategies for high and consistent quality of fermented products. In the United States, commercial sauerkraut production is usually carried out in bulk tanks without addition of starter cultures (Fleming et al., 1988). With the increasing interest in reducing waste brine disposal, low-salt fermentation is currently being developed. This will require greater control of the non-lactic population and may involve the use of starter cultures. Since the fermentation system is not sterile, the starter cultures may be susceptible to the infection by phages naturally present in these environments. Therefore, phage-control strategies are needed to ensure the viability of starter cultures until the end of the fermentation.

More research is warranted to continue to unravel the ecological role of phages and to evaluate their impact on the vegetable fermentation process. Additionally, it may

be necessary to study the fermentations at other geographic locations to examine phage diversity on a spatial scale as well as on a temporal scale.

CONLUSIONS

Results from this study demonstrated for the first time the complex phage ecology in commercial sauerkraut fermentations. The appearance of a new group of phages was correlated closely to bacterial succession. Such data strongly suggest that phages are one important factor that influences the succession of LAB species in sauerkraut fermentations, and consequent characteristic properties of the fermented products. The dynamic nature of phages and their hosts in sauerkraut fermentation provides new insights into the complex process in natural vegetable fermentations. The results indicate that phage infection is common in sauerkraut fermentations, with some phages predominating and reoccurring. This suggests that a phage-control strategy will be essential in low-salt sauerkraut fermentations, which rely on starter cultures.

REFERENCES

Atlas, R.M. 1993. Sucrose agar. In: Handbook of microbiological media. pp. 854. C. P. Lawrence, ed., CRC Press, London.

Barrangou, R., Yoon,, S.S., Breidt, F., Fleming, H.P., Klaenhammer, T.R. 2002. Identification and characterization of *Leuconostoc fallax* strains isolated from an industrial sauerkraut fermentation. Appl. Environ. Microbiol. **68**:2877-2884.

Binder, B. 1999. Reconsidering the relationship between virally induced bacterial mortality and frequency of infected cells. Aquat. Microb. Ecol. **18**:207-215.

Breidt, F. and Fleming, H.P. 1996. Identification of lactic acid bacteria by ribotyping. J. Rap. Meth. Autom. Microbiol. **4**:219-233.

Bruttin, A., Desiere, F., D'Amico, N., Guérin, J.-P., Sidoti, J., Huni, B., Lucchini, S., and Brüssow, H. 1997. Molecular ecology of *Streptococcus thermophilus* bacteriohage infections in a cheese factory. Appl. Environ. Microbiol. **63**:3144-3150.

Charbit, A. and Hofnung, M. 1985. Isolation of different bacteriophages using the LamB protein for adsorption on *Escherichia coli* K12. J. Virol. **53**:667-671.

Daeschel, M.A., McFeeters, R.F., Fleming, H.P., Kaenhammer, T.R., and Sanozky, R.B. 1984. Mutation and selection of *Lactobacillus plantarum* strains that do not produce carbon dioxide from malate. Appl. Environ. Microbiol. **47**(2):419-420.

Daeschel, M.A., Andersson, R.E., and Fleming, H.P. 1987. Microbial ecology of fermenting plant materials. FEMS Microbiol. Rev. **46**:357-367.

Everson, T.C. 1991. Control of phage in the dairy plant. Bull. Int. Dairy Fed. **263**:24-28.

Fleming, H.P. 1982. Fermented vegetables. In: Economic microbiology. Fermented foods. Vol. 7. pp. 227-258. A.H. Rose, ed., Academic Press, Inc. New York, NY.

Fleming, H.P., McFeeters, R.F., and Humphries, E.G. 1988. A fermentor for study of sauerkraut fermentation. Biotechnol. Bioeng. **31**:189-197.

Fleming HP, McFeeters RF, Daeschel MA. 1992. Fermented and acidified vegetables. In: Compendium of methods for the microbiological examination of foods. 3rd ed., pp 929-952, C. Vanderzant and D.F. Splittstoesser, eds. American Public Health Association, Washington, DC.

Gottschalk, G. 1986. Bacterial Fermentations. In: Bacterial Metabolism, pp. 220, 2nd ed., Springer-Verlag, New York.

Haggard-Ljungquist, E. Halling, C., and Calendar, R. 1992. DNA sequences of the tail fiber genes of bacteriophage P2: Evidence for horizontal transfer of tail fiber genes among unrelated bacteriophages. J. Bacteriol. **174**:1462-1477.

Jensen, E.C., Schrader, H.S., Rieland, B., Thompson, T.L., Lee, K.W., Nickerson, K. W., and Kokjohn, T.A. 1998. Prevalence of broad-host-range lytic bacteriophages of *Sphaerotilus natans*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **64**:575-580.

Lu, Z., Fleming, H.P., and McFeeters, R.F. 2001. Differential glucose and fructose utilization during cucumber juice fermentation. J. Food Sci. **66**(1):162-166.

Lu, Z., Fleming, H.P., McFeeters, R.F., and Yoon, S.S. 2002. Effects of anions and cations on sugar utilization in cucumber juice fermentation. J. Food Sci. **67**(3): 1155-1161.

Lu, Z., Fleming, H.P., and McFeeters, R.F. 2002. Fruit size effects on cucumber composition, pH, and buffer capacity, sugar utilization and bloater formation in cucumber fermentation. J. Food Sci. **67**: In press.

McDonald L.C., Fleming, H.P., and Hassan, H.M. 1990. Acid tolerance of *Leuconostoc mesenteroides* and *Lactobacillus plantarum.* Appl. Environ. Microbiol. **56**:2120-2124.

McFeeters, R.F., Fleming, H.P., and Thompson, R.L. 1982a. Malic and citric acids in pickling cucumbers. J. Food Sci. **47**(6):1859-1861, 1865.

Mundt, J.O., Graham, W.F., and McCarty, I.E. 1967. Spherical lactic acid-producing bacteria of southern-grown raw and processed vegetables. Appl. Microbiol. **15**:1303-1308.

Mundt, J.O. and Hammer, J.L. 1968. Lactobacilli on plants. Appl. Microbiol. **16**:1326-1330.

Mundt, J.O. 1970. Lactic acid bacteria associated with raw plant food material. J. Milk Food Technol. **33**:550-553.

Paul, J.H. and Kellogg, C.A. 2000. Ecology of bacteriophages in nature. In: Viral ecology. pp. 211-246. C. J. Hurst, ed.. Academic Press. New York

Pederson, C.S. and Albury, M.N. 1969. The sauerkraut fermentation. New York State Agricultural Experiment Station Technical Bulletin 824, Geneva, New York.

Peitersen, N. 1991. Practical phage control. Bull. In. Dairy Fed. **263**:1-43.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Stamer, J.R., Stoyla, B.O., and Dunckel, B.A. 1971. Growth rates and fermentation patterns of lactic acid bacteria associated with the sauerkraut fermentation. J. Milk Food Technol. **34**:521-525.

Vaughn, R.H. 1985. The microbiology of vegetable fermentations. In: Microbiology of Fermented Foods. Vol. 1. pp. 49-109. B.J.B. Wood, ed., Elsevier, New York.

Werts, C., Michel, V., Hofnung, M., and Charbit, A. 1994. Adsorption of bacteriophage lambda on the LamB protein of *E. coli* K12: point mutations in gene *J* of lambda responsible for extended host range. J. Bacteriol. **176**:941-947.

Yoon, S.S., Barrangou-Poueys, R., Breidt, F., Klaenhammer, T.R., and Fleming, H.P. 2002. Isolation and characterization of bacteriophages from fermenting sauerkraut. Appl. Environ. Microbiol. **68**:973-976.

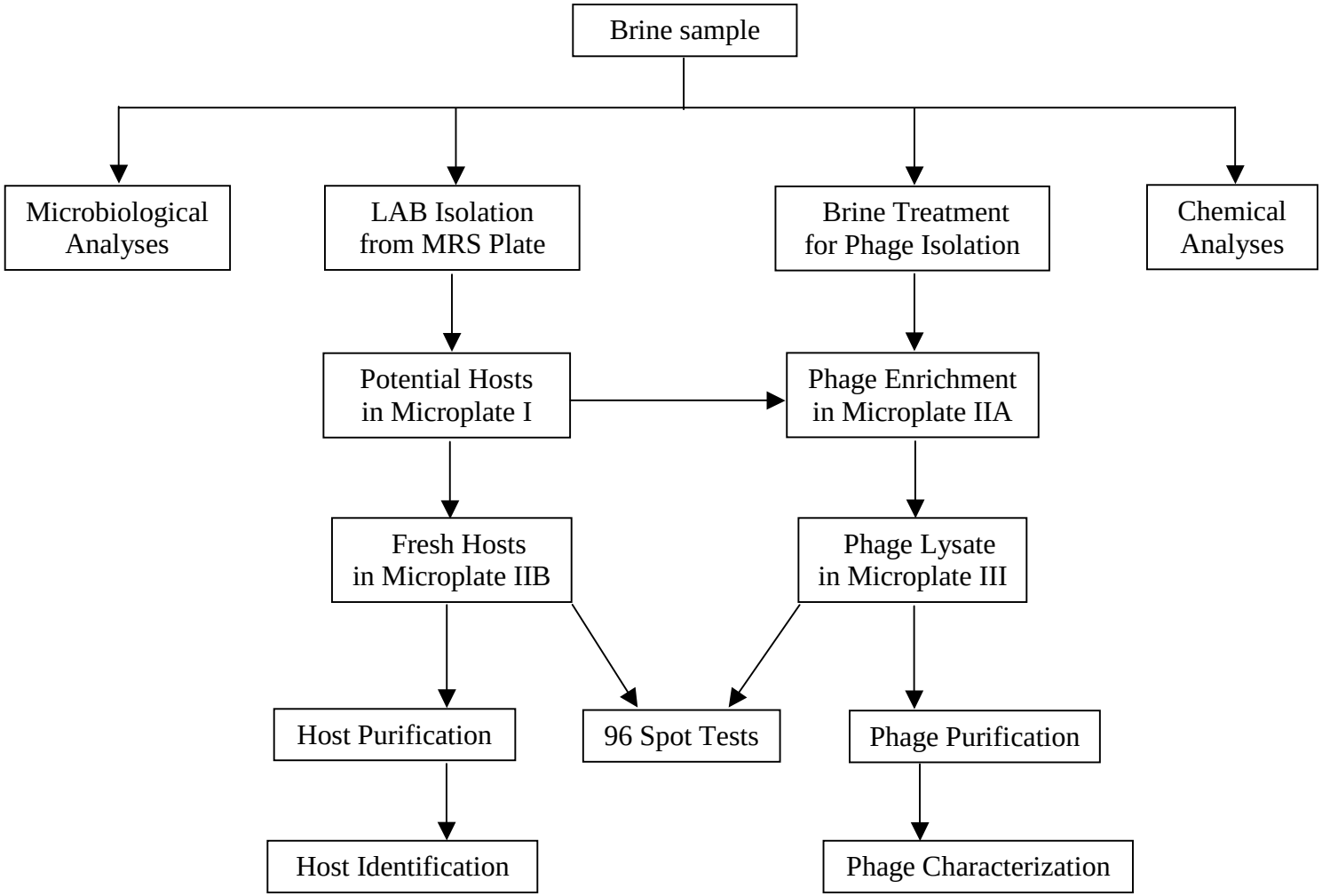

Figure 1. Flow diagram for the analyses of a brine sample and for the isolation of phages and their hosts in a commercial sauerkraut fermentation studied in 2000.

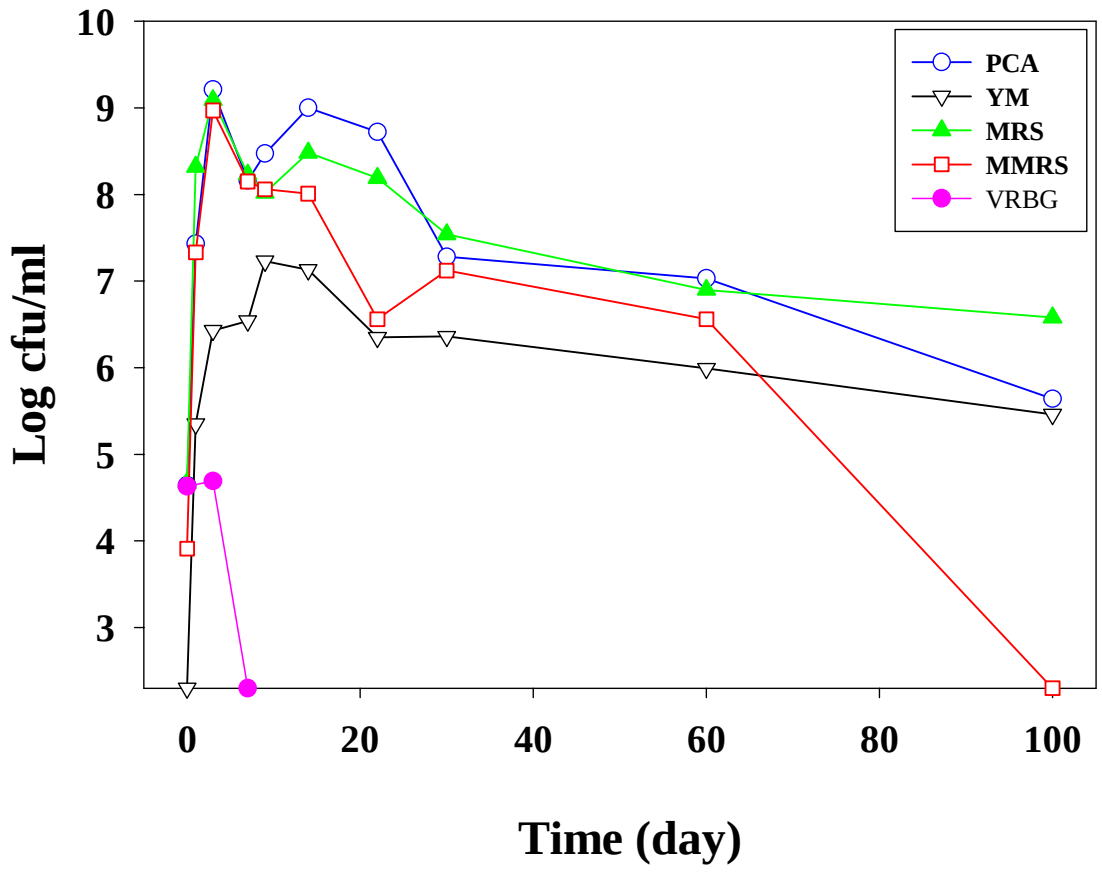

Figure 2. Microbial counts in a commercial sauerkraut fermentation tank studied in 2000.

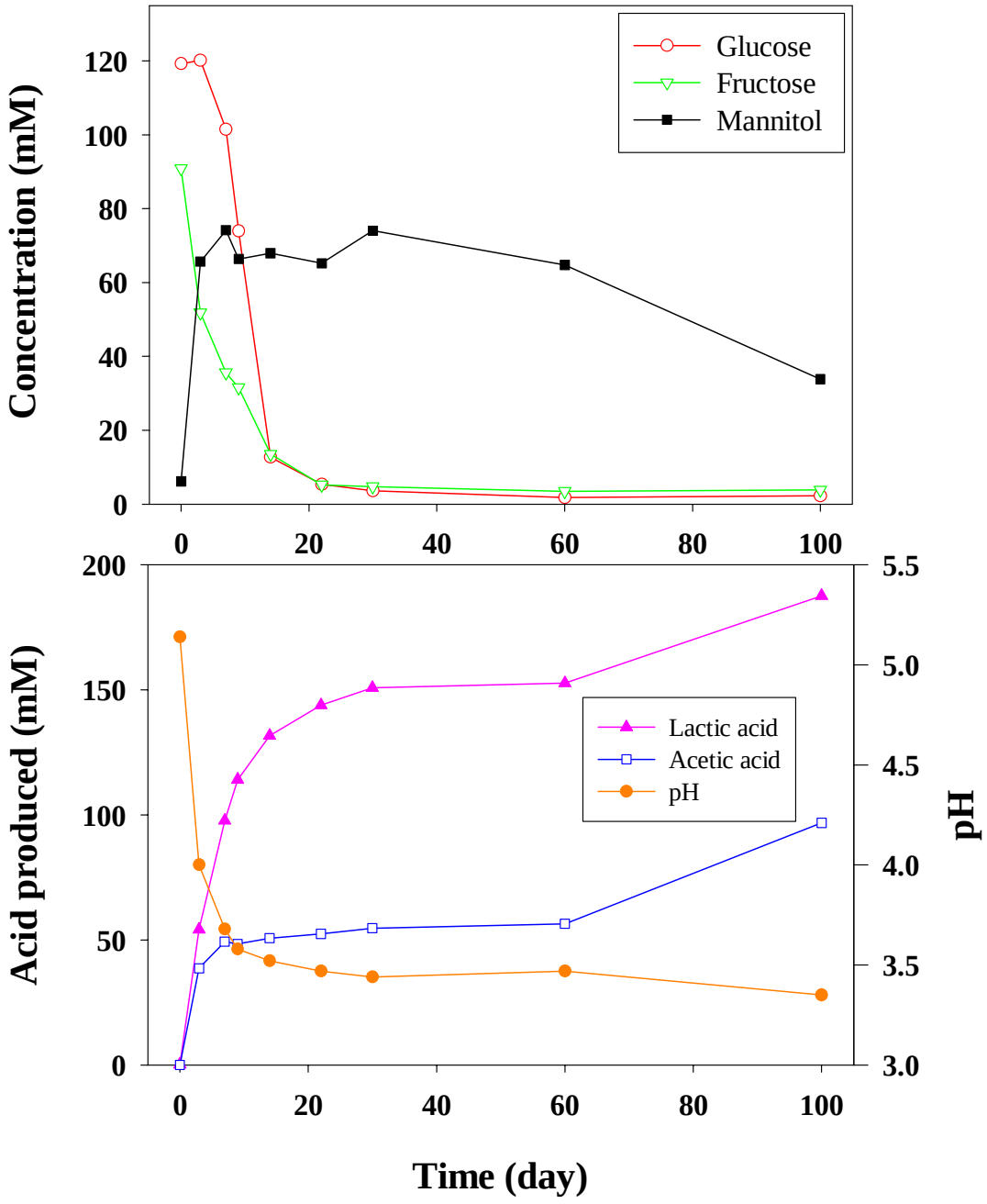

Figure 3. Changes in the concentrations of substrates and products, and pH in a commercial sauerkraut fermentation.

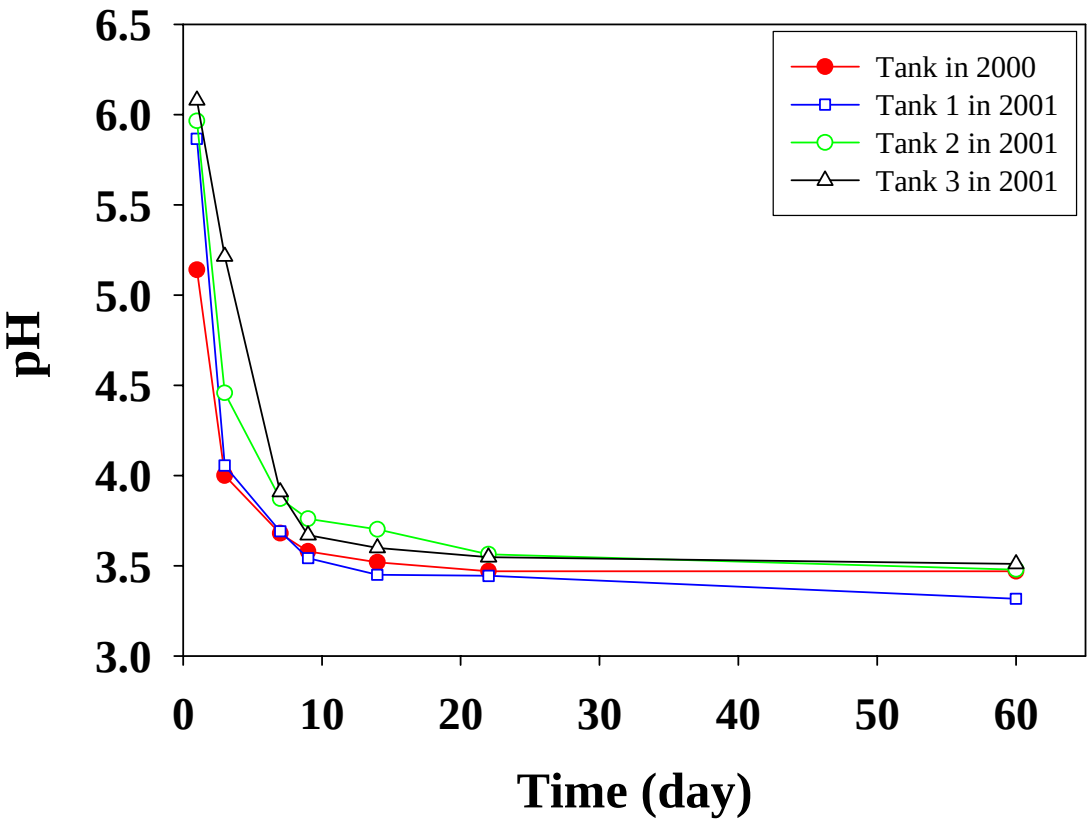

Figure 4. The pH profiles in four commercial sauerkraut fermentation tanks studied in 2000 and 2001.

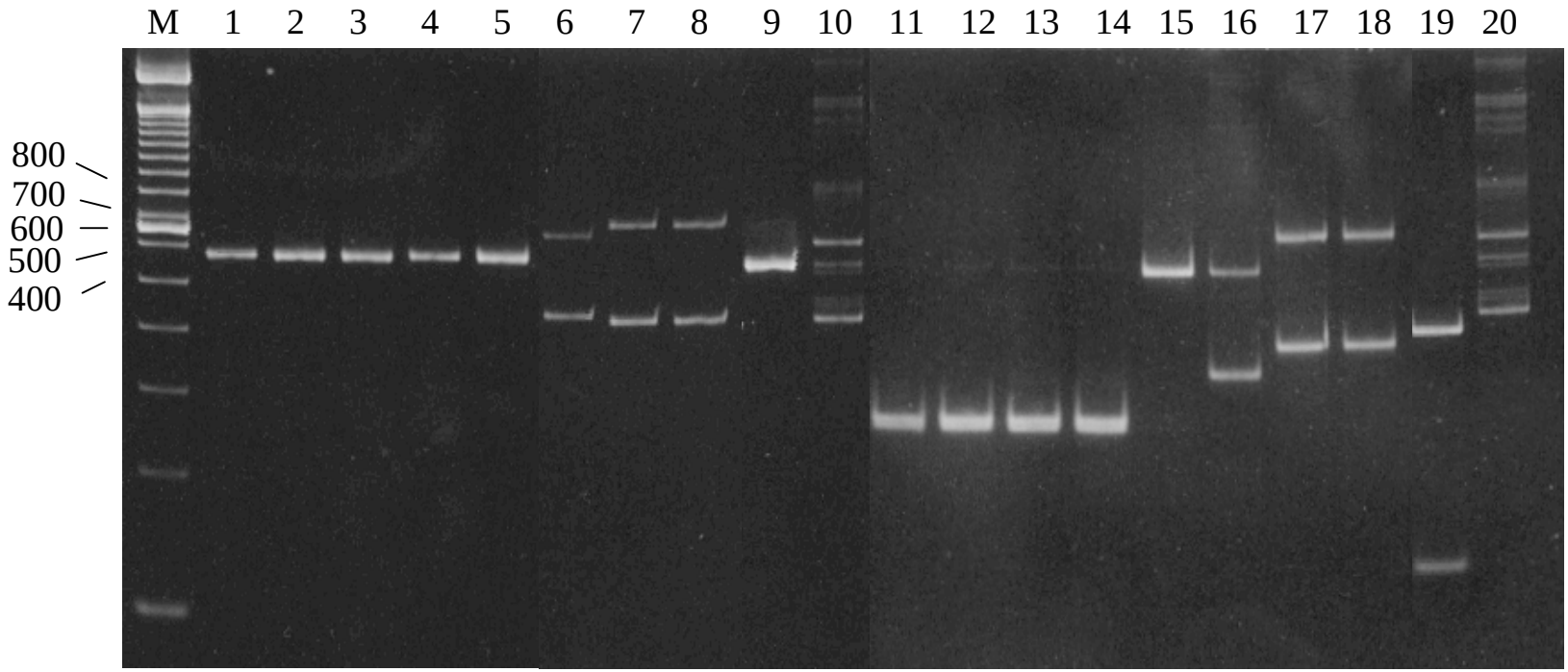

Figure 5. ITS-PCR products and ITS restriction profiles obtained from ten phage hosts. Lane M contained the 100 bp DNA ladder size standard; lanes 1-10, undigested; lanes 11-20, *Rsa*I digests. The hosts and the corresponding lane numbers are *Leu. mesenteroides* 1-A4 (1, 11), *Leu. mesenteroides* 1-F8 (2, 12), *Leu. mesenteroides* 3-A4 (3, 13), *Leu. mesenteroides* 3-B1 (4, 14), *Leu. pseudo mesenteroides* 3-B11 (5, 15), *Lb. Brevis* 7-E1 (6, 16), *Lb. plantarum* 14-C8 (7, 17), *Lb. plantarum* 22-D10 (8, 18), *Leu. fallax* 3-G1 (9, 19), and *W. kimchii* 3-H2 (10, 20).

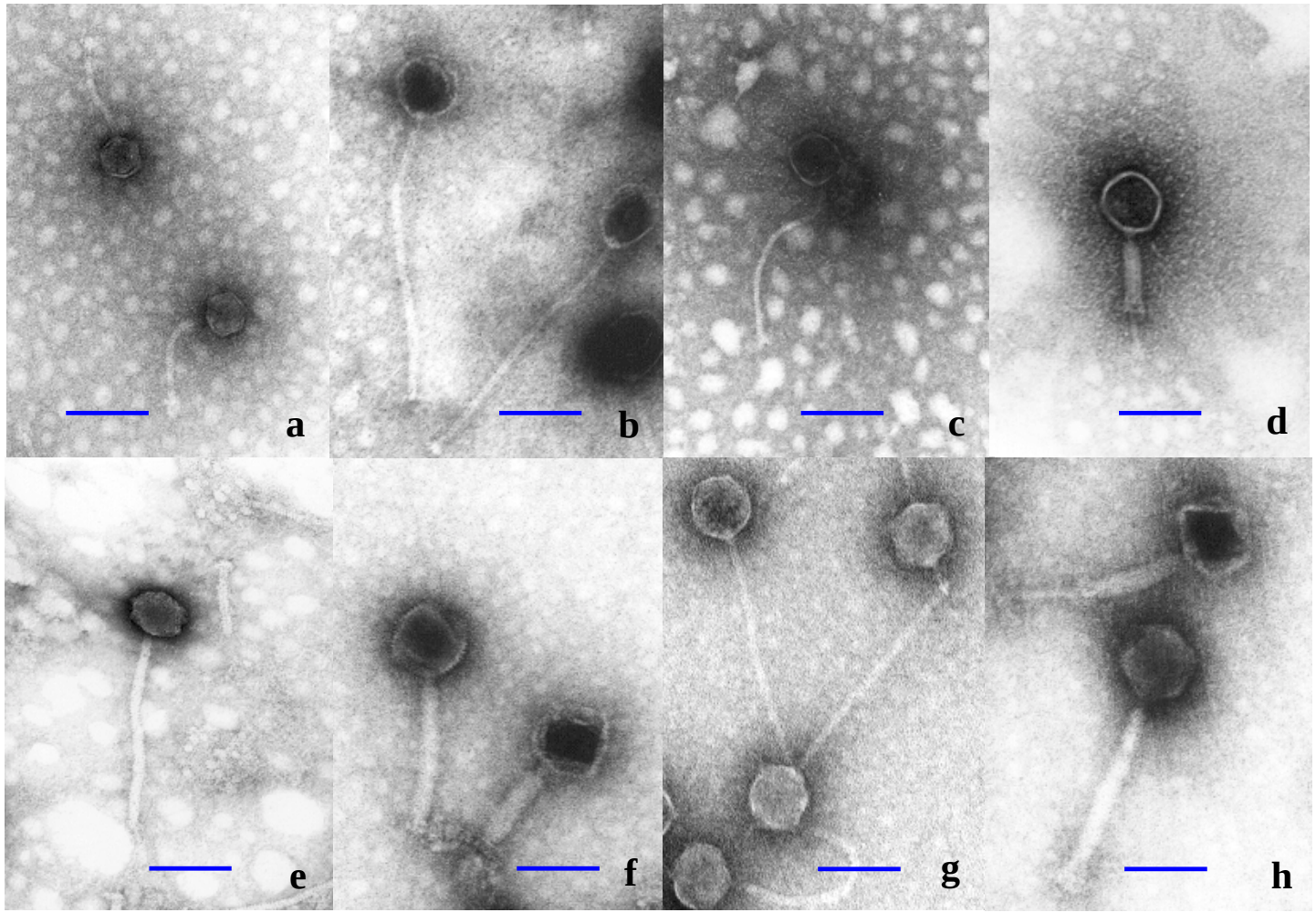

Fig. 6. Electron micrographs of eight selected phages isolated from commercial sauerkraut fermentation. CsCl-purified phage preparation was negatively stained with 2% uranyl acetate (pH 4.0). a: Φ1-A4; b: Φ1-F8; c: Φ3-A4; d: Φ3-B1; e: Φ3-B11; f: Φ7-E1 g: Φ14-C8; h: Φ22-D10. The length of the bar in each micrograph is 100 nm.

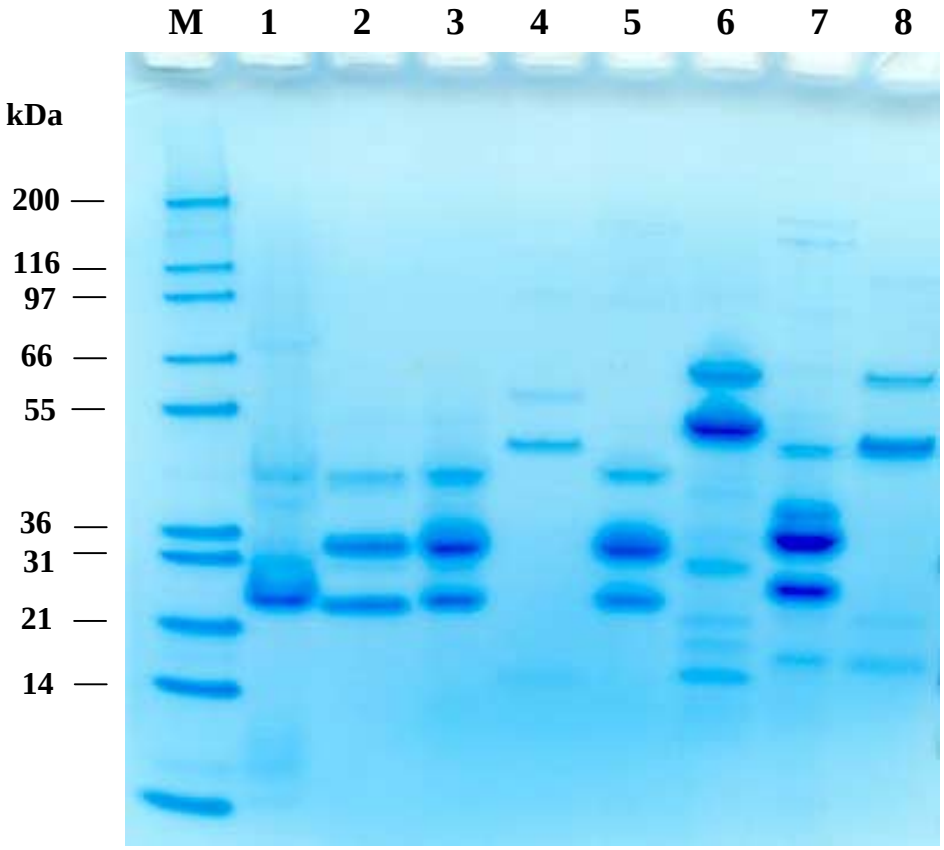

Figure 7. SDS-PAGE analysis of structural proteins from eight selected phages isolated from a commercial sauerkraut fermentation. Lane M: molecular weight standard; lane 1: Φ1-A4; lane 2: Φ1-F8; lane 3: Φ3-A4; lane 4: Φ3-B1; lane 5: Φ3-B11; lane 6: Φ7-E1, lane 7: Φ14-C8, lane 8: Φ22-D10.

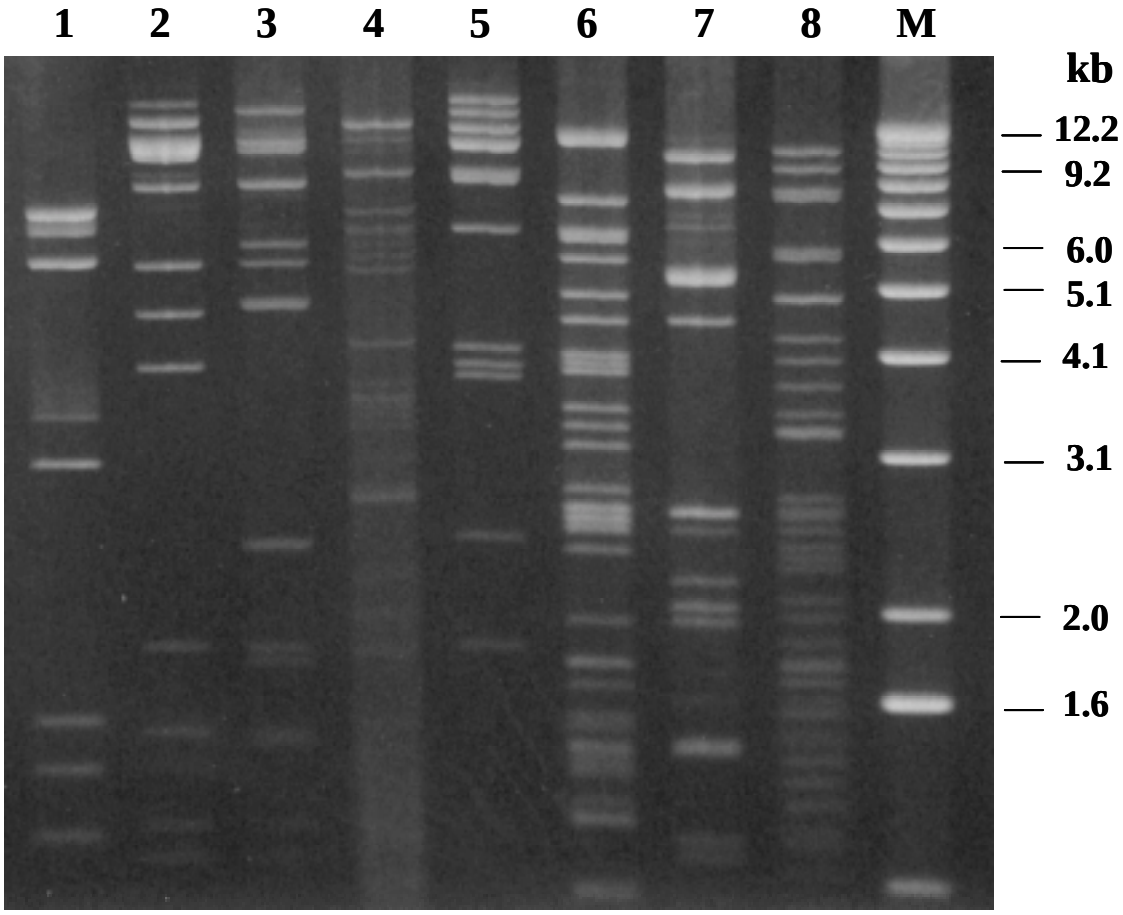

Figure 8. *Hin*dIII restriction digestion analysis of DNAs from eight selected phages isolated from a commercial sauerkraut fermentation. Lane 1: Φ1-A4; lane 2: Φ1-F8; lane 3: Φ3-A4; lane 4: Φ3-B1; lane 5: Φ3-B11; lane 6: Φ7-E1, lane 7: Φ14-C8, lane 8: Φ22-D10; lane M: a 1-kb DNA ladder.

Table 1. Fermentable substrate composition of shredded cabbage used in sauerkraut fermentations[a]

Year	Tank	Glucose		Fructose		Sucrose		Malic acid	
		mM	%	mM	%	mM	%	mM	%
2000	1	119.2	2.15	90.8	1.64	4.8	0.17	5.6	0.08
2001	1	92.4	1.66	81.7	1.47	ND[b]	ND	3.9	0.05
2001	2	90.8	1.63	83.5	1.50	ND	ND	3.5	0.05
2001	3	81.7	1.47	73.8	1.33	ND	ND	4.7	0.06

[a]mM = millimolar, percentages are by weight.
[b]ND = not detected.

Table 2. Phages and their hosts isolated from a commercial sauerkraut fermentation tank in 2000[a]

Host	Phage isolates obtained on days 1 and 3																							Phage isolates obtained on days 7, 9, 14, 22, and 60																							
	Φ 1 A 3	Φ 3 H 2	Φ 1 B 8	Φ 1 C 5	Φ 1 F 7	Φ 1 F 10	Φ 1 A 4	Φ 1 D 6	Φ 1 G 7	Φ 1 E 10	Φ 1 F 8	Φ 1 H 3	Φ 3 D 5	Φ 3 A 4	Φ 3 B 5	Φ 1 C 4	Φ 3 B 1	Φ 3 G 9	Φ 3 B 11	Φ 3 F 4	Φ 3 E 11	Φ 3 G 1	Φ 3 G 10	Φ 7 E 1	Φ 14 A 2	Φ 14 B 7	Φ 14 D 10	Φ 14 H 7	Φ 22 G 10	Φ 9 B 4	Φ 14 F 3	Φ 14 H 4	Φ 14 E 10	Φ 22 A 2	Φ 22 E 2	Φ 22 D 10	Φ 22 F 3	Φ 60 H 11	Φ 7 C 4	Φ 60 D 8	Φ 14 C 8	Φ 14 F 10	Φ 60 E 4	Φ 60 E 8
1-A3	+	+																																												
3-H2	+	+																																												
1-A4							+	+	+	+																																			
1-D6							+	+	+	+																																			
1-F9							+	+	+	+																																			
1-G7							+	+	+	+																																			
1-B8			+				+	+	+	+																																			
1-E10							+	+	+	+						+	+																												
1-C5				+							+	+	+	+	+																													
1-F8											+	+	+	+	+																													
3-B5											+	+	+	+	+																													
3-D5											+	+	+	+	+																													
1-H3											+	+	+	+	+																													
3-A4										+	+	+	+	+	+																													
1-F10						+								+	+																													
1-F7					+																																								
1-C4																+	+																												
3-B1																+	+	+																											
3-G9																+	+	+																											
3-B11																			+	+																									
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3-E11																					+																								
3-G1																						+																							
3-G10																							+																						
7-C4																																			+	+	+	+								
7-E1																								+	+	+	+	+	+				+	+											
14-A2																								+	+	+	+	+	+				+	+											
14-B7																								+	+	+	+	+	+				+	+											
14-D10																								+	+	+	+	+	+				+	+											
14-H7																								+	+	+	+	+	+				+	+											
22-G10																								+	+	+	+	+	+				+	+											
14-A4																														+			+	+											
9-B4																														+			+	+		+	+	+							
14-E10																																	+													
14-F3																															+	+	+	+											
22-A2																																		+												
14-H4																																+													
22-E2																																			+	+	+	+			+	+	+	+	
22-D10																																			+	+	+	+	+	+	+	+	+	+	
60-D8																																			+	+	+	+	+	+	+	+	+	+	
14-C8																																				+	+	+	+	+	+	+	+	+	
14-F10																																				+	+	+	+	+	+	+	+	+	
22-F3																																				+	+	+	+	+	+	+	+	+	
60-H11																																				+	+	+	+	+	+	+	+	+	
60-E4																																				+	+	+		+	+	+	+	+	
60-E8																																				+	+	+			+	+	+	+	

[a]+ indicates that the host is sensitive to the phage, or the phage is infective for the host. See Materials and Methods for the naming system used.

Table 3. Features of 26 distinct phages isolated from a commercial sauerkraut fermentation in 2000[a]

Phage ID	No. of times isolated	Days when phage isolated	Host range
Φ1-A4	3	1, 1, 1	1-A4, 1-B8, 1-E10
Φ1-B8	1	1	1-B8
Φ1-C5	1	1	1-C5, 1-F8, 3-A4
Φ1-E10	1	1	1-E10, 1-A4, 1-B8, 3-A4
Φ1-F7	1	1	1-F7
Φ1-F8	3	1, 1, 3	1-F8, 3-A4
Φ1-F10	1	1	1-F10
Φ3-A4	2	3, 3	3-A4, 1-C5, 1-F8, 1-F10
Φ3-B1	2	1, 3	3-B1, 1-C4, 1-E10
Φ3-B11	2	3, 3	3-B11
Φ3-E11	1	3	3-E11
Φ3-G1	1	3	3-G1
Φ3-G9	1	3	3-G9
Φ3-G10	1	3	3-G10
Φ3-H2	2	1, 3	3-H2
Φ7-C4	1	7	7-C4, 14-C8, 22-D10
Φ7-E1	6	7, 14, 14, 14, 14, 22	7-E1
Φ9-B4	1	9	9-B4, 14-A4
Φ14-C8	4	14, 14, 60, 60	14-C8, 22-E2, 22-D10, 60-E4, 60-E8
Φ14-E10	1	14	14-E10, 7-E1, 22-G10, 14-A4, 9-B4, 14-F3
Φ14-F3	1	14	14-F3
Φ14-H4	1	14	14-H4, 14-F3
Φ22-A2	1	22	22-A2, 7-E1, 22-G10, 14-A4, 9-B4, 14-F3
Φ22-D10	3	22, 22, 60	22-D10, 7-C4, 14-C8
Φ22-E2	1	22	22-E2, 22-D10
Φ60-D8	1	60	60-D8, 14-C8, 60-E4

[a]Red color indicates phages isolated on day 1 or day 3; green color indicates phages isolated on day 7 or thereafter.

Table 4. Characteristics of 28 distinct host strains isolated from a commercial sauerkraut fermentation in 2000[a]

Host	Genus species	Catalase test	Gas production	Dextran test	Malolactic activity	Phage typing[e]
1-A4	*L. mesenteroides*[b]	-	+	+	-	Φ1-A4, Φ1-E10
1-B8	*L. mesenteroides*	-	+	+	-	Φ1-B8, Φ1-A4, Φ1-E10
1-C4	*L. mesenteroides*	-	+	+	+	Φ1-C4, Φ3-B1
1-C5	*L. mesenteroides*	-	+	+	-	Φ1-C5, Φ1-F8, Φ3-A4
1-E10	*L. mesenteroides*	-	+	+	-	Φ1-E10, Φ1-A4, Φ1-C4, Φ3-B1
1-F7	*L. citreum*	-	+	+	-	Φ1-F7
1-F8	*L. mesenteroides*	-	+	+	-	Φ1-F8, Φ3-A4
1-F10	*L. mesenteroides*	-	+	+	-	Φ1-F10, Φ3-A4
3-B1	*L. mesenteroides*	-	+	+	+	Φ3-B1, Φ1-C4, Φ3-G9
3-A4	*L. mesenteroides*	-	+	+	-	Φ3-A4, Φ1-E10, Φ1-F8
3-B11	*L. pseudomesenteroides*	-	+	+	+	Φ3-B11
3-E11	*L. fallax*	-	+	+	-	Φ3-E11
3-G1	*L. fallax*	-	+	+	-	Φ3-G1
3-G10	*L. fallax*	-	+	+	-	Φ 3-G10
3-H2	*W. kimchii* or *confusa*[c]	-	+	+	+	Φ3-H2
7-C4	*Lb. plantarum*[d]	-	-	-	+	Φ7-C4, Φ 22-D10
7-E1	*Lb. brevis*	-	+	-	+	Φ7-E1, Φ14-E10, Φ 22-A2
9-B4	*Lb. plantarum*	-	-	-	-	Φ9-B4, Φ14-E10, Φ22-A2, Φ22-D10
14-A4	*Lb. plantarum*	-	-	-	-	Φ14-A4, Φ14-E10, Φ22-A2
14-C8	*Lb. plantarum or pentosus*	-	-	-	+	Φ14-C8, Φ7-C4, Φ 22-D10, Φ60-D8
14-E10	*Lb. plantarum*	-	-	-	+	Φ14-E10
14-F3	*Lb. paraplantarum*	-	-	-	+	Φ14-F3, Φ14-E10, Φ14-H4, Φ 22-A2
14-H4	*Lb. paraplantarum*	-	-	-	+	Φ14-H4
22-A2	*Lb. plantarum*	-	-	-	+	Φ22-A2
22-D10	*Lb. plantarum* or *pentosus*	-	-	-	+	Φ22-D10, Φ7-C4, Φ 14-C8, Φ22-E2, Φ60-D8
22-E2	*Lb. plantarum*	-	-	-	+	Φ 22-E2, Φ14-C8, Φ22-D10
60-E4	*Lb. plantarum*	-	-	-	+	Φ60-E4, Φ22-D10, Φ60-D8

[a]The first number of each host ID indicates the fermentation day when the host was isolated.
[b]*L*. represents *Leuconostoc*.
[c]*W*. represents *Weissella*.
[d]*Lb*. represents *Lactobacillus*.
[e]Phage typing is defined as the pattern of sensitivity of a bacterium to lysis by a series of phages.

Table 5. Phages isolated from 3 commercial sauerkraut fermentation tanks in 2001[a]

	Day 1 Tank				Day 3 Tank				Day 7 Tank		
Host	1	2	3	**Host**	1	2	3	**Host**	1	2	3
1-A3	+	+	+	3-A4	+	+	+	7-C4	+	+	+
1-A4	+	+	-	3-B1	+	+	+	7-E1	+	-	-
1-B8	+	+	+	3-B11	+	+	+				
1-C4	+	+	+	3-E11	+	+	+		**Day 9** Tank		
1-C5	+	+	+	3-G1	+	+	+				
1-E10	+	+	+	3-G10	+	+	+	**Host**	1	2	3
1-F7	+	+	+	3-H2	+	-	-	9-B4	-	+	+
1-F8	+	+	-	1-F8	+	+	+	7-E1	+	-	+
1-F10	+	+	+	3-B5	+	+	-				
	Day 14 Tank				**Day 22** Tank				**Day 60** Tank		
Host	1	2	3	**Host**	1	2	3	**Host**	1	2	3
14-A4	-	-	-	22-A2	-	-	-	60-E4	+	+	+
7-E1	+	+	+	7-E1	+	+	+	7-E1	+	+	+
14-C8	+	+	+	14-C8	+	+	+	14-C8	+	+	+
14-E10	-	-	-	22-F3	+	+	+	22-F3	+	+	+
14-F3	-	-	-	22-D10	+	+	+	22-D10	+	+	+
14-H4	+	+	+	22-E2	+	+	+	60-D8	+	+	+
14-A2	+	+	+	22-G10	+	+	+	60-E8	+	+	+
								60-H11	+	+	+

[a]+ indicates that a phage was isolated against the host in a specific tank on a particular day;
- indicates that no phage was isolated aginst the host in the specific tank on a particular day.

Table 6. Morphological features of eight selected phages isolated from a commercial sauerkraut fermentation[a]

Phage	Family	Head	Tail	
		diam (nm)	Length (nm)	Width (nm)
Φ1-A4	Siphoviridae	53	131	10
Φ1-F8	*Siphoviridae*	71	351	12
Φ3-A4	*Siphoviridae*	60	288	9
Φ3-B1	*Myoviridae*	78	155	23
Φ3-B11	*Siphoviridae*	60	273	13
Φ7-E1	*Myoviridae*	87	149	26
Φ14-C8	*Siphoviridae*	70	292	13
Φ22-D10	*Myoviridae*	85	271	25

[a]Each value represents the mean of five independent measurements.

Chapter 3

Isolation and Characterization of *Lactobacillus plantarum* Bacteriophage ΦJL-1 from a Cucumber Fermentation

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 the derivative mutant MU45, which have been evaluated as starter cultures for controlled cucumber fermentation and as biocontrol microorganisms for minimally processed vegetable products. We report here genome sequencing data for ΦJL-1; the genome size was 36.7 kbp. SDS-PAGE profiles indicated that ΦJL-1 contains six structural proteins (22, 34, 45, 50, 58, and 82 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 per cell. An adsorption experiment showed that 90% of phage particles were adsorbed to the host cells 20 min after infection. It was observed that calcium supplementation (up to 30 mM $CaCl_2$) in MRS media did not affect the first cycle of phage adsorption, but greatly 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, ΦJL-1, *Lactobacillus plantarum*, lactic acid bacteria, vegetable fermentation, cucumber fermentation

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 may greatly affect the quality and microbial stability of the final product. Many physical, chemical, and biological factors, 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.

Bacteriophage 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* are by far the most frequent phage group (61.7%), followed by the *Myoviridae* (24.5%) and *Podoviridae* (13.9%) (Ackermann, 1999). Most *Lactobacillus* phage, including several reported *L. plantarum* phage 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* phage 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 phage specific for *L. plantarum* MU45, which has been evaluated for use in low-salt controlled fermentations. 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). To our knowledge, this is the first report of isolation and characterization of a *L. plantarum* phage from a cucumber fermentation.

MATERIALS AND METHODS

Bacterial strains and culture media

Lactobacillus plantarum strain MU45 was used as the primary host for the isolation, propagation, and characterization of the bacteriophage, named ΦJL-1. Thirty two strains 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 phage 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.

Phage isolation and enrichment

Brine samples were obtained from a commercial cucumber fermentation tank (Mt. Olive, NC) containing size no. 1 (2.4-2.7 cm diameter) cucumbers. The samples were adjusted to pH 6.5 with 3N NaOH and centrifuged (5,000 x 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 $CaCl_2$, to which an early log-phase host culture was previously added. After incubation at 30°C for 16-18 h, the medium was centrifuged at 4,000 x 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.

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. Three ml of soft agar (MRS broth with 0.7% agar) was seeded with 0.1 ml of actively growing culture (10^9 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. The plate was allowed to stand for 30 min before incubation 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.

Plaque purification, lysate preparation, and bacteriophage titering

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 as described above, and plaqued again, repeating the cycle three additional times. After plaque purification, phage lysate was prepared. 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 4,000 x g for 10 min at 4°C (Sorvall RC-5B centrifuge, Wilmington, DE). The pH of the supernatant was adjusted to 6.5 with 3N 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.

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 corresponded to an initial cell count of approximately 1 x 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 9,000 x g for 3 min. The supernatant was filtered (0.45-μm pore size syringe filter) and assayed to determine the phage titer by using the double layer agar plate method described above. Viable cell counts were determined by using a spiral plater (Autoplate 4000; Spiral Biotech, Inc., Bethesda, MD) for plating samples on MRS agar, and a colony 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 to demonstrate the absence of contamination. 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.

Large-scale phage production

One liter pre-warmed (30°C) MRS broth was inoculated with an overnight *L. plantarum* MU 45 culture to an initial cell level of approximately 8 x 10^7 cfu/ml. The cells were grown to approximately 2 x 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 the predetermined optimum MOI (0.01-0.02). The incubation was continued until complete lysis was observed (about 4.5 h after infection).

Concentration and purification of phage lysates

A large-scale phage lysate (1 liter, as described above) was centrifuged at 8,000 x g for 20 min. The supernatant was 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. Phage were then precipitated using modification of a method described by Yamamoto et al. (1970). Polyethylene glycol (Sigma-Aldrich) 8,000 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 phage were pelleted by centrifugation at 10,000 x 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 x g for 6 h at 15°C (Sorvall micro-ultra-centrifuge 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 6,000-8,000 dalton pore size membrane (Spectrum, Houston, TX).

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 50,000x and printed at 85,000x (V. Knowlton, Center for Electron Microscopy, NC State University, Raleigh, NC). The phage size was determined from the average of five independent measurements.

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 and pelleted with a microcentrifuge. 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 carried out at the Department of Energy Joint Genome Institute (JGI) sequencing facility (Walnut Creek, CA), and sequence data are available on the JGI web site at http://www.jgi.doe.gov. 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 under UV light (300 nm) after stained with ethidium bromide (1 µg/ml).

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 (≈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 a 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 tested for unadsorbed phages by the double-layer agar plate method. MRS broth containing phage only was used as a control.

Percent adsorption of the phage was calculated as [(control titer - residual titer)/control titer] x 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 ml of an early log phase culture of the host cells (2 x 10^8 cfu/ml) was harvested by centrifugation (12,000 x 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 x 10^9 pfu/ml) to give 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.

Calcium effect on phage propagation

Calcium effect on phage propagation was determined in five 15-ml tubes. Ten ml of early log-phase host culture (≈ 1 x 10^8 cfu/ml) in MRS broth were 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 obtained from each tube at selected intervals and filtered immediately. Plaque-forming units were determined by the double-layer agar plate method.

One-step growth

For one-step growth experiments a modification of the method 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 sec (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 titered 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 sigmoidal model with five parameters was used for fitting the one-step growth curve (SigmaPlot for Windows, Version 5.00, Jandel Scientific, Chicago, IL).

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

An aliquot (26 µl) of CsCl-purified ΦJL-1 sample was mixed with 10 µl of sample buffer and 4 µl of reducing agent (NuPAGE LDS system, Novex, San Diego, CA). The mixture was heated in a boiling water bath 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, Piscataway, NJ), 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.

Thermal inactivation

A temperature-controlled water bath (M3 Lauda, Brinkman Instrument Co., Westbury, NY) was used in the thermal inactivation experiment to determine the D values of phage ΦJL-1. A 1.5-ml microcentrifuge tube containing 900 µl of sterile, de-ionized water was preheated to a desirable temperature, ranging from 70 to 100°C. One hundred µl of phage solution (10^6 pfu/ml in water) was added to the tube. After heating at intervals between 15 sec to 3 min, the tube was placed in an ice-water bath. Samples were assayed to determine plaque forming units by the double-layer agar plate method. D values were calculated as the time required for one log reduction in pfu/ml.

RESULTS

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 Figure 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 overall appearance of this phage suggests that the phage could be classified as morphotype B1 according to Ackermann (1996), or to the family *Siphoviridae* according to the International Committee on Taxonomy of Viruses (Matthews, 1982). The optimal MOI was determined to be 0.01-0.03 (data not shown).

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 Figure 2. About 75% of ΦJL-1 phage particles were adsorbed to the host cells in 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, indicating that excess of Ca^{2+} did not affect the initial adsorption rate. However, rapid phage propagation occurred in calcium-supplemented media (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 to 30 mM. The phage titer in the medium without calcium supplementation reached the same level as those in calcium-supplemented media in 4 h. The percent adsorption of the phage ΦJL-1 in calcium-free BES buffer was similar to those in MRS media with or without calcium supplementation.

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* MU45 and a related isogenic strain BI7 (B17 is able to carry out malolactic fermentation, while MU45 does not). No other LAB tested were sensitive to ΦJL-1. The infection cycle of ΦJL-1 in MRS was characterized by its one-step growth kinetics at 30°C. Three experiments were carried out with strain MU45 to determine the one-step growth curve of ΦJL-1. Figure 4 showed that the latent period was about 35 min, the rise period was 40 min. The average burst size was calculated to be 22 pfu per cell. Thermal lability was demonstrated by thermal inactivation of the ΦJL-1 population (10^6 pfu/ml) at pH 6.5 within 3 min at 70, 80, 90, and 100°C. Survivor curves of the phage at 70 and 80°C are shown in Figure 5. The D values of the phage were calculated as 2.7 min at 70°C and 0.2 min at 80°C. The phage titer decreased below the detection limit (20 pfu/ml) after heating for longer than 60 sec at 80°C, or 15 sec at 90 or 100°C.

Protein and sequence analysis

The structural proteins of phage ΦJL-1 were analyzed by SDS-PAGE. The resulting protein profile (Fig. 6) reveals six structural proteins of apparent molecular masses of 82, 58, 50, 45, 34 and 22 kDa. Chromosomal DNA was submitted to the Department of Energy Joint Genome Institute (Walnut Creek, CA). Preliminary sequence data showed two major contiguous sequences, Contigs 1 and 2. Contig 2 was constructed from 1,216 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 ΦJL-1 (Fig. 7) was constructed based on this primary sequencing data and confirmed by our restriction analyses (data not shown).

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 ΦPL1-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 is also dependent on the presence of certain cations in the media. Many bacteriophage require higher concentration 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 to 30 mM) in MRS media did not affect the adsorption rate in the first 30 min, but promoted rapid phage propagation and cell lysis. The result suggested that calcium and/or other cations in MRS media were sufficient for the adsorption during the first cycle of phage infection but not for the subsequent cycle. Watanabe and Takesue (1972) reported that calcium ions were required for the penetration of the phage genome into the host cells of *Lactobacillus casei*. It is likely that as phage titer increased exponentially, higher calcium concentration was required for rapid propagation in the subsequent cycle. The phage titer in MRS medium without calcium supplementation gradually increased and eventually reached the same level as those in calcium-supplemented media after 4 h of infection (Fig. 3), indicating that calcium greatly stimulated the rate of phage propagation, but did not affect the final phage titer. 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 ions were not required for phage adsorption in BES buffer. 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 phage 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 seemed to be sufficient to facilitate phage adsorption. Watanabe and Takesue (1972) studied *L. casei* phage using 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.

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 per cell, larger than 12-14 pfu per cell for *L. plantarum* 8014 phage, B2 (Nes et al., 1988), but almost 10 times smaller than that for another *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 that for ΦJL-1 (59 nm, 182 nm, respectively). Both phage B2 and fri had 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 *L. plantarum* ATCC 14917, while SC912 was. High intraspecies 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). Phage sensitivity can be related to cell wall composition in some strains of *L. plantarum* (Douglas and Wolin, 1971).

Like most tailed phage, ΦJL-1 had a genome consisting of 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 *L. sake* phage PWH2 (35 kbp), *L. bulgaricus* phage ch2 (35 kbp), and *L. casei* phage J-1 (37 kb), but smaller than that of *L. gasseri* phage phi adh (43.8 kbp; Table 2). The restriction digestion fragment sizes reported for these phage 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 molecular weight ranging from 22 kDa to 82 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 susceptible to temperatures above 70°C. The D value at 80°C and pH 6.5 was 0.2 min. The information may be useful for designing a procedure to prepare

vegetables prior to starter culture addition. Breidt et al. (2000) reported that blanching whole pickling cucumbers for 15 sec (0.25 min) at 80°C reduced microbial cell counts by 2 to 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, 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 characterization of this phage provides valuable information for the development of the procedure for controlled cucumber fermentation and for the biocontrol system using *L. plantarum* MU45. Further research is needed to evaluate the impact of ΦJL-1 on these systems.

REFERENCES

Ackermann, H.-W. 1996. Frequency of morphological phage descriptions in 1995. Arch. Virol. **141**:209-218.

Ackermann, H.-W. 1999. Tailed bacteriophage: the order Caudovirales. Adv. Virus Res. **51**:135-201.

Adams, M.H. 1959. Bacteriophage. Interscience Publishers, Inc., New York. pp. 450 -456.

Birge, E.A. 2000. Bacterial and bacteriophage genetics. 4th ed. Springer-Verlag. New York.

Breidt, F., Hayes, J.S., Fleming, H.P. 2000. Reduction of microflora of whole pickling cucumbers by blanching. J. Food Sci. **65**:1354-1358.

Caso, J. L., de los Reyes-Gavilán, C.G., Herrero, M., Montilla, A., Rodriguez, A., Suarez, J.E. 1995. Isolation and characterization of temperate and virulent bacteriophage of *Lactobacillus plantarum*. J. Dairy Sci. **78**:741-750.

Chopin, M.C.,Chopin, A., Roux, C. 1976. Definitions of bacteriophage groups according to their lytic action on mesophilic lactic streptococci. Appl. Environ. Microbiol. **32**: 741-746.

Chow, J.J., Batt, C.A., Sinskey, A.J. 1988. Characterization of *Lactobacillus bulgaricus* bacteriophage ch2. Appl. Environ. Microbiol **54**:1138-1142.

de Man, J. C., Rogosa, M., Sharpe, M.E. 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. **23**:130-135.

Douglas, L.J., Wolin, M.J. 1971. Cell wall polymers and phage lysis of *Lactobacillus plantarum*. Biochem. **10**:1551-1555.

Durmaz, E. 1992. A fourth mechanism of bacteriophage resistance in *Lactococcus lactis* subsp. *lactis* ME2. M.S. Thesis. NC State University, Raleigh, NC.

Durmaz, E., Klaenhammer, T.R. 2000. Genetic analysis of chromosomal regions of *Lactococus lactis* acquired by recombinant lytic phage. Appl. Environ. Microbiol. **66**: 895-903.

Ellis, E.L., Delbruck, M**.** 1939. The growth of bacteriophage. J. Gen. Physiol. **22**:365 -384.

Etchells, J.L., Bell, T.A., Fleming, H.P., Kelling, R.E., Thompson, R.L. 1973. Suggested procedure for the controlled fermentation of commercially brined pickling cucumbers – the use of starter cultures and reduction of carbon dioxide accumulation. Pickle Pak Sci. **3** (1):4-14.

Fleming, H.P. 1982. Fermented vegetables. In: Rose, A.H. (Ed.), Economic Microbiology. Fermented Foods, vol. 7, Academic Press, Inc., New York, NY. pp. 227-258.

Fleming, H.P. 1984. Developments in cucumber fermentation. J. Chem. Tech. Biotechnol. **34B**:241-252.

Foschino, R., Perrone, F., Galli, A. 1995. Characterization of two virulent *Lactobacillus fermentum* bacteriophage isolated from sour dough. J. Appl. Bacteriol. **79**:677-683.

Fremaux, C., De Antoni, G.L., Raya, R.R., and Klaenhammer, T.R. 1993. Genetic organization and sequence of the region encoding intergrative functions from *Lactobacillus gasseri* temperate bacteriophage phi adh. Gene. **126**(1):61-66.

Jarvis, A.W. 1989. Bacteriophage of lactic acid bacteria. J. Dairy Sci. **72**:3406-3428.

Khosaka, T. 1977. Physicochemical properties of virulent *Lactobacillus* phage containing DNA with cohesive ends. J. Gen. Virol. **37**:209-214.

Leuschner, R. G. K., Arendt, E.K., Hammes, W.P. 1993. Characterization of a virulent *Lactobacillus sake* phage PWH2. Appl. Microbiol. Biotech. **39:**617-621.

Luria, S.E., Darnell, Jr., J.E., Baltimore, D., Campbell, A. 1978. Phage-bacterium interaction: General features. In: General virology, 3rd ed., John Wiley & Sons. New York. pp. 135-156.

Mathews, R.E.F. 1982. Classification and nomenclature of viruses. Fourth report of the International Committee on Taxonomy of Viruses. Intervirol. **17**:1-10.

Nes. I. F., Brendehaug, J., von Husby, K.O. 1988. Characterization of the bacteriophage B2 of *Lactobacillus plantarum* ATCC 8014. Biochimie. **70**:423-427.

Nes, I. F., Sorheim, O. 1984. Effect of infection of a bacteriophage in a starter culture during the production of salami dry sausage. J. Food Sci. **49**:337-340.

Pederson, C. S., Albury, M.N. 1969. The sauerkraut fermentation. New York State Agricultural Experiment Station Technical Bulletin No. 824. NY State Agric. Exper. Sta., Geneva, NY.

Romick, T. L. H.P., 1994. Biocontrol of *Listeria monocytogenes*, a psychrotrophic pathogen model, in low salt, non-acidified, refrigerated vegetable products. Ph.D.

Dissertation. North Carolina State University.

Sechaud, L., Cluzel, P.-J., Rousseau, M., Baumgartner, A., Accolas, J.-P. 1988. Bacteriophage of lactobacilli. Biochimie. **70**:401-410.

Topley, W.W.C., Wilson, G.S. 1990. Principles of bacteriology, virology and immunity. In: Collier, L.H., Timbury, M.C. (Ed.). vol. 4. B.C. Decker Publisher, London, UK. pp. 52-53.

Trevors, K. E., Holley, R.A., Kempton, A.G. 1983. Isolation and characterization of a *Lactobacillus plantarum* bacteriophage isolated from a meat starter culture. J. Appl. Bacteriol. **54**:281-288.

Watanabe, K., Takesue, S. 1972. The requirement for calcium in infection with *Lactobacillus* phage. J. Gen. Virol. **17**:19-30.

Yamamoto, K.R., Alberts, B.M., Benzinger, R., Lawhorne, L., Treiber, G. 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. Virol. **40**:734-744.

Yoon, S-S., Kim, J.W., Breidt, F., Fleming, H.P. 2001. Characterization of a lytic *Lactobacillus plantarum* bacteriophage and molecular cloning of a lysin gene in *Escherichia coli*. Intern. J. Food Microbiol. **65**:63-74.

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	-	LA 23
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	-	LA272A
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).

Table 2. Genome sizes of several *Lactobacillus* bacteriophages

Phage	DNA size (kb)	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*	Fremaux et al., 1993
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

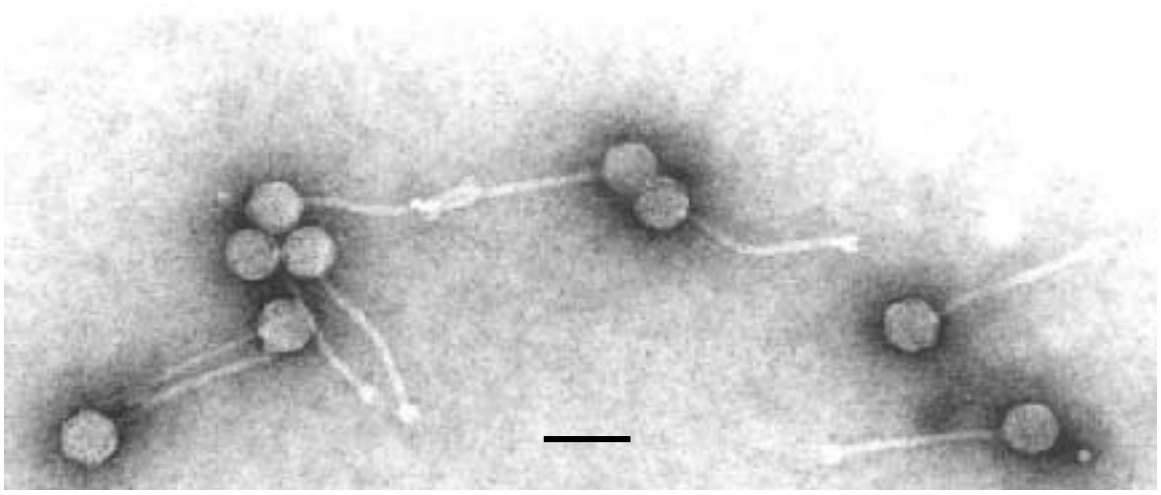


Figure 1. Electron micrograph of *L. plantarum* phage ΦJL-1. CsCl-purified bacteriophage preparation was negatively stained with 2% uranyl acetate (pH 4.0). Magnification, 85,000X. Bar, 100 nm.

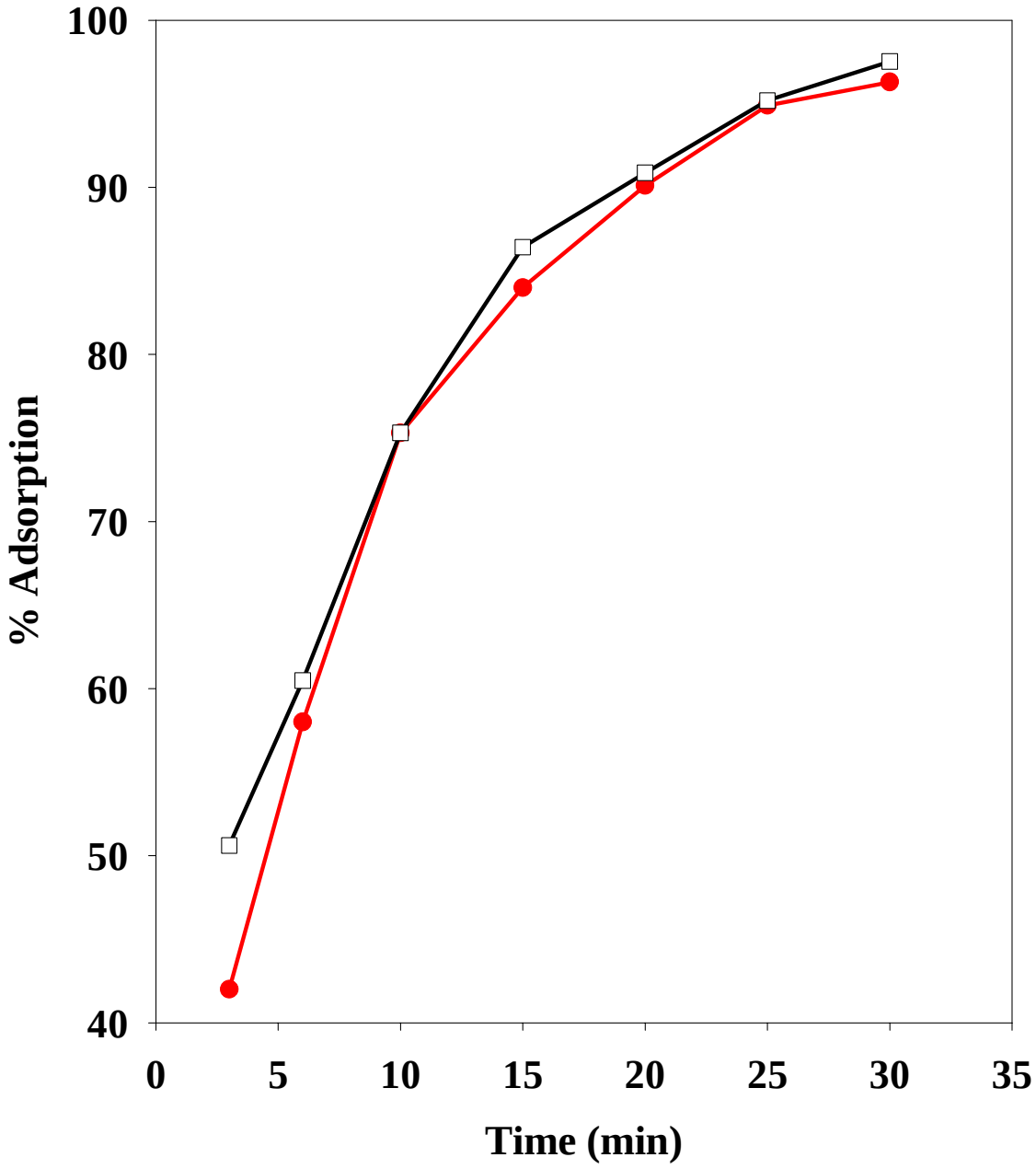


Figure 2. Adsorption curves of *L. plantarum* phage ΦJL-1 in MRS medium without calcium supplement (●) or with 20 mM calcium supplement (□).

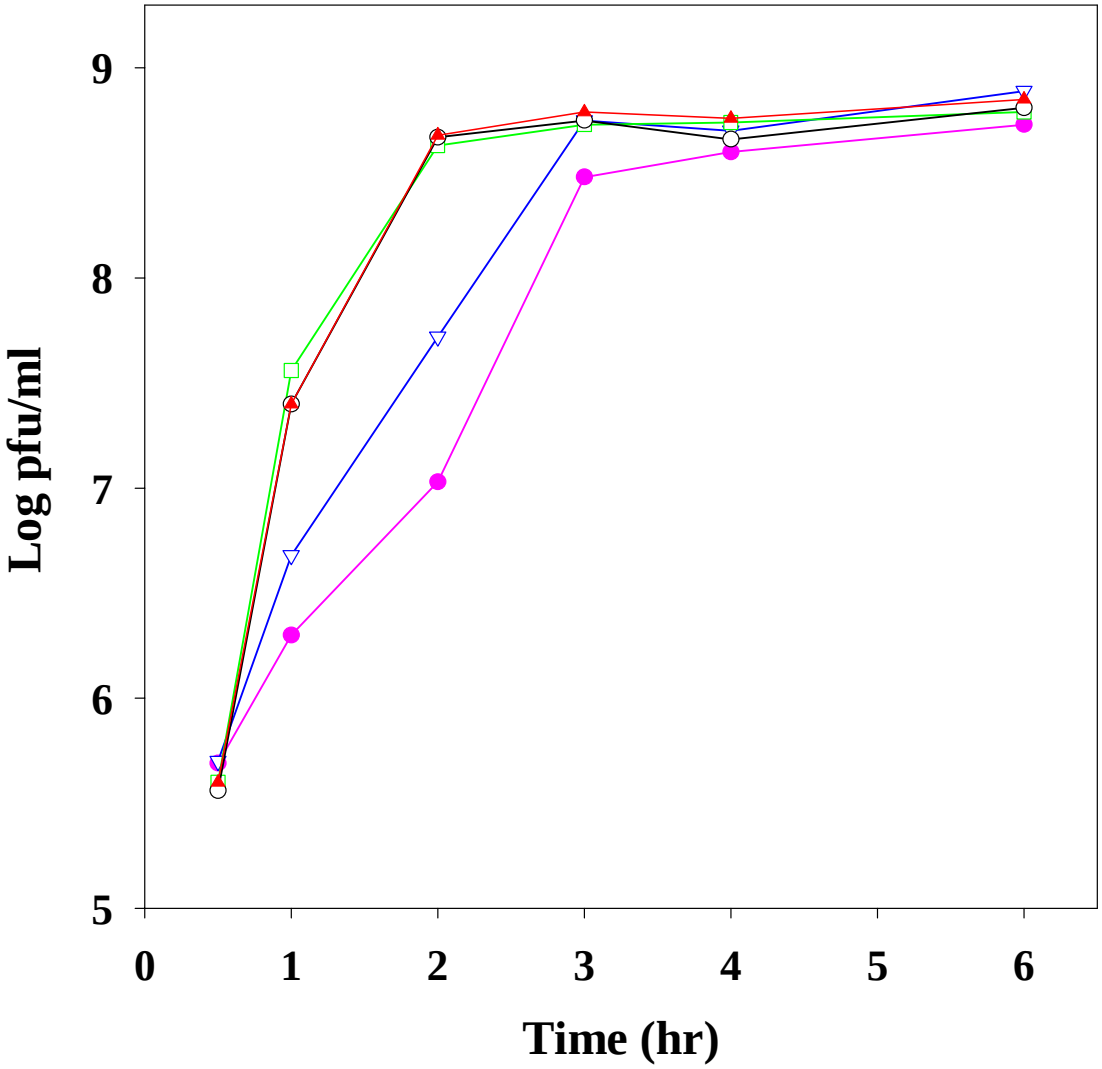


Figure 3. Calcium effect on phage ΦJL-1 propagation in MRS media at 30°C. ●, without calcium supplement; ▽, with 1 mM calcium supplement; ▲, with 10 mM calcium supplement; □, with 20 mM calcium supplement; ○, with 30 mM calcium supplement.

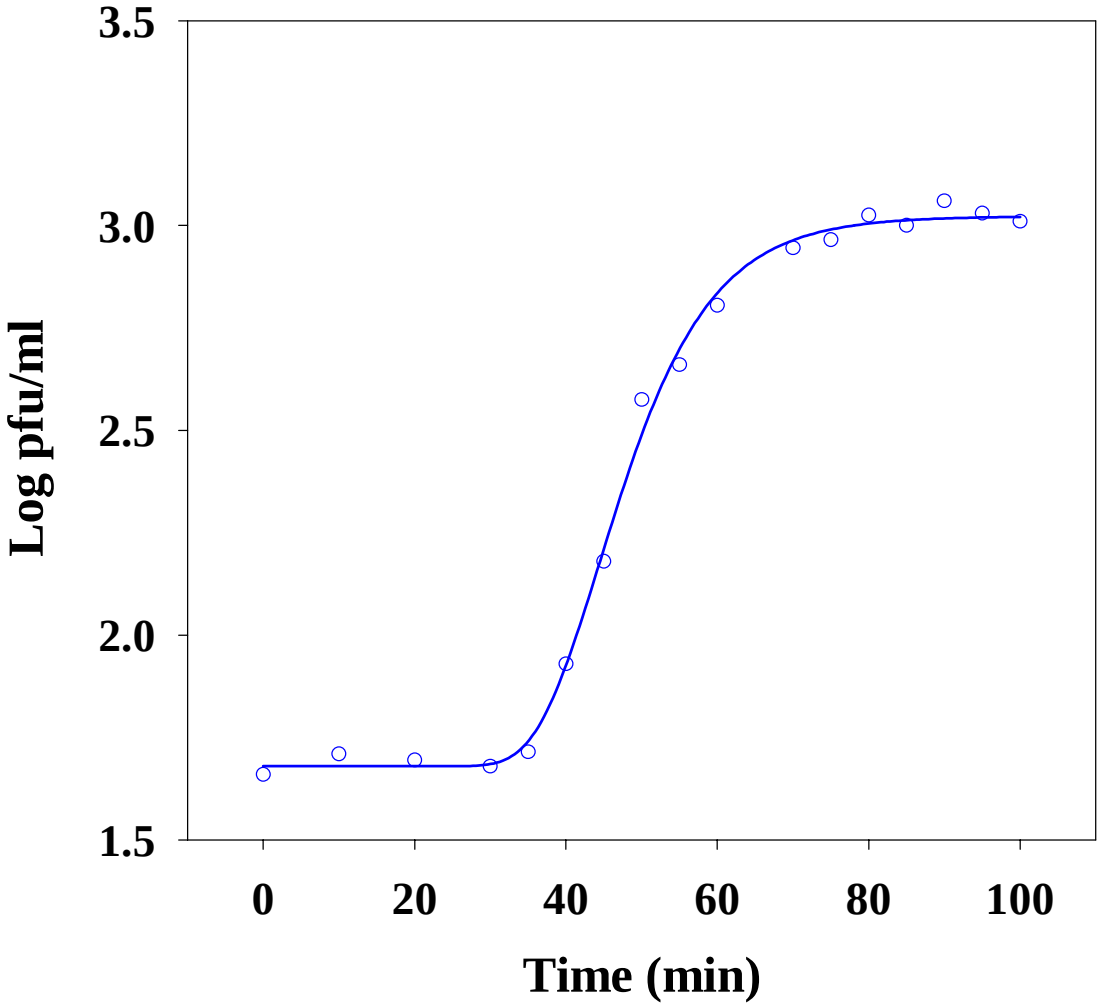


Fig. 4. One-step growth curve of *L. plantarum* phage ΦJL-1in MRS broth at 30°C. Each point is the mean of three independent determinations.

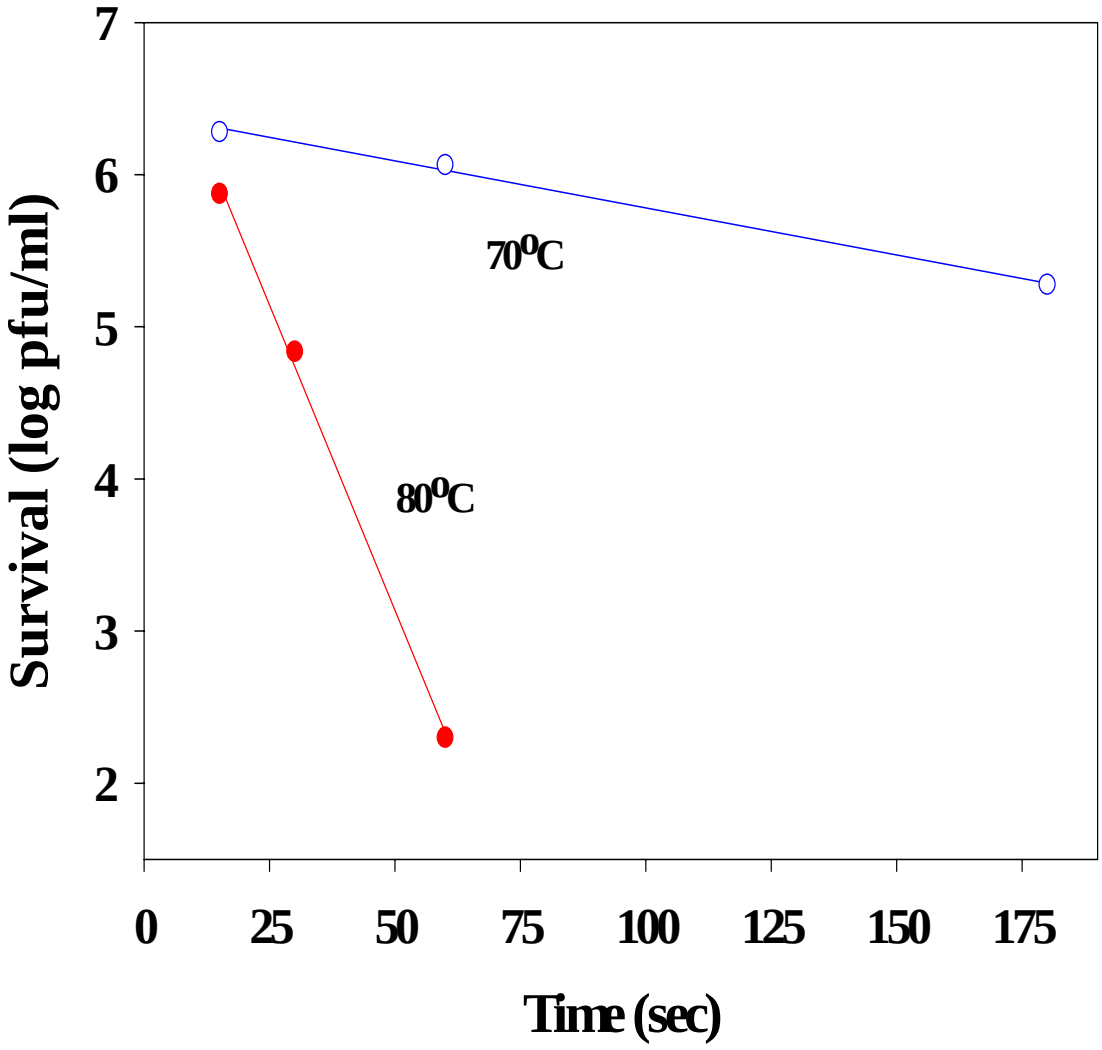


Figure 5. Phage ΦJL-1 population over time at 70 and 80°C. The initial pH was 6.48.

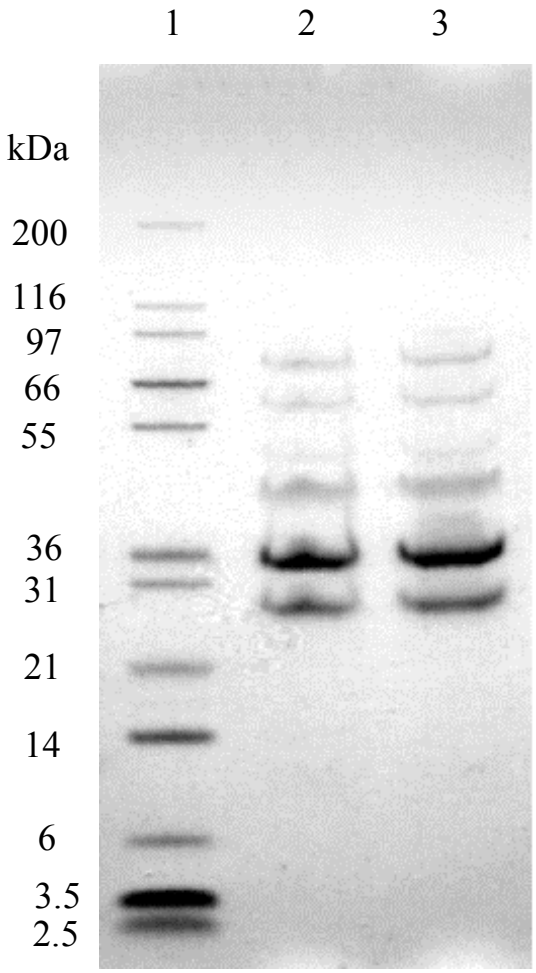


Figure 6. SDS-PAGE of *L. plantarum* phage ΦJL-1 structural proteins (lanes 2 and 3) and molecular mass markers (lane 1). Mark 12 unstained standard was used as molecular weight standard.

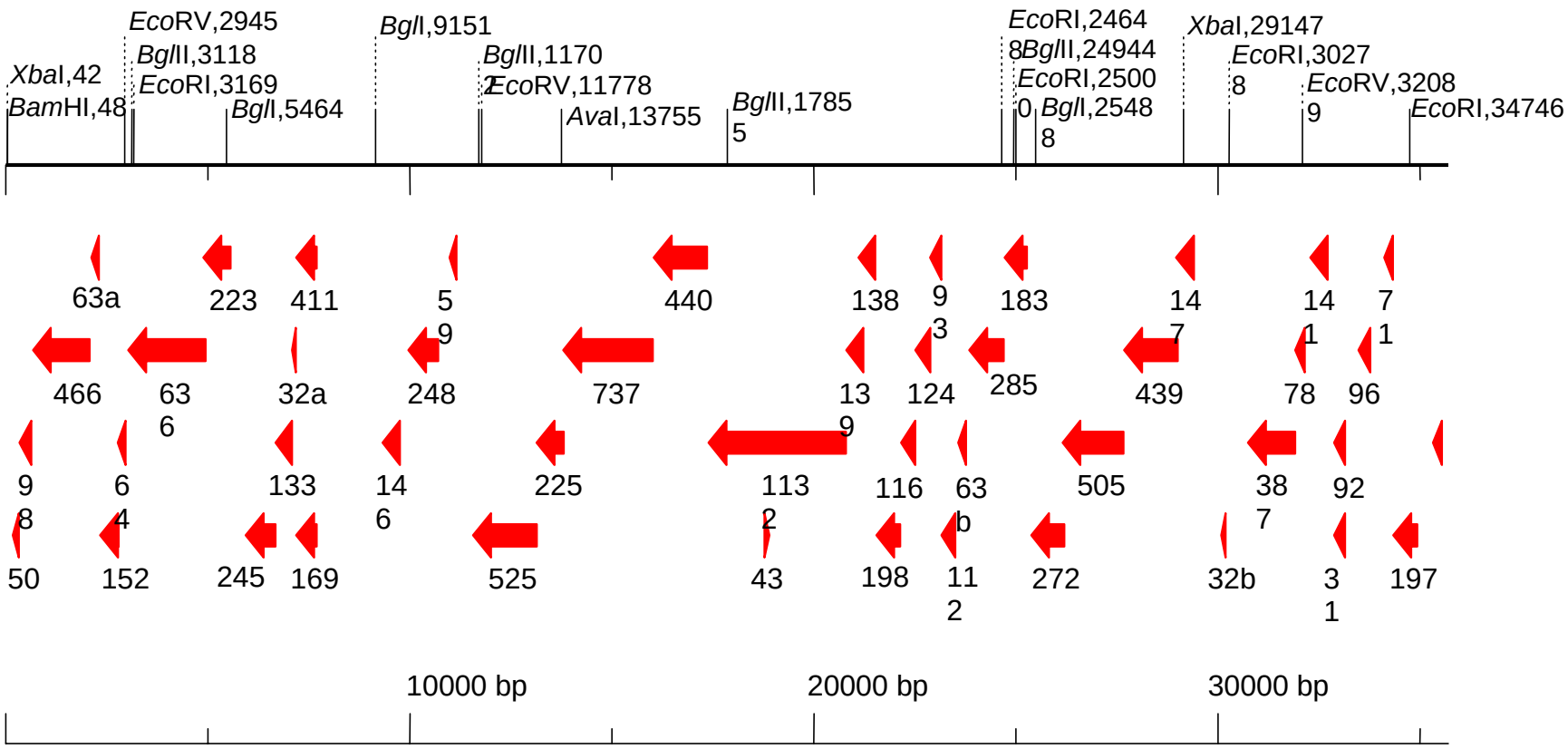


Figure 7. Restriction and ORF map of phage ΦJL-1 DNA. Locations (in bp) of the restriction endonuclease (*Ava*I, *Bam*HI, *Bgl*I, *Bgl*II, *Eco*RI, *Eco*RV, and *Xba*I) sites are as indicated. The orientations of the ORFs are indicated by arrows. The number below each arrow represents the number of amino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

Chapter 4

Sequence analysis of *Lactobacillus plantarum* phage ΦJL-1

ABSTRACT

The complete genomic sequence of a *Lactobacillus plantarum* virulent phage ΦJL-1 was determined. The phage possesses a linear double-stranded DNA genome consisting of 36,674 bp with a G+C content of 39.4%. Forty-six possible open reading frames (ORFs) were identified. According to the N-terminal amino acid sequencing and bioinformatic analyses, proven or putative functions were assigned to 17 ORFs (39%), including 6 structural protein genes. The ΦJL-1 genome shows that functionally related genes were clustered together, resulting in a modular genome structure: DNA packaging, head and tail morphogenesis, lysis, DNA replication, and transcriptional regulation modules. This type of modular genomic organization was similar to several other phages infecting lactic acid bacteria. The structural gene maps revealed that the late gene order is highly conserved among the genomes of *Siphoviridae* phages, allowing the assignment of probable functions to certain uncharacterized ORFs from phage ΦJL-1 and other *Siphoviridae* phages. The genetic information from this study is essential for the understanding of the phage-host interaction in vegetable fermentations and for the development of phage-control strategies for controlled vegetable fermentations relying on *L. plantarum* starter cultures.

Keywords: Bacteriophage, ΦJL-1, sequence analysis, structural proteins, *Lactobacillus plantarum*

INTRODUCTION

Most vegetable fermentations occur under non-sterile conditions. Phages can be a component of the microflora on vegetables, and may attck lactic acid bacteria which are responsible for driving vegetable fermentations. A virulent phage, ΦJL-1, infecting *Lactobacillus plantarum*, was isolated from a commercial cucumber fermentation. Some of its biological properties were described previously (Lu et al., 2002). The phage has an isometric head and a long non-contractile tail, and belongs to morphotype B1 within the *Siphoviridae* family. Tail fibers were not observed. ΦJL-1 has a linear double-stranded DNA genome of 36.7 kb. SDS-PAGE revealed 6 structural proteins. The host range of ΦJL-1 was limited to two related strains of *L. plantarum*, BI7 and a mutant strain MU45, deficient in malolactate fermenting ability. Both strains have been evaluated as starter cultures for controlled cucumber fermentation and as biocontrol microorganisms for minimally processed vegetable products. Using *L. plantarum* MU45 as a host, the phage ΦJL-1 had an average burst size of 22 and a latent period of 35 min. However, little is known about the functions of the ΦJL-1 genes and the genomic organization of these genes. A better understanding of the genetics and biological properties of the lactobacillus phage would be fundamental to the development of phage-control strategies for controlled vegetable fermentations and biocontrol systems using *L. plantarum* BI7 or MU45 .

Many new insights have been recently obtained from detailed analysis of new phage genomes available in databases. Comparative genomic analysis has provided substantial knowledge on phage evolution, genetic diversity, horizontal/vertical gene transfer, module similarity, and lytic/lysogenic cycles. A recent survey of the available functional maps shows that in most phages, genes with related functions are clustered together (Ackermann, 1999; Brøndsted et al., 2001; Kodaira et al, 1997). Functional clustering of genes provides finer levels of regulation because genes whose products interact with each other occupy adjacent positions. This would constitute a powerful mechanism to ensure evolutionary stability (Ackermann, 1999). Comparative genomics of phages infecting lactic acid bacteria (LAB) revealed that several LAB phages have a

fixed, modular structure in their genomes, each module having a set of genes involved in a specific phase of the phage life cycle (Venema et al., 1999; Desiere et al., 1999; Brüssow and Desiere, 2001; Desiere et al., 2001; Lucchini et al., 1999), supporting a modular evolution theory. The term "functional modules" was defined as a stretch of genes with related functions (Botstein, 1980; Casjens et al., 1992). In tailed phages, gene orders are more conserved than nucleotide sequences. Morphopoietic genes are generally located at the left end or the center of the genome. Head genes usually precede tail genes (Ackermann, 1999). Many tailed phages with very similar morphology have little sequence similarity to each other in their structural genes (Ackermann, 1999). Comparative sequence analysis allowed the establishment of associations between phenotype and genotype and thus the attribution of possible functions to new ORFs (Desiere et al., 1999).

The objectives of this study were to determine and analyze the complete genome sequence of the *L. plantarum* phage ΦJL-1, to identify the structural genes, including the major head and tail protein genes, and to explore the genomic organization of the phage.

MATERIALS AND METHODS

Bacterial strain, phage, and media

L. plantarum MU45 was grown in MRS broth (Difco Laboratories, Detroit, MI) at 30°C. Phage ΦJL-1 was propagated on *L. plantarum* MU45 (MOI = 0.02) in MRS medium supplemented with 10 mM $CaCl_2$ at 30°C.

Purification of ΦJL-1 and isolation of phage DNA

Phage ΦJL-1 particles were concentrated from 1L of phage lysate by PEG precipitation and then resuspended in 6 ml of 10 mM Tris-HCl buffer (pH 7.4). The phage suspension was intentionally vortexed (Daigger Vortex-Genie 2, A. Daigger and Company, Inc., Vernon Hills, IL) at highest speed for 2 min in an attempt to generate defected phages. The mixture of intact and defected phage particles was separated and purified by CsCl density gradient centrifugation at 600,000 x g for 6 h at 15°C. The intact

phage band and the defective phage band were collected separately, and dialyzed against 3L 10 mM Tris-HCl buffer (pH 7.4). Phage DNA was isolated as described by Lu et al. (2002).

Electron microscopy

CsCl-purified phage samples were 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 50,000x and printed at 85,000x (V. Knowlton, Center for Electron Microscopy, NC State University, Raleigh, NC).

Sequence and analysis of ΦJL-1 DNA

DNA sequencing was carried out at the Department of Energy Joint Genome Institute (JGI) sequencing facility (Walnut Creek, CA) using shotgun cloning and primer walking sequencing strategies. Sequence analysis was performed using software GAMOLA (Altermann, 2002). Briefly, a number of ORFs were manually determined. Based on these ORFs, a training model was built for use with glimmer to identify the rest of ORFs in the ΦJL-1 genome. The non-redundant database from NCBI (Oct. 2001) was used to BLASTP the identified ORFs. The subsequent results were used to establish automated computer annotation. Sequence alignments were performed using Clone Manager 6, Plasmid Map Enhancer v. 3 (Scientific Educational Software, Durham, NC), and ClustalW (Thompson et al., 1994). Molecular BioComputing Suite (Muller et al., 2001) was used to calculate the molecular mass and isoelectric point of predicted proteins of ΦJL-1 and other LAB phages from databases.

Restriction enzyme 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 and visualized under UV-light (300 nm).

Analysis of structural proteins

An aliquot (78 μl) of CsCl-purified ΦJL-1 sample was mixed with 30 μl of sample buffer and 12 μl of reducing agent (NuPAGE LDS system, Novex, San Diego, CA). The mixture was heated in a boiling water bath for 10 min. The heated sample (36 μl for each well) was loaded to a NuPAGE precast gradient minigel (4-12% Bis-Tris, Invitrogen Corporation, Carlsbad, CA), and then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using a NOVEX Xcell II SureLock Mini-Cell electrophoresis system with NuPAGE morpholineethanesulfonic acid (MES) running buffer (50 mM MES, 50 mM Tris base, 3.5 mM SDS, and 1 mM EDTA, Invitrogen). The SDS-PAGE was run at 200 V for 1 hr. After electrophoresis, the gel was soaked briefly in NuPAGE transfer buffer supplemented with 10% methanol prior to electroblotting.

Xcell II Blot Module (Invitrogen) was used to electroelute the proteins in SDS-PAGE gel onto a polyvinylidene difluoride (PVDF) membrane (0.2 μm pore size), which was pre-wetted for 30 sec in 100% ethanol according to the protocol suggested by Invitrogen. Electroblotting was conducted at 25 V (constant voltage) for 2 h in a transfer buffer containing 12 mM Tris base, 96 mM glycine, and 10% methanol (v/v). After rinsing with deionized water, the PVDF membrane was stained with 1% Amido Black in 40% methanol and 10% acetic acid (v/v) for 5 min, and then destained for 5 min in 40% methanol and 10% acetic acid at room temperature. The immobilized protein bands were excised from the membrane, placed in 1.5-ml microfuge tubes, air-dried for 1.5 h, and stored at -20°C until ready to ship for commercial N-terminal protein sequencing (Proseq, Inc., Bosford, MA) by automated Edman degradation. Ten to eleven amino acids of each protein were determined to identify the corresponding open reading frame. A broad range protein marker (Mark 12, Invitrogen, Carlsbad, CA) and prestained multicolor molecular mass markers (Invitrogen) were used to estimate the molecular weights of phage ΦJL-1 structural proteins.

RESULTS AND DISCUSSION

Complete nucleotide sequence and genomic organization of ΦJL-1

The complete nucleotide sequence of ΦJL-1 was determined by combining shotgun cloning with a primer walking sequencing strategy and presented in GenBank format in Appendix 1. The sequence reported here is also available on the JGI web site (http://www.jgi.doe.gov). ΦJL-1 has a linear double-stranded DNA genome consisting of 36,674 bp with a G+C content of 39.4%, which is lower than that (43.1%) of *Lactobacillus plantarum* phage ϕgle (Kodaira et al, 1997), but higher than that (35.3%) of *Lactobacillus* gassiri phage ϕadh (Altermann et al., 1999). Bioinformatic analysis of the ΦJL-1 genome revealed 46 possible ORFs (listed in Table 1) based on several criteria: (i) the ORF begins with either an ATG, GTG, or TTG, and ends with either TTA, TGA, TAG, or TAA; (ii) the ORF contains at least 30 codons; (iii) the codon usage of the ΦJL-1 OFRs was determined by the training model (data not shown); and (iv) with a few exceptions, the ORF is preceded by an identifiable ribosomal binding site (RBS). The ORFs were named according to the number of amino acids (aa) in the deduced proteins. All ORFs were oriented in the same direction (Fig. 1). Of the 46 ORFs, 37 are initiated with the start codon ATG, 5 with TTG, and 4 with GTG. A potential RBS, complementary with the 3' ends of 16S rRNAs of various bacteria, can be identified upstream of the 46 ORFs. In most of the sites listed in Table 1, the core consensus sequence (AGGAGG) of RBS from the *Lactobacillus delbruckii* ssp. *lactis* phage LL-H (Mikkonen et al., 1994) is highly conserved. A search for restriction sites in the nucleotide sequence agreed well with the experimentally determined restriction pattern (Fig. 2).

The deduced amino acid sequences of all the ORFs were compared with protein sequences in a nonredundant peptide sequence database encompassing Pfam, SWISS-PROT and Protis using BlastP or FASTA comparison programs. Twenty three (50%) ORFs showed homologies with previously characterized genes in databases. In most cases homologies were found to phages infecting gram-positive bacteria, primarily lactic acid bacteria (Table 1). Five ORFs displayed homologies to unknown functions in the

databases. Only those ORFs for which a putative function can be attributed are discussed below.

Early transcribed genes

The predicted protein products of ORF467 and ORF224 (Table 1, Fig. 1) are homologous to the helicase from *Streptococcus thermophilus* phage ϕO1205 and from *L. plantarum* phage ϕgle (Kodaira, 1997), respectively, suggesting that these two gene products (gp) might be involved in DNA replication. The derived protein product of ORF64a shows very low overall similarities with several database entries. However, a specific portion of the sequence (aa 10-57) shared a number of well-conserved amino acids with a DNA polymerase of *Methanococcus voltae* (Konisky et al., 1994). This feature indicates that gpORF64a is probably required for initiation or elongation steps of DNA synthesis. The deduced protein from ORF627 exhibits sequence similarity (identified by PSI-Blast searching) to putative replication protein from *Streptococcus pyogenes* M1 GAS and putative DNA primase from *Streptococcus pyogenes* MGAS315, suggesting that protein may be involved in DNA replication.

Late transcribed genes and DNA packaging

The predicted protein from ORF398 was nearly identical to lysin of *Oenococcus* (previously *Leuconostoc*) *oenos* phage 10MC (Table 1), and similar to several phage lysins (data not shown). Similarity searches did not identify a holin for ΦJL-1. However, holin genes are often located immediately upstream of the lysin, implying that ORF147 may encode the holin of ΦJL-1.

The product derived from ORF148 exhibited homology to the small terminase subunit from *Bacillus subtilis* prophage (Krogh et al., 1996). ORF440 resembles to the putative large terminase subunit found in *Streptococcus pyogenes* prophage M1 GAS (Table 1; Ferretti, 2001) and large terminase subunit in *Lactobacillus johnsonii* prophage Lj771 (data not shown). These database results suggested that the two proteins (gpORF148 and gpORF440) are likely to be involved in phage DNA packaging. In tailed phages, a small terminase subunit is responsible for specific DNA binding, and a large

terminase subunit is responsible for cleaving the phage DNA into genome units and prohead binding. Generally, the DNA-interaction sites (*pac* or *cos*) of the terminases are located within or close to the structural genes (Black, 1989). The *pac* or *cos* site of ΦJL-1 has not yet been detected by sequence analysis, and remains to be identified.

Experimentally determined structural proteins of ΦJL-1

During purification of phage ΦJL-1, several bands, including two grayish bands (band 1 and band 2), appeared in the CsCl density gradients (Fig. 3A). Samples from the two bands were individually analyzed by electron microscopy and SDS-PAGE. The electron micrograph (Fig. 3B) shows that band 1 contained only phage heads (tail-less phage particles), and band 2 contained intact phage particles. The morphology (with an average length of about 182 nm) of the intact phage particles was consistent with the initial description of ΦJL-1 in our previous study (Lu et al., 2002).

SDS-PAGE of the phage head sample identified 3 head proteins, whereas SDS-PAGE of the intact phage sample revealed six structural proteins, including the 3 head proteins (Fig. 3C), suggesting that the 3 proteins absent in the phage head sample were tail proteins. In order to identify the corresponding ORFs in ΦJL-1 DNA, the 6 protein bands from the intact phage sample were transferred to a PVDF membrane, and the N-terminal sequences were determined (Fig. 4).

The 3 head proteins present in both intact and defective phage samples have molecular weights (MW) of 34 kDa, 45 kDa, and 61 kDa, respectively, as estimated by SDS-PAGE (Fig. 4). The 61-kDa protein appeared to be a minor head protein as it was much less abundant than the other two head proteins (Fig. 4). The first 11 amino acids (MDYDLTEHKQA) of this protein (61-kDa) exactly matched amino acids 1 to 11 of ΦJL-1 gpORF506 with predicted molecular mass of 57.7 kDa. This minor head protein showed very strong homology to the portal protein of *Streptococcus thermophilus* phage Sfi11 (Table 1). The 34-kDa and 45-kDa proteins (Fig. 4) appear to be major head proteins according to their abundance shown in Figure 2. They shared identical N-terminal sequence (ATTNNDLPVR), which perfectly matched the residues 2 to 11 of the ΦJL-1 gpORF286. The 34-kDa protein observed on the SDS-PAGE corresponded to the

predicted molecular mass of gpORF35 (30.4 kDa). However, one of the observed proteins (45-kDa) had much a higher MW than the predicted value (30.4 kDa) of gpORF286. The discrepancy may be a result of post-translational modification or reflect a programmed translational frameshifts at some point of the translation of ORF286. Translational frameshifts can be caused by ribosomal slippage or unusual anticodons on tRNA molecules which pair with two bases instead of the usual three (Farabaugh, 1996; Birge, E.A. 2000). Sequencing a longer section of the 45-kDa protein or the entire protein may be needed to confirm the attribution of this protein. The first methionine residue was absent in the two major head proteins, which was in accordance with the rule that the N-terminal methionine is generally processed when the second amino acid residue is alanine (Ben-Bassat et al., 1987). Processing of the initiation methionine during protein maturation has been observed in many phages and occurs via the host methionine aminopeptidase activity (Mahanivong et al., 2001; Lowther and Matthews, 2000). The product deduced from ORF286 exhibits a striking sequence similarity (37% or 39% overall identity) with the experimentally determined major head protein of *L. lactis* phage ul36 (Labrie and Moineau, 2002) and with the hypothetical protein (gpORF36) of *Streptococcus pneumoniae* phage MM1 (Fig. 5, Table 1). This bioinformatic link suggested that gpORF36 of *S. pneumoniae* phage MM1 may also be a major head protein.

The three tail proteins reoccurred from the intact phage sample had observed MW of 28 kDa, 50 kDa, or 76 kDa, respectively (Fig. 4). The observed 28-kDa protein appears to be a major tail protein according to its abundance shown in SDS-PAGE (Fig. 4). The first 10 amino acids of the protein were VAVNNGNKFV. This sequence was identical to residues 2 to 11 (except residue 8) of the ΦJL-1 ORF199. Residue 8 of ORF199 was V instead of N. The discrepancy may reflect an error in either the N-terminal sequencing or nucleotide sequencing. The N-terminal methionine is not present in the mature protein. The predicted MW of gpORF199 is 21.5 kDa, lower than observed (28 kDa). The major tail protein displayed a strong homology to gpORF21 of *Bacillus subtilis* phage SPP1.

The N-terminal peptide sequence (MDLLIEKDGKR) of the 50-kDa protein revealed on the SDS-PAGE matched amino acid positions 12 to 22 of the predicted

gpORF441, suggesting that the protein was proteolytically processed during maturation. Processing of the gpORF441 (49.7 kDa) at position 12 predicts a protein with MW of 48.5-kDa, slightly lower than the observed (50 kDa). Similar proteolytic cleavage of N-terminal amino acids during phage morphogenesis has also been observed in other LAB phages such as *L. lactis* phage BK5-T (Mahanivong et al., 2001), *S. thermophilus* phages (Desiere et al., 1998), and *L. gasseri* ϕadh (Altermann et al., 1999). This protein is probably a minor tail protein because it was much less abundant than the other two tail proteins (Fig. 4). The result from database search showed that the minor tail protein exhibited a weak sequence similarity with a tail component protein from *Lactobacillus casei* phage A2 (Table 1).

N-terminal sequence analysis of another tail protein (76-kDa, observed from SDS-PAGE) revealed the sequence AIRTYDILLDS, which is identical to amino acids 2-12 of the protein (82.4-kDa) derived from ORF749. Again, the N-terminal methionine was absent in the mature protein. This protein, apart from its function as a tail protein, may also be responsible for host specificity because it shows homology with putative anti-receptors from several dairy phages (Brøndsted et al., 2001; Desiere et al., 1999; Lucchini et al., 1999), as well as with ORF112 of *L. lactis* phage bIL170 (Crutz-Le Coq et al., 2002), which was possibly involved in host range determination.

Other ΦJL-1 genes with putative functions

ORF1133 is the longest ORF in the ΦJL-1 genome. The predicted product of this ORF showed strong sequence homology to the tail tape measure protein of *L. lactis* phage TP901-1, suggesting that the protein may be responsible for determining phage tail length. In phage lambda, tail tape measure protein is used as a template for tail polymerization and remains inside the tail tube (Katsura and Hendrix, 1984).

Generally, the genes located between the major head and major tail genes are involved in formation and connection of the head and tail structures and in DNA packaging (Brøndsted et al., 2001). In this region of the ΦJL-1 genome, ORF125 and ORF113, respectively, showed homology to head-tail joining and DNA packaging

proteins from *Lactococcus lactis* phage TP901-1 (Table 1), suggesting that ORF125 and ORF113 products may be involved in phage assembly.

The protein specified by ORF97 exhibited high homology with the putative DNA binding protein from *S. thermophilus* phage Sfi18. The product of ORF198 shares homology with HNH homing endonuclease of *L. lactis* phage bIL170 (Crutz-Le Coq et al., 2002). HNH homing endonucleases confer mobility to their own genes or to host intervening sequences, either an intron or intein, by catalyzing a highly specific, double-strand break in a cognate allele lacking the intervening sequence (Chevalier and Stoddard, 2001). These endonucleases can be found as free-standing ORFs between genes or encoded within introns or inteins. The function of HNH homing endonucleases in the phage cycle and/or the reason for their maintenance in such compact phage genomes is intriguing (Crutz-Le Coq et al., 2002).

DNA packaging and structural gene map of ΦJL-1

A partial gene map of ΦJL-1, surrounding the major head and tail protein genes, is aligned with corresponding genome sections of 5 other *Siphoviridae* LAB phages including 2 Lactobacillus phages (ϕgle, and ϕadh), 2 lactococcal phages (TP901-1, ul36), and 1 streptococcal phage (Sfi21) (Fig. 6). The alignment demonstrated that these phages shared a highly conserved structural gene order, supporting the notion that the structural gene map is highly conserved among *Siphoviridae* phages (Luccini et al., 1998; 1999). In addition, the size (in aa or kDa) and isoelectric point (pI) value of structural proteins with the same function appear to be relatively conserved. These physical properties can be useful for extrapolating and predicting gene functions for closely or distantly related phages even showing little or no regions of sequence homology. The alignment of the ΦJL-1 structural gene map with other LAB phages (Fig. 6) predicts that the experimentally determined minor head protein (gpORF506) of ΦJL-1 may be also a portal protein because the gene location (immediately downstream of large subunit terminase), the size (506 aa) and pI value (4.6) of the gene product were very similar to a portal protein (gene) from Sfi21, TP901-1, ul36, and ϕgle. This prediction was supported by the database search result (Table 1) as discussed above. Portal proteins are generally

responsible for forming the entrance to the head during packaging and determining the amount of DNA to be packaged (Dube et al., 1993). Similarly, we predict that ORF397 of ϕadh is probably a portal protein as well. The derived protein from ORF184 shows limited sequence similarity with a putative scaffold protein from *S. thermophilus* phage Sfi11 (Lucchini et al., 1998). However, the size (184 aa) of the protein and its pI value (4.7) were very similar to scaffold proteins of several LAB phages, including ul36, TP901-1, ϕgle (Fig. 6), and Sfi11. These features supported the prediction that gpORF184 might have a scaffolding function. In the ϕadh genome, the function of ORF159b is unknown. But, its physical properties (the location in the gene map, and the size and pI value of the gene product) are similar to putative head-tail joining protein gene from phages ΦJL-1, Sfi21, and TP901-1 (Fig. 6). Thus, ORF159b of ϕadh may encode a head-tail joining protein. As mentioned earlier, the derived product from ORF36 of S. *pneumoniae* phage MM1 (Obregon et al., unpublished) shared a striking sequence similarity (Fig. 5) with the major head proteins from ΦJL-1 and ul36. The structural gene map of phage MM1 (data not shown) reveals that immediate upstream of ORF36 is a gene encoding a putative scaffold protein. Downstream of ORF36 are several small (77-130 aa) protein genes, including 2 small putative minor capsid protein genes (114-123aa). Furthermore, the deduced protein of ORF36 consists of 295 aa and has pI value of 5.7. These features are very similar to those of the major head protein from ΦJL-1, TP901-1, ul36, and ϕgle (Fig. 6), strongly supporting our prediction that ORF36 of phage MM1 may encode a major head protein gene.

Functional modules and genomic organization in ΦJL-1

In many phages the genes encoding related biological functions are clustered. Inspection of the proven and putative gene functions, and the locations of individual ORFs from phage ΦJL-1 reveal that the phage genome is highly modular with functionally-related genes clustered together. Thus, the following functional modules are proposed and indicated in Fig. 1: DNA packaging, head morphogenesis, head-tail joining, tail morphogenesis, host specificity, cell lysis, and DNA replication.

The DNA packaging module contains ORFs encoding two putative terminase subunits and a DNA packaging protein (Fig. 1). The head morphogenesis module includes ORFs encoding the experimentally determined major head protein, minor head-portal protein, putative scaffold protein, and a few small ORFs nearby (Fig. 1). The head-tail joining module consists of ORF125. The tail morphogenesis module includes genes encoding the experimentally identified major and minor tail proteins, putative tape measure protein, and several other ORFs between the cell lysis and head-joining modules (Fig. 1). The host specificity module consists of ORF749 encoding an anti-receptor, experimentally identified as a minor tail protein. The cell lysis module contains of putative lysin gene and ORF147 which is likely to encode holin protein. The replication module contains genes encoding two putative helicases, a putative primase, a putative DNA polymerase, and a few other ORFs as indicated in Figure 1. Besides these modules, a regulation module was also assigned in Figure 1, based upon extrapolations from other *Siphoviridae* phages (Brüssow and Desiere, 2001). Further analysis and experimental evidence are needed to confirm these modules.

Notably, genes involved in packaging of the genome into the phage head are located at the left of the genome. Immediately following are structural modules. Head genes are clustered together and precede the tail genes, which are also clustered together. These genes are followed by gene clusters required for lysis of the host, and genes involved in DNA replication. Although some regions have not been assigned to any functional module due to lack of information regarding the biological functions of the encoded genes, the overall organization of functional modules within ΦJL-1 revealed a striking correlation with those observed in many other *Siphoviridae* LAB phages, such as the virulent phage ul36 (Labrie and Moineau, 2002), and temperate phages TP901-1 (Brøndsted et al., 2001), ϕgle (Kodaira et al., 1997), ϕadh (Altermann et al., 1999), and O1205 (Brüssow and Desiere, 2001), except that a lysogeny module was absent in the ΦJL-1 genome.

Further studies on gene structure, expression, and function in phage ΦJL-1 are needed to better understand the biology of the phage and to develop phage-control strategies in vegetable fermentations relying on *L. plantarum* starter cultures.

REFERENCES

Ackermann, H-W. 1999. Tailed bacteriophages: the order Caudovirales. In: Advances in virus research. Vol. 51. pp. 135-201. K. Maramorosch, F.A. Murphy, and A.J. Shatkin, eds., Academic Press, New York.

Altermann, E. 2002. http://fsweb2.schaub.ncsu.edu/TRKwebsite/index.htm.

Altermann, E., Klein, J.R., and Henrich, B. 1999. Primary structure and features of the genome of the *Lactobacillus gasseri* temperate bacteriophage ϕadh. Gene. **236**:333-346.

Ben-Bassat, A., Bauer, K., Chang, S.Y., Myambo, K., Boosman, A, and Chang, S. 1987. Processing of the initiation methionine from proteins: Properties of the *Escherichia coli* methionine aminopeptidase and its gene structure. J. Bacteriol. **169**:751-757.

Birge, E.A. 2000. Transcription and translation: Processes and basic regulation. In: Bacterial and bacteriophage genetics. 4th ed. pp. 103-141. Springer, New York.

Black, L.W. 1989. DNA packaging in dsDNA bacteriophages. Annu. Rev. Microbiol. **43**:267-292.

Botstein, D. 1980. A theory of modular evolution for bacteriophages. Ann. N.Y. Acad. Sci. **3554**:484-490.

Brøndsted, L., Ostergaard, S., Pedersen, M., Hammer, K., and Vogensen, F.K. 2001. Analysis of the complete DNA sequence of the temperate bacteriophage TP901-1: evolution, structure, and genome organization of Lactococcal bacteriophages. Virology. **283**:93-109.

Brüssow, H. and Desiere, F. 2001. Comparative phage genomics and the evolution of *Siphoviridae*: insights from dairy phages. Mol. Microbiol. **39**:213-222.

Casjens, S., Hatfull, G., and Hendrix, R. 1992. Evolution of dsDNA tailed-bacteriophage genomes. Semin. Virol. **3**:383-397.

Chevalier, B.S. and Stoddard, B.L. 2001. Homing endonucleases: structural and functional insight into the catalysts of intron/intein mobility. Nucleic Acids Re. **29**:3757-3774.

Crutz-Le Coq, A.M., Cesselin, B., Commissaire, J., and Anba, J. 2002. Sequence analysis of the lactococcal bacteriophage bIL170: insights into structural proteins and HNH endonucleases in dairy phages. Microbiology **148** (4):985-1001.

Desiere, F., Lucchini, S. and Brüssow, H.1998. Evolution of *Streptococcus thermophilus*

bacteriophage genomes by modular exchanges followed by point mutations and small deletions and insertions.Virology. **241**(2):345-356.

Desiere, F., Lucchini, S., and Brüssow, H. 1999. Comparative sequence analysis of the DNA packaging, head and tail morphogenesis modules in the temperate *cos*-site *Streptococcus thermophilus* bacteriophage Sfi21. Virology. **260**:244-253.

Desiere, F., Mahanivong, C., Hillier, A.J., Chandry, P.S., Davidson, B.E., and Brüssow, H. 2001. Comparative genomics of lactococcal phages: insight from the complete genome sequence of *Lactococcus lactis* phage BK5-T. Virology. **283**:240-252.

Dube, P., Tavares, P., Lurz, R., and van Heel, M. 1993. The portal protein of bacteriophage SPP1: a DNA pump with 13-fold symmetry. EMBO J. **12**:1303-1309.

Farabaugh, P.J. 1996. Programmed translational frameshifting. Microbiol. Rev. **60**:103-134.

Ferretti, J.J., McShan, W.M., Adjic, D., Savic, D., Savic, G., Lyon, K., Primeaux, C., Sezate, S.S., Surorov, A.N., Kenton, S., Lai, H., Lin, S., Qian, Y., Jia, H.G., Najar, F.Z., Ren, Q., Zhu, H., Song, L., White, J., Yuan, X., Clifton, S.W., Roe, B.A., and McLaughlin, R.E. 2001. Complete genome sequence of an M1 strain of *Streptococcus pyogenes*. Proc. Natl. Acad. Sci. U.S.A. **98**(8):4658-4663.

Gindreau, E. and Lonvaud-Funel, A. 1999. Molecular analysis of the region encoding the lytic system from *Oenococcus oeni* temperate bacteriophage phi 10MC. FEMS. Microbiol. Lett. **171** (2):231-238.

Katsura, I and Hendrix, R.W. 1984. Length determination in bacteriophage lambda tails. Cell. **39**:691-698.

Kodaira, K.I., Oki, M., Kakikawa, M., Watanabe, N., Hirakawa, M., Yamada, K., and Taketo, A. 1997. Genome structure of the *Lactobacillus* temperate phage ϕgle: the whole genome sequence and the putative promoter/repressor system. Gene. **187**:45-53.

Konisky, J., Paule, S.M., Carinato, M.E., and Kansy, J.W. 1994. The DNA polymerase gene from the methanogenic archaeon *Methanococcus voltae*. J. Bacteriol. **176** (20): 6402-6403.

Krogh, S., O'Reilly, M., Nolan, N. and Devine, K.M. 1996. The phage-like element PBSX and part of the skin element, which are resident at different positions on the *B. subtilis* chromosome, are highly homologous. Unpublished.

Labrie, S. and Moineau, S. 2002. Complete genomic sequence of bacteriophage ul36: Demonstration of phage heterogeneity within the P335 quasi-species of lactococcal phages. Virology. **296**:308-320.

Lowther, W.T. and Mathews, B.W. 2000. Structure and function of the methionine aminopeptidases. Biochim. Biophys. Acta. **1477**:157-167.

Lu, Z., Breidt, F. Jr., Fleming, H.P., Altermann, E., and Klaenhammer, T.R. 2002. Isolation and characterization of a *Lactobacillus plantarum* bacteriophage, ΦJL-1, from a cucumber fermentation. Int. J. Food Microbiol. In press.

Lucchini, S., Desiere, F., and Brüssow, H. 1998. The structural gene module in *Streptococcus thermophilus* bacteriophage ϕSfi1 shows a hierarchy of relatedness to Siphoviridae from a wide range of bacterial hosts. Virology. **246**:63-73.

Lucchini, S., Desiere, F., and Brüssow, H. 1999. Comparative genomics of *Steptococcus thermophilus* phage species supports a modular evolution theory. J. Virol. **73**:8647-8656.

Mahanivong, C. Boyce, J.D., Davidson, B.E., and Hillier, A.J. 2001. Sequence analysis and molecular characterization of the *Lactococcus lactis* temperate bacteriophage BK5-T. Appl. Environ. Microbiol. **67**:3564-3576.

Mikkonen, M., Vuoristo, J., Alatossava, T. 1994. Ribosome binding site consensus sequence of *Lactobacillus delbrueckii* subsp. *lactis* bacteriophage LL-H. FEMS Microbiol. Lett. **116**:315-320.

Muller, P.Y., Studer, E., and Miserez, A.R. 2001. Molecular Biocomputing Suite: a word processor add-in for the analysis and manipulation of nucleic acid and protein sequence data. BioTechniques. **31**:1306-1313.

Obregon, V., Garcia, P., Garcia, E., Lopez, R., and Garcia, J.L. Complete nucleotide sequence and analysis of the temperate bacteriophage MM1 genome of *Streptococcus pneumoniae*. Unpublished.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**:4673-4680.

Venema, G., Kok, J. and Sinderen, D.V. 1999. From DNA sequence to application: possibilities and complications. Antonie van Leeuwnehoek. **76**:3-23.

Table 1. ORFs and genetic features of phage ΦJL-1[a]

ORF or feature[b]				Predicted product						
			Putative RBS[d] and start codon	Size (aa)	Mass[f] (kDa)	pI[g]	Database search results[h]			
ORF	Star	End[c]	3′-AUCUUUCCUCCACUAGGUC…[e]				Predicted function	Organism matched	E[i]	Reference[j]
77	345	575	agaAAAGGAtaTGAaaaAGaatg	77	8.7	9.6				
198	959	1552	ttAaattAGGAGGaatcgtAatg	198	22.6	9.2	HNH homing endonuclease	*Lactococcus lactis* phage bIL170	3e-15	AF009630
72	1560	1775	TtactgGGAGagatgatAagatg	72	8.3	4.3				
97	2125	2415	TttatAGGAGaaaATaaAaaatg	97	11.1	4.8	DNA binding	*Streptococcus thermophilus* phage Sfi18	4e-15	AF158601
32	2390	2485	tcgcgatGgtAAGAtGatcgatg	32	3.3	10.1				
93	2738	3016	aaataAttGGAGGaataatcatg	93	11.1	9.0				
142	3177	3602	agtaAaAttGGAGGaagcCgatg	142	16.1	4.9				
79	3749	3985	tcTAGtAAGGAGaTaaaacatg	79	9.0	5.5				
388	3985	5148	tgtGGgGtaGcTattaaatcatg	388	44.2	6.1				
33b	5703	5801	taAAtGcAGaTGATaacGccgtg	33	3.6	8.4				
148	6481	6924	tggtAGGAGGTGtataaGccttg	148	17.0	8.1	Terminase, small subunit	*Bacillus subtilis* PBSX phage	0.08	Z99110
440	6890	8209	tgatagtcAAGtAGtcGtgaatg	440	51.0	8.4	Terminase, large subunit	*Streptococcus pyogenes* prophage M1 GAS	9e-98	AE006544
506	8223	9740	aggActAtAGGAGGcttagCatg	506	57.7	4.6	Minor head protein			This work
							Portal protein	*Streptococcus thermophilus* phage Sfi11	1e-70	AF158600
273	9691	10509	ccAGAAcGGgGtaatTagtaatg	273	30.8	9.0	Unknown (SPy0975)	*Streptococcus pyogenes* prophage M1 GAS	8e-10	AE006544
184	10616	11167	gtcggGAtAGGAGGAttaCCatg	184	20.1	4.7	Scaffold protein	*Streptococcus thermophilus* phage Sfi11	0.08	AF158600
286	11190	12047	AAaAAAcGAGGTttaaaAttatg	286	30.4	4.6	Major head protein			This work
								Streptococcus pneumoniae phage MM1	3e-53	AJ302074
64b	12128	12319	gcgAtactcGTaATattaccgtg	64	6.0	3.6				
113	12391	12729	tacgaAAAGGAaGTGATtaaatg	113	12.7	4.6	DNA packaging	*Lactococcus lactis* phage TP901-1	2e-07	AF304433
94	12732	13013	gctGgAAtGAGGTtcatataatg	94	10.5	5.7				
125	13006	13380	GAAAGtgacggtgttaatctgtg	125	14.1	9.1	Head to tail joining	*Lactococcus lactis* phage TP901-1	3e-08	AF304433
117	13380	13730	AatAAGGcGGcGAagttttcatg	117	13.3	5.0				
199	13746	14342	caAtttAAGGAGGatAaaacatg	199	21.6	4.2	Major tail protein			This work
								Bacillus subtilis phage SPP1	1e-21	X97918

ORF or feature[b]				Predicted product						
			Putative RBS[d] and start codon	Size	Mass[f]		Database search results[h]			
ORF	Star	End[c]	3′-AUCUUUCCUCCACUAGGUC...[e]	(aa)	(kDa)	pI[g]	Predicted function	Organism matched	E[i]	Reference[j]
139	14367	14783	caAtAGGAGGatAaCgAGacatg	139	15.9	4.9				
140	14669	15088	ttccgtcgcgccgcgaaacattg	140	16.1	8.1				
1133	15095	18493	atcAcGGAGGTGAataatatatg	1133	112.1	9.6	Tape measure protein	*Lactococcus lactis* phage TP901-1	8e-25	AF304433
441	18531	19853	gaattAAAGcctGccAgtgtatg	441	49.7	5.4	Minor tail protein			This work
							Tail component protein	*Lactobacillus casei* phage A2	0.6	AJ251790
738	19871	22084	GcGtAGGAGGTGActaattaatg	738	81.2	4.4	Unknown (ORF977)	*Lactobacillus johnsonii* prophage Lj965	1e-30	AF195900
749	22077	24323	aTAtAAAGGtGGTaATgtAGatg	749	82.4	4.8	Minor tail protein			This work
							Host specificity	*Lactococcus lactis* phage bIL170	6e-05	AF009630
60	24725	24904	gTgGtgAGGgGcTGAaatAattg	60	6.8	5.8				
249	25177	25923	gaatttaTcatAAtGAttatttg	249	27.5	6.4				
147	26127	26567	aAGAAAGcAGGaaAataAtcatg	147	15.3	4.6	Unknown (P163)	*Oenococcus oenos* phage 10MC	1e-17	AF049087
398	26572	27765	accagcAAcGGAGGaatagtatg	398	43.6	9.7	Lysin	*Oenococcus oenos* phage 10MC	1e-85	AF049087
170	28189	28698	gcaaccaTAGAAAGGAaGTaatg	170	19.2	4.7				
33a	28704	28802	ttctacggGGAGGcataaacatg	33	3.7	8.0				
134	28805	29206	ccAGAAgGGAaGcGtaaataatg	134	14.9	9.5	Unknown (ORF6)	*Lactococcus lactis* phage phi31	5e-04	AJ292531
246	29209	29946	tcaaAAAGGAGtgctgaataatg	246	28.4	5.1				
224	30325	30996	TtGAAAGGtGaTGtTtttaaatg	224	24.5	5.8	Helicase (NTP-binding)	*Lactobacillus plantarum* phage ϕgle	2e-06	X98106
627	30941	32851	TAaAAAGcAatcaATtaAaaatg	627	72.2	5.5	Primase	*Streptococcus pyogenes* MGAS315	6e-20	NP_665246
65b	32916	33110	aGttgAAGGAGGTtAgCtAaatg	65	7.7	9.5				
153	33097	33555	ActcAAGGAGGaaATtaAaaatg	153	17.3	4.8				
64a	33573	33764	ttctaAtgGGAGaTGATtaaatg	64	7.6	4.2	DNA polymerase	*Methanococcus voltae*	1.6	L33366
467	33811	35211	ttattgtagggAGAAAtagtatg	467	53.1	8.6	Helicase	*Streptococcus thermophilus* phage ϕO1205	4E-93	U88974
99	35246	35542	taatatattaAGAAAGGttagtg	99	11.7	4.3				
114	35564	35905	taAGAAAGGAtagGtaaagcatg	114	12.7	9.9	Unknown (orf106 gp)	*Streptococcus thermophilus* phage Sfi19	9e-29	AF115102
47	35915	36055	tgttGGAGGactaatAaattatg	47	5.2	6.2				
65a	36039	36233	gttagtTgctAtGGctGcGgttg	65	6.9	9.3				

[a]See the text for the details.
[b]ORFs were designated according to the number of amino acids of the corresponding coded proteins.
[c]The end position does not include the stop codon.
[d]The sequence shown includes the immediate up stream 20 nucleotides of the putative start codon.
The nucleotide that is complementary to the one found at the 3' end of the 16S rRNA is shown in upper case letters.
[e]The nucleotides complementary to the 3' end of 16S rRNA of *Lb. delbruckii* (3'-AUCUUUCCUCCACUAGGUC …).
(Mikkonen et al., 1994).
[f]Molecular weights were calculated with Molecular Biocomputing Suite (Muller et al., 2001).
[g]Isoelectric points were calculated with Molecular Biocomputing Suite.
[h]Database searches based on homologies of deduced amino acid sequences were performed with gapped BlastP algorithm.
[i]Probabilities derived from Blast scores for obtaining a match by chance.
[j]Accession number or this work.

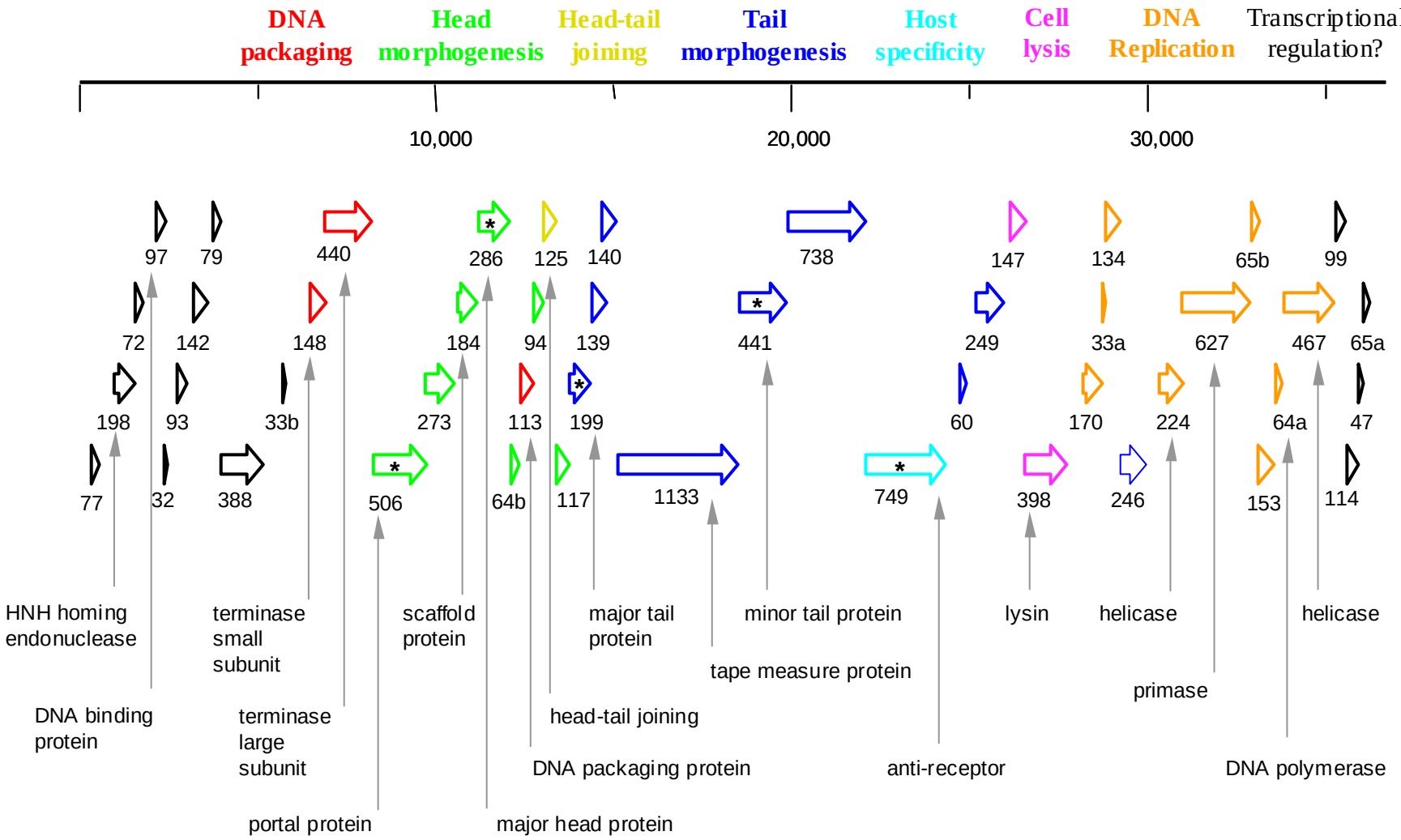

DNA packaging
Head morphogenesis
Head-tail joining
Tail morphogenesis
Host specificity
Cell lysis
DNA Replication
Transcriptional regulation?
10,000
20,000
30,000
97
79
440
286
125
140
738
147
134
65b
99
72
142
148
184
94
139
441
249
33a
627
467
65a
198
93
33b
273
113
199
60
170
224
64a
47
77
32
388
506
64b
117
1133
749
398
246
153
114
HNH homing endonuclease
DNA binding protein
terminase small subunit
terminase large subunit
portal protein
scaffold protein
major head protein
major tail protein
head-tail joining
DNA packaging protein
tape measure protein
minor tail protein
anti-receptor
lysin
helicase
primase
helicase
DNA polymerase

Figure 1. Modular genomic organization of the phage ΦJL-1 genome. The double-stranded DNA is shown as a thick black line. The open arrows represent ORFs predicted from the genomic sequence. The direction of the arrows corresponds to the direction of transcription. The numbers below the open arrows represent the ORF designation and size (in aa). Proven or putative functions of individual ORFs (see Table 1) are indicated by gray upward arrows. The experimentally determined structural genes are indicated with asterisks inside open arrows. The proposed functional modules are indicated at the top of the figure. ORFs belonging to the same module are shown in the same color. The regulation module is based on an extrapolation from other *Siphoviridae* phages. ORFs in black may or may not belong to regulation module.

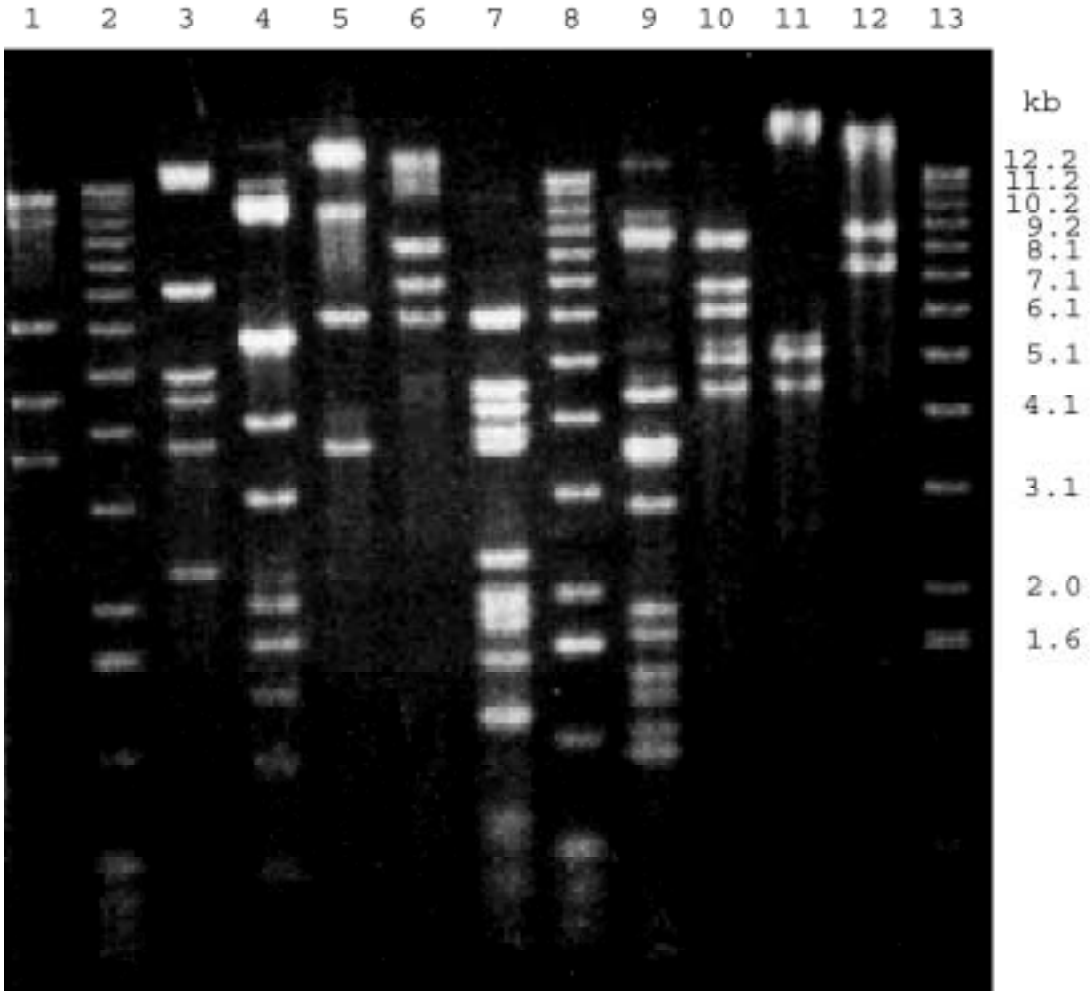


Figure 2. Restriction analysis of the phage JL-1 DNA. The phage DNA was digested with restriction endonucleases *Ava*I + *Bgl*I (lane 1), *Sal*I (lane 3), *Hind*III (lane 4), *Bgl*I (lane 5), *Bgl*II (lane 6), *Cla*I (lane 7), *Pvu*II (lane 9), *Bgl*II + *EcoR*I (lane 10), *EcoR*I (lane 11), *EcoR*V (lane 12). 1 kb DNA ladder was used as the molecular weight standard (lanes 2, 8, and 13).

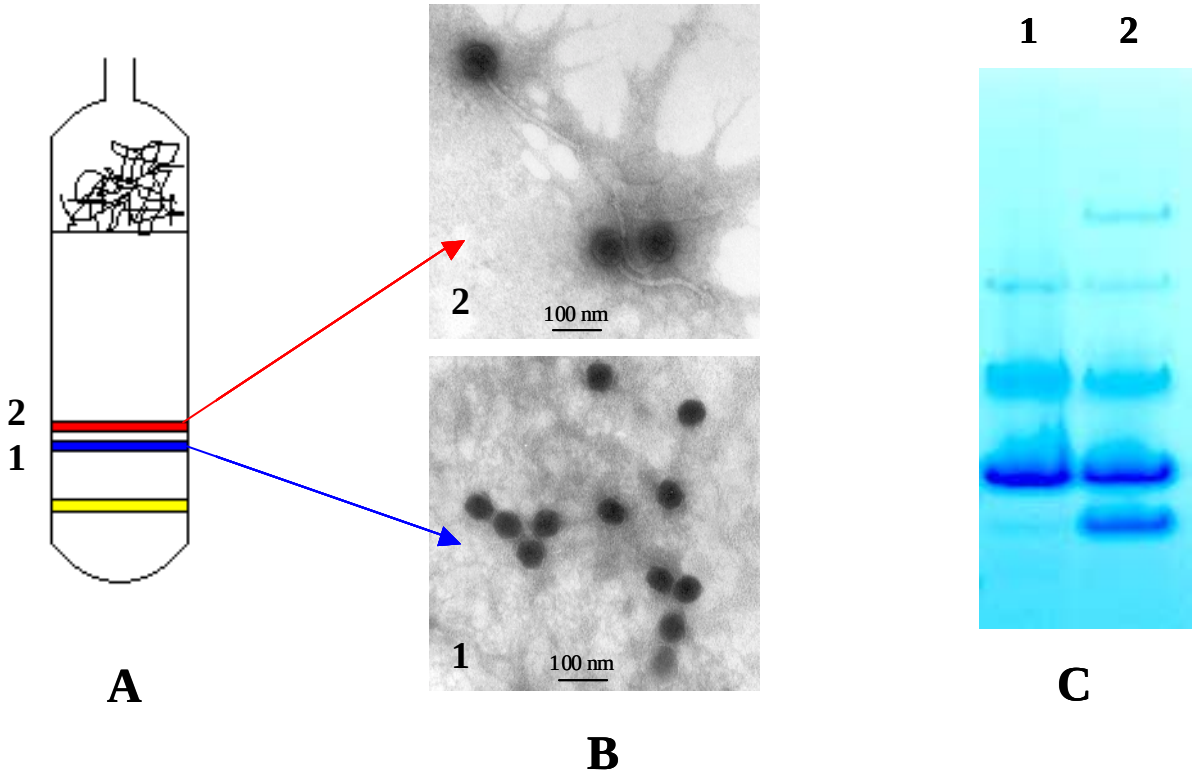
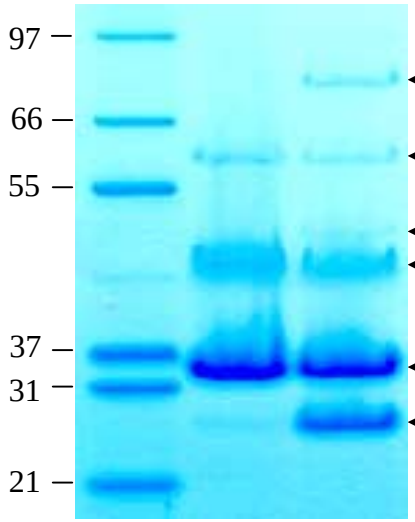


Figure 3. Analysis of the phage ΦJL-1samples from CsCl density gradient. (A). Schematic representation of CsCl gradient tube containing ΦJL-1 phage head band (1) and intact phage particle band (2) after ultra-centrifugation. (B). Electron micrographs of phage heads and intact phage particles. (C). SDS-PAGE gel of the ΦJL-1 structural proteins.



	MW (kDa) Estimated	MW (kDa) Predicted	N-terminal sequence	ORF matched	aa position of matching ORF	Proposed function
←	76	82.4	AIRTYDILLDS	ORF749	2-12	Minor tail protein
←	61	57.7	MDYDLTEHKQA	ORF506	1-11	Minor head protein
←	50	49.7	MDLLIEKDGKR	ORF441	12-22	Minor tail protein
←	45	30.4	ATTNNDLPVR	ORF286	2-11	Major head protein
←	34	30.4	ATTNNDLPVR	ORF286	2-11	Major head protein
←	28	21.5	VAVNNGNKFV	ORF199	2-11	Major tail protein

Figure 4. Analysis of the phage ΦJL-1 structural proteins. SDS-PAGE profile is shown on the left. Lane M, molecular mass marker; lane 1, proteins from ΦJL-1 heads; lane 2, proteins from the intact ΦJL-1 particles. The N-terminal amino acid sequences of the 6 structural proteins were determined. The position of the N-terminal sequence in the corresponding ΦJL-1 ORF and the proposed function of the ORF are indicated.

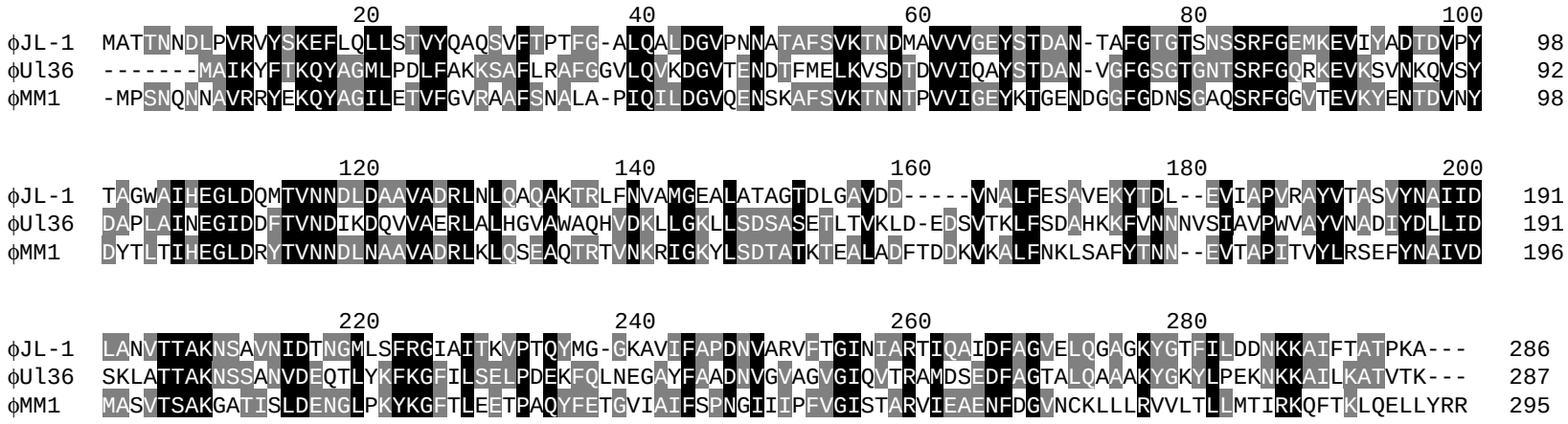


Figure 5. Multiple sequence alignment of the major head proteins from *Lactobacillus plantarum* phage ΦJL-1 and *Lactococcus lactis* phage ul36 with ORF36 of *Streptococcus pneumoniae* phage MM1. Residue numbers of the proteins are given on the right. Perfectly conserved residues are highlighted in black boxes. Residues that are conserved in 2 of the aligned sequences are shaded in gray. Numbers refer to the amino acid position.

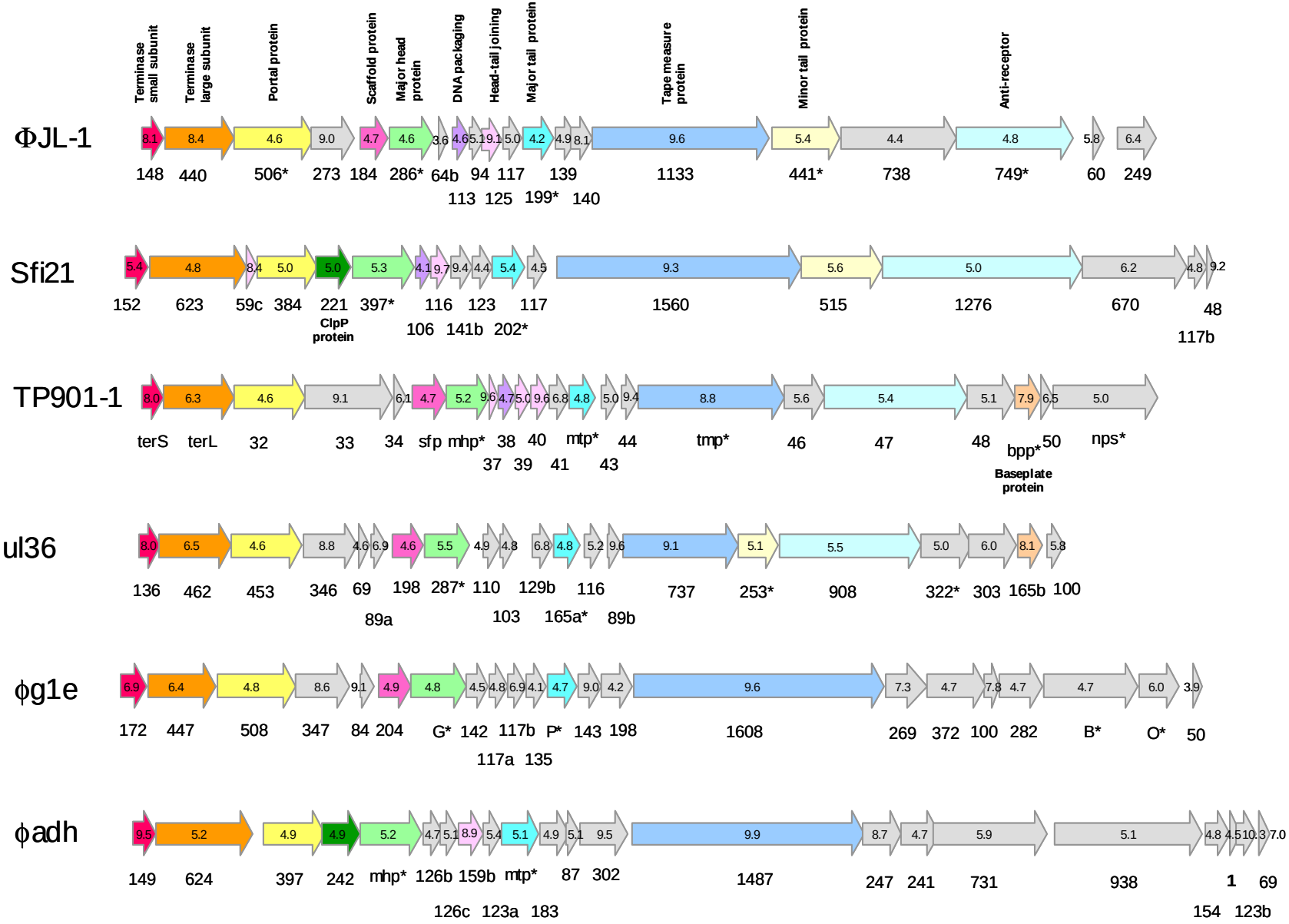

Terminase small subunit
Terminase large subunit
Portal protein
Scaffold protein
Major head protein
DNA packaging
Head-tail joining
Major tail protein
Tape measure protein
Minor tail protein
Anti-receptor
ΦJL-1
8.1 8.4 4.6 9.0 4.7 4.6 3.6 4.6 5.1 9.1 5.0 4.2 4.9 8.1 9.6 5.4 4.4 4.8 5.8 6.4
148 440 506* 273 184 286* 64b 94 117 139 1133 441* 738 749* 60 249
113 125 199* 140
Sfi21
5.4 4.8 8.4 5.0 5.0 5.3 4.1 9.7 9.4 4.4 5.4 4.5 9.3 5.6 5.0 6.2 4.8 9.2
152 623 59c 384 221 397* 116 123 117 1560 515 1276 670 48
ClpP protein
106 141b 202*
117b
TP901-1
8.0 6.3 4.6 9.1 6.1 4.7 5.2 9.6 4.7 5.0 9.6 6.8 4.8 5.0 9.4 8.8 5.6 5.4 5.1 7.9 6.5 5.0
terS terL 32 33 34 sfp mhp* 38 40 mtp* 44 tmp* 46 47 48 bpp* 50 nps*
37 39 41 43
Baseplate protein
ul36
8.0 6.5 4.6 8.8 4.6 6.9 4.6 5.5 4.9 4.8 6.8 4.8 5.2 9.6 9.1 5.1 5.5 5.0 6.0 8.1 5.8
136 462 453 346 69 198 287* 110 129b 116 737 253* 908 322* 303 165b 100
89a 103 165a* 89b
ϕg1e
6.9 6.4 4.8 8.6 9.1 4.9 4.8 4.5 4.8 6.9 4.1 4.7 9.0 4.2 9.6 7.3 4.7 7.8 4.7 4.7 6.0 3.9
172 447 508 347 84 204 G* 142 117b P* 143 198 1608 269 372 100 282 B* O* 50
117a 135
ϕadh
9.5 5.2 4.9 4.9 5.2 4.7 5.1 8.9 5.4 5.1 4.9 5.1 9.5 9.9 8.7 4.7 5.9 5.1 4.8 4.5 10.3 7.0
149 624 397 242 mhp* 126b 159b mtp* 87 302 1487 247 241 731 938 1 69
126c 123a 183 154 123b

Figure 6. Comparison of the partial genome of phage ΦJL-1 with other *Siphovaridae* phages. The numbers below the maps refer to ORFs of ΦJL-1 (Table 1), Sfi21 (Desiere et al., 1999), TP901-1 (Brønsted et al., 2001), ul36 (Labrie and Moineau, 2002), ϕgle (Kodaira et al., 1997), and ϕadh (Altermann et al., 1999). Sizes of individual ORFs are reflected by the lengths of the arrows. The pI values predicted for individual proteins are indicated inside the arrows representing the respective ORFs. Corresponding genes are indicated with the same color code. ORFs whose structural function has been experimentally verified are indicated by asterisks.

Chapter 5

Identification of Anti-receptors on Bacteriophage ΦJL-1 via Phage Display Technology

ABSTRACT

All tailed phages adsorb to bacteria by their tails. Anti-receptors in tailed phages are the receptor-binding proteins at the tip of the phage tail, which are responsible for host specificity. Identification of anti-receptors is fundamental to the understanding of the biological process in phage infection. The T7 phage display system was used to clone and identify tail proteins of phage ΦJL-1 which attacks *L. plantarum* BI7 and MU45, starter cultures for controlled cucumber fermentation and biocontrol microorganisms for minimally processed vegetable products. Fourteen genes in the genome of ΦJL-1, including 6 structural protein genes, were amplified by PCR and treated with *Eco*RI and *Hind*III endonucleases. The resulting products were individually subjected to directional cloning into T7Select10-3b vector. The result shows that 12 of the display phages contained inserts with expected sizes. Two large inserts (>2.2 kb) did not appear to be in display phages. Seven phage clones were obtained after 2-round cell-based panning in LB medium with display phage mix against *L. plantarum* MU45 cells. Four of the clones seemed to contain the inserts of interest. More work is needed to confirm these inserts. The results from 1-round biopanning in MRS medium with individual display phage or phage mix were not conclusive. Optimization of biopanning conditions or application of phage adsorption assay may be needed in order to see if significant difference in binding affinity or specificity exists among different display phages.

Keywords: bacteriophage, ΦJL-1, phage display, cloning, T7Select, *Lactobacillus plantarum*

INTRODUCTION

Most lactic acid fermentations occur under non-sterile conditions. Thus, lactic acid bacteria are susceptible to infection by lytic phages naturally present in these environments. Phage infection can create havoc in food fermentations, especially in the dairy industry. A virulent phage, ΦJL-1, has been recently isolated from a commercial cucumber fermentation and extensively characterized (Chapters 3 and 4). The phage was active against two related strains of *Lactobacillus plantarum*, BI7 and the derivative mutant MU45, both of which have been evaluated as starter cultures for controlled cucumber fermentation (McDonald et al., 1993; Passos et al., 1994; Lu et al., 2001, 2002a, 2002b), and as biocontrol microorganisms for minimally processed vegetable products (unpublished data). Electron microscopy revealed that the phage has a long non-contractile tail, belonging to the *Siphoviridae* family. Like other tailed phages, ΦJL-1 had the genome consisting of a linear double-stranded DNA. The complete nucleotide sequence (36,674 bp) and N-terminal amino sequences of 6 structural proteins of ΦJL-1 have been determined (Chapter 4).

In the phage life cycle, the first step in the infection is the adsorption of the phage to the host cell. This process involves a very specific interaction between the phage anti-receptor and the host receptor (Duplessis and Moineau, 2001). All tailed phages adsorb to bacteria by their tails (Ackermann, 1999). Most adsorb to the cell wall. Some tailed phages adsorb to cell pili, flagellae, or capsules, but all eventually reach the cell wall by pilus retraction, sliding along the flagellae, or digestion of the capsule (Ackermann, 1999). Once adsorbed, tailed phages digest the cell wall using specialized enzymes located at the tail tip and inject their DNA through the cytoplasmic membrane. Typically, phage-binding receptors may be outer membrane proteins such as LamB protein on *E. coli* (Wang et al., 1998), other proteins (usually glycoproteins) (Puig et al, 2001), carbohydrate residues present on glycoproteins or on cell wall (Quiberoni et al. 2000), or techoic acids (Wendlinger et al., 1996). Anti-receptors in tailed phages are the receptor-binding proteins at the tip of the phage tail, which confer the molecular specificity in the recognition of host cells, mediating phage infection (Wang et al., 1998; Weiss and Sidhu, 2000; Schouler et al., 1994). There have been no tail proteins found to be common to all

tailed phages (Ackermann, 1999). It is of great interest to identify the anti-receptor proteins on ΦJL-1 that specifically bind to *L. plantarum* BI7 and MU45. The identification is important for better understanding the critical early events in virus infection at the molecular level. The understanding would aid in identifying cellular receptors, in controlling virus entry, and in designing a strategy to increase phage-resistance of the starter culture, such as genetically engineering the receptors so that the phage is no longer able to adsorb to the host. The identified tail proteins may provide a molecular basis for development of a probe to track and quantify *L. plantarum* BI7 or MU45 in mixed culture systems, such as vegetable fermentation and biocontrol systems. In addition, the identified tail proteins may be used to mask or block cellular receptor, thereby preventing phage attachment and subsequent infection.

At present, very few structural and biophysical data on phage-host interactions are available, the major reason being that both reaction partners (cellular receptor and viral anti-receptor) are usually water-insoluble and thus difficult to purify (Wang et al., 1998). Phage-host interactions remain poorly understood in lactic acid bacteria and essentially in all Gram-positive bacteria. Although several phage tail proteins have been proposed to be anti-receptors in *Lactococcus lactis* phages bIL67 and c2 (Schouler et al., 1994; Lubbers et al., 1995), few anti-receptors have been characterized (Duplessis and Moineau, 2001). The reported studies mainly used hybridoma technology and neutralization assay to identify anti-receptors on phages. A phage protein was injected into rabbits or mice for the production of anti-receptor monoclonal antibodies which were then used in neutralization assay. If the antibodies recognized and inactivated the phage, the injected phage protein would be considered as an anti-receptor protein and would be further confirmed by dot blot or Western blot assay to test its ability to react with purified receptor (Wang et al., 1998; Krummenacher et al., 2000). Hybridoma technology is a time-consuming, cumbersome, and costly process (Sheets et al., 1998). A few researchers have used phage display to identify prokaryotic or eukaryotic receptors. Stricker et al.(1997) screened ligands with unknown receptors using display phage in an effort to identify the receptor. Prokaryotic receptor genes have been cloned directly from chromosomal DNA of *Staphylococcus aureus* into a phage display system, without any prior knowledge of the receptor, thus eliminating the need for probes in the identification

of receptor genes (Jacobsson and Frykberg, 1996). However, using phage display technology to identify receptor-binding protein of phage has not been reported in literature.

Phage display is a powerful new technology for the isolation of proteins or peptides that bind with high specificity and affinity to virtually any target molecule of interest. The main advantage of phage display is to facilitate the screening of very large numbers of different molecules by simple selection methods ("panning"), finding a needle in a vast molecular haystack (Rodi and Makowski, 1999). Vast numbers (e.g. $>10^9$) of different proteins or peptides and their coding sequences can be simultaneously screened. The time required to isolate proteins (e.g. antibodies) from phage display libraries can take as little as several weeks compared to the several months required for conventional methodologies. Bypassing the hybridoma system, phage display can produce the proteins in large quantities with the simple, rapid, and inexpensive process of viral replication and with no need for extensive purification (Felici et al., 1995).

Phage display of varying formats has been developed and applied successfully in a large number of studies to identify molecules with desired binding properties for research, medical, and industrial application. These include λ (Santini et al., 1998), T4 (Ren and Black, 1998), T7 (Danner and Belasco, 2001), and phagemid (Bass, S. and Wells, 1990; Sidhu, 2000) display systems.

T7 phage display system has many attractive features (Novagen, 2002):

- T7 replicates very fast (plaques form within 3 hr at 37°C and cultures lyse 1-2 hr after infection)
- T7 is a lytic phage. Therefore, displayed peptides or proteins do not need to be capable of export through the cell membrane to the periplasmic space
- Peptides up to 50 aa can be displayed in high copy number (415 per phage)
- Peptides or large proteins up to 1200 amino acids can be displayed in low copy number (0.1-1 per phage), suitable for the selection of proteins that bind strongly to their targets
- T7 is very stable to many harsh conditions, expanding the variety of agents that can be used in biopanning

T7 phage display has been used to study drug and gene delivery vehicles (Sokoloff et al., 2000), to isolate RNA-biding proteins from a complex cDNA library(Danner and Belasco, 2001), and to determine monoclonal antibody specificity (Houshmand et al., 1999).

The objective of this study was to identify the gene of ΦJL-1 that encodes the tail protein involved in host recognition via phage display technology. Using the powerful technology to identify ΦJL-1 tail proteins that mediate host recognition can be instrumental in the study of phage-host interaction in fermentation and biocontrol systems. The unique approach may be applicable to identify other functional phage proteins and/or to study other phage-host systems such as phage ecology in sauerkraut fermentation.

MATERIALS AND METHODS

Bacterial strains and culture media

Lactobacillus plantarum strain MU45 was obtained from the culture collection of the USDA Food Fermentation Laboratory (Raleigh, NC). It was propagated in MRS agar or broth (Difco) and used as the target for biopanning. *E. coli* BLT5403 purchased from Novagen (Madison, WI) was propagated in Luria-Bertani (LB) broth or agar (Difco) supplemented with 50 μg/ml carbenicillin (LB/carbenicillin), and used as the host for amplifying recombinant T7 phage. All bacterial stock cultures were stored at -84°C in MRS or LB broth containing 16% or 8% (v/v) glycerol. Soft agar was prepared with LB broth supplemented with 0.6% agar.

Phage JI-1 and ΦJL-1 DNA

ΦJL-1 DNA was extracted and purified by phenol/chloroform and ethanol precipitation as described in Materials and Methods in Chapter 3. The DNA was stored at 4°C.

Growth and storage of T7 lysate

LB/carbenicillin broth was used for preparation of the T7 lysate according to the method recommended by Novage (2002). The lysate was stored at 4°C for short term or at -80°C for long term after mixed with 0.1 volume of sterile 80% glycerol.

Titering phages

Phage titers were determined by plaque assays using the BLT5403 *E. coli* strain as a host, as described in T7Select System Manual (Novagen, 2002) with some modifications. About 250 µl of host cells and 100 µl of the phage dilution were added to a tube containing 3 ml molten agar (0.6% LB agar). After a brief mixing, the content was poured onto a prewarmed (37°C) LB/carbenicillin agar plate. The plate was allowed to sit for a few min, and then incubated for 4 h at 37°C or overnight at room temperature.

Enzymes and chemicals

All enzymes were purchased from Promega (Madison, WI) and/or Novagen (Madison, WI) and stored at -20°C until use. T7 Cloning Vector, DNA Ligation Kit, T7 Packaging Extracts, and T7 primers were purchased from Novagen. Chemicals for phage preparation, DNA extraction and other preparations were purchased from Sigma-Aldrich (St. Louis, MO).

T7 vector

T7Select10-3b Vector (Fig. 1) was purchased from Novagen and used to display 14 selected proteins from ΦJL-1. The vector contains *EcoR* I and *Hind* III arms, ready for directional cloning of appropriately prepared inserts (Fig. 2). The vector displays an average of 5-15 copies of peptides or proteins up to 1200 amino acids on the surface of the T7 capsid. The target inserts were fused to the C-terminus of the 10B capsid protein near amino acid 348, which precedes a series of multiple cloning sites (Fig. 2). T7Select10-3b phage was grown on a complementing host (BLT5403) that supplies large

amount of the 10A capsid protein from a plasmid. Capsids thus contain mostly 10A protein, along with 5-15 copies of 10B fusion protein per virion.

Design of PCR primers for amplifying the open reading frames of interest

For the sake of convenience, ORFs discussed in this chapter were designated along the genome (Table 1). There were 15 ORFs (ORFs 18, 19, 20, 21, 22, 23, 25, 27, 28, 29, 30, 31, 32, 33, and 34) between lysin gene and major capsid protein genes followed by terminase gene (Table 1). Since ORF31 contains more than one *Hind*III site and can not be cloned into T7Select vector, the ORF was not considered in primer design. Although ORF 25 contains one *Hind*III site, the ORF was still considered in primer design because the first *Hind*III fragment was big (encoding 342 amino acids). A total of 14 pairs of PCR primers was designed to amplify ORFs 18, 19, 20, 21, 22, 23, 25, 27, 28, 29, 30, 32, 33, and 34 (Table 2). ORFs 21, 23, and 29 encode 3 identified tail proteins whose N-terminal amino acid sequences had been determined (Chapter 4).

Each forward primer starts with 5 extra nucleotides (in order to enhance the efficiency of restriction digestion) and an *EcoR* I restriction site, GAATTC (to provide compatibility with the vector arms), followed by an extra nucleotide (to ensure the expression in-frame with the 10B protein such that recombinant fusion proteins are displayed), the start codon (ATG, TTG or GTG), and gene-specific nucleotides (Table 2). Each reverse primer starts with 6 extra nucleotides and a *Hind* III site, AAGCTT, followed by TTA (complementary to the stop codon on the top strand), and gene-specific sequence (Table 2). The 14 pairs of primers were synthesized by Genosys Biotechnologies Inc. (The Woodlands, TX).

Preparing inserts for cloning

A total of 14 PCR reactions was performed to amplify the selected ORFs. The amplification was performed with a thermal cycler (RoboCycler Gradient 96, Stratagen) in a total volume of 100-µl. Each reaction mixture contained 80.5 µl water, 2 µl template ΦJl-1 DNA (8 ng), 10 µl 10X High Fidelity PCR buffer (Invitrogen, Carlsbad, CA), 4 µl 50 mM $MgSO_4$, 1 µl of each primer (20 µM), 2 µl of dNTP mixture (10 mM each dNTP),

and 0.5 µl Platinum *Tag* High fidelity DNA polymerase (5 Unit/µl, Invitrogen). Samples were overlaid with 75 µl mineral oil and subjected to an initial denaturation step (94°C for 5 min), followed by 30 cycles (30 sec at 94°C, 0.5 or 1 min at 55°C, 1 or 2.5 min at 68°C), and a final step of 8 min at 68°C. The PCR products were purified with QIAquick PCR purification kit (QIAgene Inc., Valencia, CA). To generate sticky ends, 10 µl purified PCR product was mixed with 70 µl water, 10 µl 10X buffer E (Promega), 5µl BSA(1 mg/ml), and 5µl *Hind*III endonuclease (10 U/µl, Promega). After overnight incubation at 37°C, each reaction was mixed with 5 µl *Eco*RI (12 U/µl, Promega), and then incubated for 4 h at 37°C. To check molecular size, the digested PCR products (5 µl each) were electrophoresed on 1% (w/v) agarose gel in TAE buffer at a constant 85 V for 1.2 hr. A 100 bp DNA ladder-size standard (Invitrogen, Carlsbad, CA) was used as a standard. If a digested PCR product contained extra bands, the band with expected molecular size was excised and gel-purified with QIAquick Gel Extraction Kit (Qiagen). After estimation of concentration by measuring absorbance at 260 nm, the concentration of each digest (also known as insert) was adjusted to 0.04 pmol/µl with nuclease-free water prior to cloning.

Ligation of inserts and vector arms

After *Eco*RI and *Hind*III restriction digestion, the insert was ligated into T7Select10-3b vector. Each ligation reaction contained 1.5 µl diluted digest (0.04 pmol/µl), 1µl T7Select Vector Arms (0.5 µg; 0.02 pmol), 0.5 µl 10X ligase buffer, 0.5 µl 10 mM ATP, 0.5 µl 100 mM DTT, and 1 µl T4 DNA ligase (diluted 10 times in advance). A positive control contained 1µl positive control insert, whereas a negative control contained no insert. Each ligation reaction was incubated at 18°C for 16 h.

***In vitro* Packaging of ligated DNA**

An aliquot of ligation reaction was added directly to T7 Packaging Extract (Novagen) for *in vitro* packaging at the volume ratio of 1 to 5 (ligation reaction : packaging extract, total 25µl). For a packaging control, packaging control DNA was used. After gentle mix, each mixture was incubated at room temperature for 2 h. To stop

the reaction, 270 µl LB medium was added to the reaction. The packaging reactions were temporarily stored at 4°C. Plaque assay was performed to determine the titer of each sample. Packaging efficiency was calculated based on the following formula:

Packaging efficiency = (Total pfu in the packaging reaction) / (µg of vector used)

Liquid lysate amplification

Recombinant phages were amplified once in liquid culture (50 ml) according to a modified protocol developed by Novagen. Briefly, a freshly grown BL5403 culture in LB/carbenicillin medium was infected with recombinant phage at an MOI of 0.001-0.01 and then incubated on a flask shaker at 37°C until cell lysis (3-4 h). After centrifugation (8,000 x g, 10 min), the supernatant was transferred into a sterile bottle. Plaque assay was performed to determine the titer of the amplified phage lysate. Each individual amplified lysate was stored at 4°C for subsequent panning, or at -84°C for long-term storage (> several months) after mixed with 0.1 volume sterile 80% glycerol.

The insert sizes of the 14 recombinant phages were analyzed by PCR with a pair of T7Select primers that flank the site of insert: 5'-GGAGCTGTCGTATTCCAGTC-3' (T7SelectUP primer) and 5'-GGCTGATACCACCCTTCAAG-3' (T7SelectDOWN primer, Novagen, 2002). Each PCR reaction contained 76.5 µl water, 3 µl phage lysate which had been heated in boiling water for 10 min, 10 µl 10X High Fidelity PCR buffer (Invitrogen), 4 µl 50 mM $MgSO_4$, 2 µl T7SelectUP primer (Novagen), 2 µl T7SelectDOWN primer (Novagen), 2 µl dNTP mix (10 mM each dNTP), and 0.5 µl Platinum *Tag* High fidelity DNA polymerase (5 Unit/µl, Invitrogen). Samples were subjected to an initial denaturation step (94°C for 5 min), followed by 35 cycles of denaturation (94°C for 50 sec), annealing (55°C for 1 min), and extension (68°C for 1 min), and finished with a final extension step at 68°C for 8 min. The PCR products were subjected to electrophoresis on 1% agarose gel at 85 V for 1.2 hr.

Naming system

Inserts and display phages (recombinant phages) were named according to the corresponding ORF in ΦJL-1. For example, if an insert resulted from ORF18, the insert was designated as insert 18, and the resulting display phage was called display phage 18.

Cell-based biopanning

Phage-binding experiments were preformed with *L. plantarum* MU45 cells by using two methods. The first method was based on two alternating rounds of affinity selection and viral replication, according to the protocol developed by Stephenson et al. (1998) with some modifications. Phage mix was prepared by mixing the 14 amplified phage lysates at appropriate ratio so that the phage mix contained 1 x 10^5 pfu/ml of each type of recombinant phages. One ml phage mix (total 1.4 x 10^6 pfu) was mixed with the target MU45 cell pellet resulting from 1 ml fresh culture (1 x 10^8 cfu). After vortexing and 30-min incubation at room temperature, the binding reaction was micro-centrifuged at 12,000 rpm for 30 sec. After decanting, the cell pellet with bound phages was re-suspended (by vortex for 10 sec.) in 1 ml LB medium. The washing step was repeated 4 more times (totally 5 times) to remove non-specifically bound phage prior to the amplification with *E. coli* BLT5403 in LB medium supplemented with 50 μg/ml carbenicillin. The amplified phage lysate was used for the next round of panning on MU45 cells. After the secondary panning, a plaque assay was carried out and 7 individual phage clones were picked from the plate. To evaluate the effectiveness of the cell-based panning, the packaging control phage served as a negative control in the panning on MU45 cells.

The second method included one round of 16 separate pannings using 14 individual display phage lysates, packaging control lysate, and phage mix. LB medium was replaced with MRS medium supplemented with 10 mM $CaCl_2$ (MRS/Ca). Each display phage stock was serial-diluted with MRS/Ca medium (from 10^{10} to 10^7 pfu/ml). A phage mix was prepared by mixing 14 diluted display phage lysates and packaging control phage (the final titer of each phage in the mix was approximately 10^7 pfu/ml). One ml of diluted phage lysate (10^7 pfu) or packaging control phage or phage mix was

mixed with MU45 cell pellet resulting from 1 ml fresh culture (2 x 10^8 cfu). After 30-min incubation at room temperature, the phage/cell mixture was micro-centrifuged at 12,000 rpm for 30 sec. After decanting, the cell pellet with bound phages was re-suspended (by a brief vortex) in 1 ml MRS/Ca medium, and micro-centrifuged (at 12,000 rpm for 30 sec). The washing step was repeated one more time. After removing supernatant, the cell pellet with bound phages was re-suspended in 1 ml MRS/Ca medium. The titer was determined by plaque assay.

Characterization of selected phage clones

The insert size of 7 individual recombinant phage clones were examined by PCR amplification using T7Select UP and Down primers (see above). A portion of the phage plaque was dispersed in a tube containing 100 µl of 10 mM EDTA (pH 8.0). After a brief vortex, the tube was heated in boiling water for 10 min, and then centrifuged at 14,000 x g for 3 min. The supernatant (phage lysate) was used for direct PCR amplification of the displayed insert DNA in each clone. The assembly of PCR reaction and PCR condition were the same as the phage lysate PCR described above.

RESULTS AND DISCUSSION

The 14 genes of interest in ΦJL-1 were successfully amplified by PCR. All the inserts revealed expected molecular sizes (Fig. 3A). After the ligation of inserts and T7 vector arms, the cloned inserts were *in vitro* packaged. The titer of display phages after packaging varied between 3.2 x 10^5 and 2.1 x 10^9 pfu/ml, higher than that of negative control insert, but lower than that of packaging control (Table 3). Accordingly, packaging efficiency was calculated based on the total number of pfu and the µg of vector used in the ligation reaction. The highest packaging efficiency (1.3 x 10^9 pfu/µg) was obtained by using Packaging Control DNA, whereas the lowest packaging efficiency (5.2 x 10^4 pfu/µg) was obtained when a negative control insert was used. Packaging efficiency for the positive control insert was 1.4 x 10^8 pfu/µg. The packaging efficiencies for the 14 cloned inserts were similar to or slightly lower than that of the positive control insert

(Table 3). Inserts 21, 22, and 23 resulted in lower packaging efficiency because their initial concentrations used for ligation were lower presumably due to the sample loss in the extra purification step prior to ligation.

Liquid lysate amplification after *in vitro* packaging resulted in high phage titer (10^{10} pfu/ml), regardless of phage types (data not shown). The PCR products of the inserts from the 14 display phages are shown in Fig. 3B. All these PCR products, except insert 21 and 22, had expected sizes which were 98 bp larger than the corresponding ones in Fig. 3B, because T7Select primers, instead of the primers listed in Table 2, were used in PCR amplification, resulting in longer sequences. The two largest inserts 21 and 22 (> 2.3 kb) did not appear in PCR products (Fig. 3B), suggesting that they might not be successfully cloned into the T7Select10-3b vector due to their large sizes. The unexpected much smaller products (< 300 bp) amplified from sample 21 or 22 were probably from non-specific amplification.

The 2-round affinity selection using display phage mix containing 14 display phages against *L. plantarum* MU45 cells was carried out in LB medium. Each round included 5 vigorous washes with LB medium. After the 2-round panning, 7 individual phage clones (c1, c2, c3, c4, c5, c6, and c7) in a LB/carbenicillin plate were picked and amplified with T7Select primers. The PCR product of c1, c2, or c3 was around 150 bp in size, which was smaller than any of the expected inserts, including positive, negative control inserts, and insert from packaging control (Fig. 4). It was unclear where those bands originated. The bands from c4 and c5 seemed to match the size of insert 18, 27, or 28, whereas c6 band appeared to match the size of insert 30 (Fig. 3 and 4). The c7 band had the similar size as insert 29, encoding the major tail protein. Further work such as restriction mapping or sequencing is needed to confirm these inserts. The PCR products from positive, negative control inserts, and packaging control had identical size (approximately 250 bp), which was different from that of inserts in any display phages.

The result from 1-round panning (with 2 washes) in MRS/Ca medium using individual display phage, or phage from packaging control, or phage mix plus packaging control phage is shown in Table 4. There was no significant difference among different treatments. Perhaps more wash is needed to remove non-specific bound display phages in order to see the difference.

The results from biopanning with phage mix or individual display phage were not conclusive. More work is needed to optimize the panning conditions in order to see the significant difference in binding affinity among different display phages (if the difference really exists). Since attachment of phages to bacterial cells is a first-order reaction with respect to the concentrations of both phage and bacteria (Luria et al., 1978), higher concentrations of phage and host cells may be needed in biopanning. Phage adsorption is a very complex, highly coordinated, and reversible process. MOI, the time allowed for adsorption, panning temperature, ionic strength of the medium, absence of carbenicillin, vortex speed and time, and number of times of washing all can influence phage adsorption and retention on host cells. If the binding affinity of phage on host cell is not very strong, the binding could be easily disrupted during the panning procedure. If this is the case, phage adsorption assay may be a better approach than the affinity selection for the identification of anti-receptor proteins. Phage adsorption assay does not require the washing step which could disrupt phage binding. If a displayed phage interferes with the adsorption of ΦJL-1 on MU45, the displayed protein is likely to be the anti-receptor protein of ΦJL-1 conferring molecular specificity in the recognition of host receptor site.

There are a number of possibilities that may make the attempt using phage display to identify anti-receptor unsuccessful. First of all, not all genes can be cloned into the T7 vector. When an insert is too large, such as inserts 21 and 22 in this study, the cloning and packaging efficiencies can be very low due to biological intolerance. When an insert is small, there is a chance of cloning more than one insert into the same site of the vector, resulting in displaying a non-natural protein without desired functions. Even if a gene is fused to a capsid protein gene, the gene product may not be successfully displayed due to defects in viral particle assembly, stability, and infectivity. Furthermore, a foreign gene may not be properly expressed because *E. coli*, *L. plantarum*, T7 phage, ΦJL-1 may use different codon systems. Thus, the same gene in different biological systems may result in different proteins. In addition, if a protein is displayed on phage surface, but the protein is not folded properly, the binding specificity and affinity of the protein would not be the same as those properly folded. It is unknown how many proteins or how many

copies of the same protein are involved in phage adsorption process. Currently, phage display can only display a single type of protein on the same phage particle. In the T7Select system, the target inserts were fused to the C-terminus of a capsid protein, thus the displayed protein does not have a free N-terminus. Also, if the recognition of host receptor requires a free N-terminus on a tail protein, other format of display system such as the phagemid system should be used. So far, no single display format has proven universally applicable. Each display system has its own advantages and limitations.

REFERENCES

Ackermann, H. -W. 1999. Tailed bacteriophages: The Order Caudovirales. *In* "Advances in Virus Research", Vol. 51, pp. 135-201. Academic Press, New York.

Bass, S. and Wells, J.A. 1990. Hormone phage: an enrichment method for variant proteins with altered binding properties. Proteins. **8**:309-314.

Danner, S. and Belasco, J.G. 2001. T7 phage display: a novel genetic selection system for cloning RNA-binding proteins from cDNA libraries. Proc. Natl. Acad. Sci. USA. **98**(23):12954-12959.

Duplessis, M. and Moineau, S. 2001. Identification of a genetic determinant responsible for host specificity in *Streptococcus thermophilus* bacteriophages. Mol. Biol. **41**:325-336.

Felici, F., Luzzage, A., Monaci, P., Nicosia, A., Sollazzo, M., and Traboni, C. 1995. Peptide and protein display on the surface of filamentous bacteriophage. Biotechnol, Annu. Rev., **1**:149-183.

Houshmand, H., Fröman, G., and Manusson, G. 1999. Use of bacteriophage T7 displayed peptides for determination of monoclonal antibody specificity and biosensor analysis of the binding reaction. Anal. Biochem. **268**:363-370.

Jacobsson, K. and Frykberg, L. 1996. Phage display shot-gun cloning of ligand-binding domains of prokaryotic receptors approaches 100% correct clones. Biotechniques. **20**:1070-1076.

Krummenacher, C., Baribaud, I., Ponce De Leon, M., Whitbeck, J. C., Lou, H., Cohen, G. H., and Eisenberg, R. J. 2000. Localization of a binding site for herpes simplex virus glycoprotein D on herpesvirus entry mediator C by using antireceptor monoclonal antibodies. J. Virol. **74**: 10863-10872.

Lu, Z., Fleming, H.P., and McFeeters, R.F. 2001. Differential glucose and fructose utilization during cucumber juice fermentation. J. Food Sci. **66**(1):162-166.

Lu, Z., Fleming, H.P., McFeeters, R.F., and Yoon, S.S. 2002a. Effects of anions and cations on sugar utilization in cucumber juice fermentation. J. Food Sci. **67**(3): 1155-1161.

Lu, Z., Fleming, H.P., and McFeeters, R.F. 2002b. Fruit size effects on cucumber composition, pH, and buffer capacity, sugar utilization and bloater formation in cucumber fermentation. J. Food Sci. **67**(4): In press.

Lubbers, M.W., Waterfield, N.R., Beresford, T.P.J., Le Page, R.W.F., and Jarvis, A.W. 1995. Sequencing and analysis of the prolate-headed lactococcal bacteriophage c2 genome and identification of the structural genes. Appl. Environ. Microbiol. **61**:4348-4356.

Luria, S.E., Darbel, J. E., Jr., Baltimore, D., and Campbell. 1978. Phage-bacterium interaction: General features. In: General virology, pp. 135-156, 3rd ed. John Wiley & Sons. New York.

McDonald, L.C., Shieh, D.-H., Fleming, H.P., McFeeters, R.F., and Thompson, R.L. 1993. Evaluation of malolactic-deficient strains of *Lactobacillus plantarum* for use in cucumber fermentations. Food Microbiol. **10** (6): 489-499.

Novagen, 2002. T7Select System Manual. Novagen, Madison, WI.

Quiberoni, A., Stiefel, J., and Reinheimer, J. 2000. Characterization of phage receptors in *Streptococcus thermophilus* using purified cell walls obtained by a simple protocol. J. Appl. Micro. **89**:1059-1065.

Passos, F.V., Fleming, H.P., Ollis, D.F., Felder, R.M., and McFeeters, R.F. 1994. Kinetics and modeling of lactic acid production by *Lactobacillus plantarum*. Appl. Environ. Microbiol. **60**(7):2627-2636.

Puig, A., Araujo, R., Jofre, J. and Frias-Lopez, J. 2001. Identification of cell wall proteins of *Bacteroides fragilis* to which bacteriophage b40-8 binds specifically. Microbiology. **147**:281-288.

Ren, Z. and Black, L.W. 1998. Phage T4 SOC and HOC display of biologically active, full-length proteins on the viral capsid. Gene. **215**:439-444.

Rodi, D.J. and Makowski, L. 1999. Phage display technology - finding a needle in a vast molecular haystack. Curr. Opin. Biotechnol. **10**:87-93.

Santini, C., Brennan, D., Mennuni, C., Hoess, R.H., Nicosia, A., Cortese, R., and Luzzago, A. 1998. Efficient display of an HCV cDNA expression library as C-terminal fusion to the capsid protein D of bacteriophage lambda. J. Mol. Biol. **282**:125-135.

Schouler, C., Dusko Ehrlich, S., and Chopin, M.C. 1994. Sequence and organization of the lactococcal prolate-headed bIL67 phage genome. Microbiology. **140**:3061-3069.

Sheets, M.D., Amersdorfer, P., Finnern, R., Sargent, P., Lindqvist, E., Schier, R., Hemingsen, G., Wong, C., Gerhart, J.C., and Marks, J.D. 1998. Efficient construction of a large nonimmume phage antibody library: The production of high-affinity human single-chain antibodies to protein antigens. Proc. Natl. Acad. Sci. USA. **95**:6157-6162.

Sidhu, S.S. 2000. Phage display in pharmaceutical biotechnology. Curr. Opin. Biotechnol. **11**:610-616.

Sokoloff, A. B., Bock, I., Zhang, G. Sebestyén, M. G., and Wolff, J.A. 2000. The interactions of peptides with the innate immune system studied with use of T7 phage peptide display. Mol. Therapy. **2**(2):131-139.

Stricker, N.L., Christopherson, K.S., Yi, B.A., Schatz, P.J., Raab, R.W., Dawes, G., Bassett, D.E. Jr., Bredt, D.S., and Li, M. 1997. PDZ domain of neuronal nitric oxide synthase recognizes novel C-terminal peptide sequences. Nat. Biotechnol. **15**:336-342.

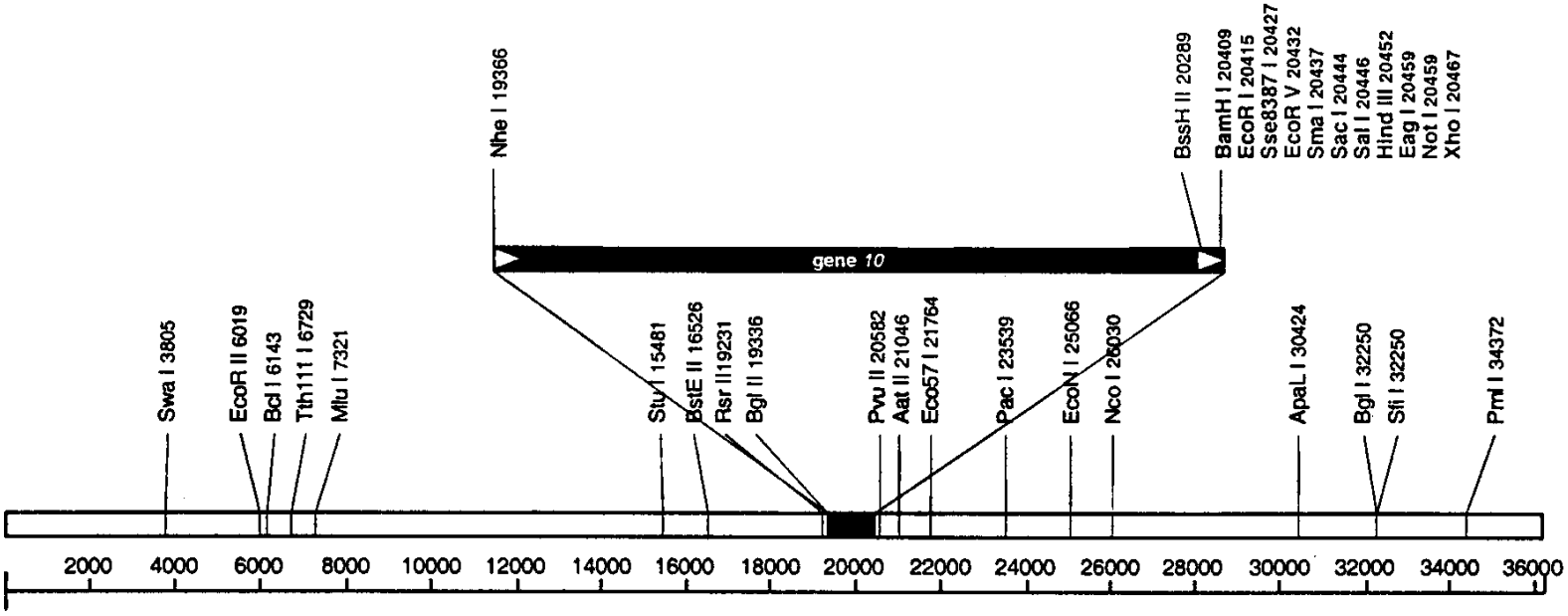
Wang, J., Michel, V., Hofnung, M., and Charbit, A. 1998. Cloning of the J gene of bacteriophage lambda, expression and solubilization of the J protein: first *in vitro* studies on the interactions between J and LamB, its cell surface receptor. Res. Microbiol. **149**:611-624.

Weiss, G.A. and Sidhu, S.S. 2000. Design and evolution of artificial M13 coat proteins. J. Mol. Biol. **300**:213-219.

Wendlinger, G., Loessner, M.J., and Scherer, S., 1996. Bacteriophage receptors on *Listeria monocytogenes* cells are the N-acetylglucosamine and rhamnose substituents of techoic acids or the peptidoglycan itself. Microbiology. **142**:985-992.

T7Select10-3b sequence landmarks

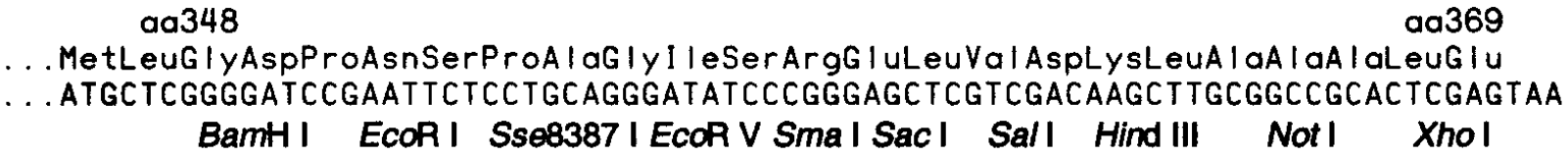
10B translation start	19363
T7SelectUP priming site	20374-20393
Multiple cloning region (*Bam*HI – *Xho*I)	20409-20472
T7SelectDOWN priming site	20498-20517



T7Select10-3b Vector (36,249 bp)

Figure 1. T7Select10-3b vector and its sequence landmarks. (Adapted from Novagen, 2002.)

T7Select10-3b cloning region



Reading frame of insert into *EcoR*I/*Hind*III T7Select10-3b vector arms

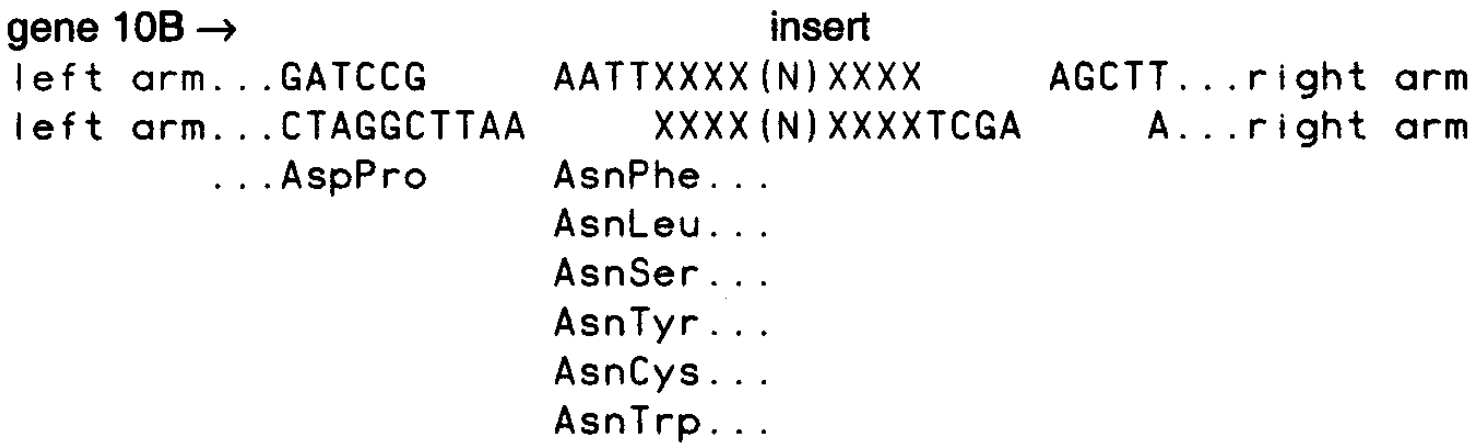


Figure 2. T7Select10-3b cloning region and reading frame. (Adapted from Novagen, 2002.)

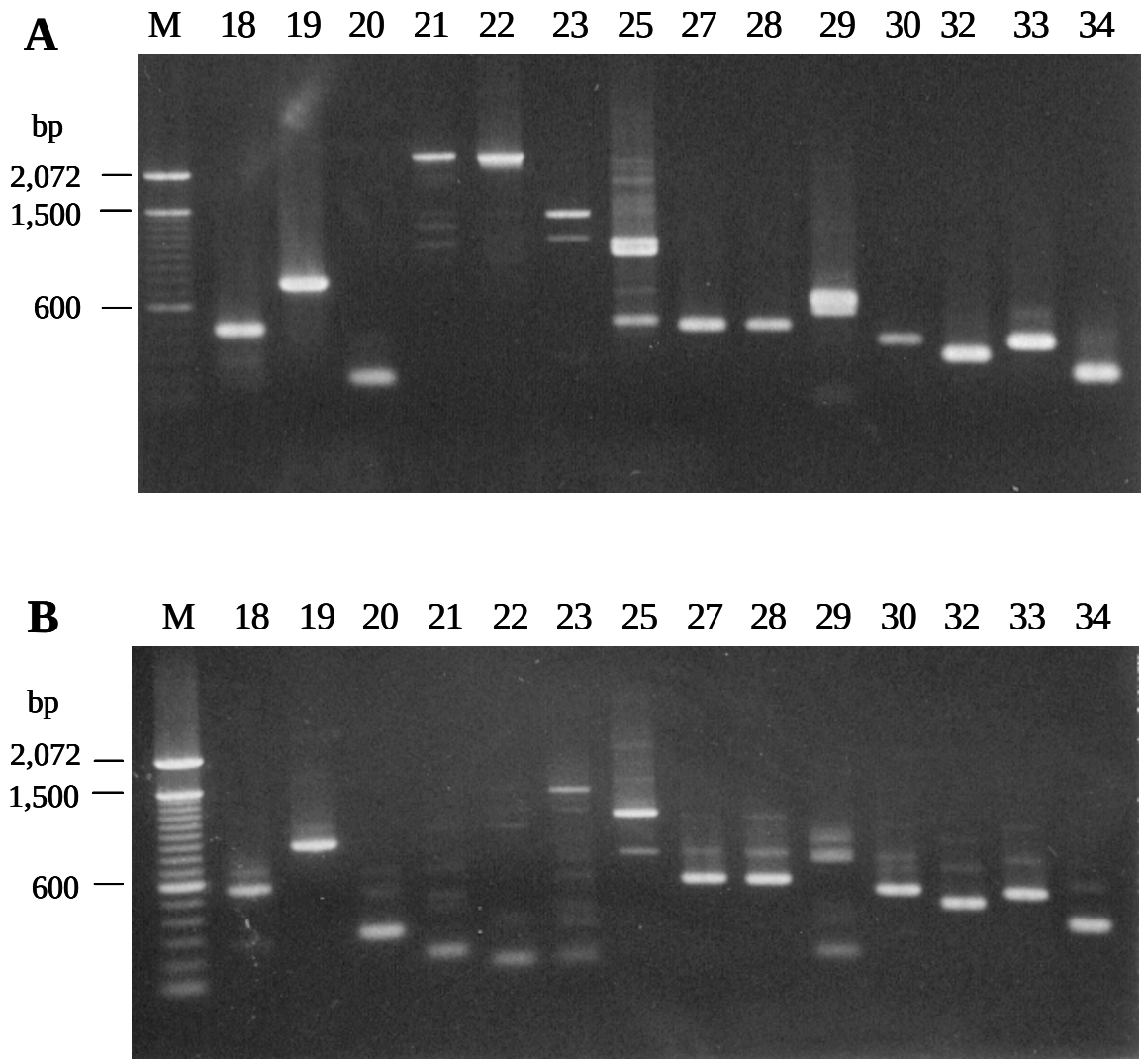


Figure 3. PCR products from 14 inserts before and after cloning. (A) The 14 inserts generated by PCR amplification followed by *Eco*RI/*Hin*dIII restriction digestion before ligation. The primers listed in Table 2 were used to generate these inserts. (B) PCR products from 14 display phages right after *in vitro* packaging. T7Select UP and DOWN primers were used in PCR amplification. Lane M, 100-bp DNA ladder; lane 18, insert 18; lane 19, insert 19; lane 20, insert 20; lane 21, insert 21; lane 22, insert 22; lane 23, insert 23; lane 25, insert 25; lane 27, insert 27; lane 28, insert 28; lane 29, insert 29; lane 30, insert 30; lane 32, insert 32; lane 33, insert 33; lane 34, insert 34.

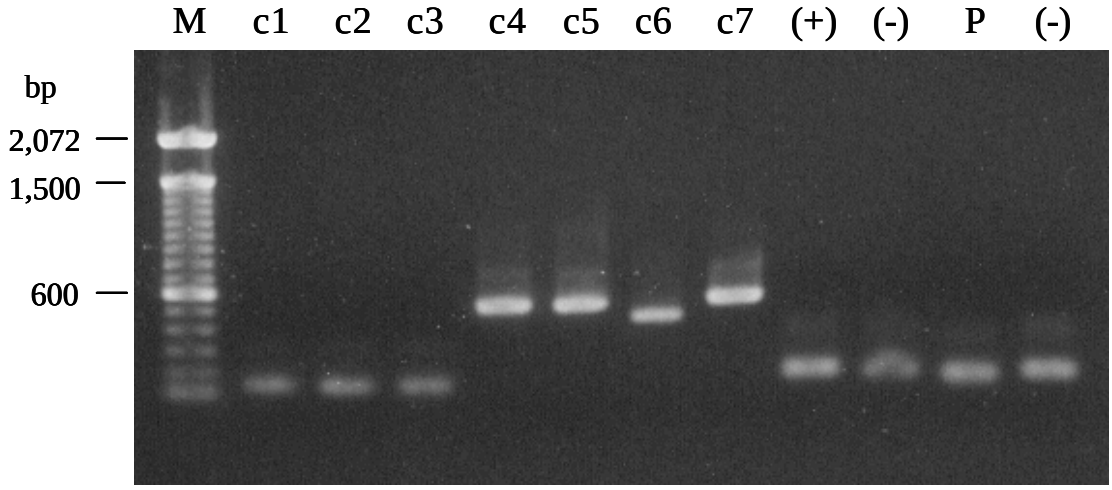


Figure 4. PCR products of inserts from the preparations of 7 display phage clones obtained after the 2-round panning using phage mix in LB/carbenicillin medium. T7Select UP and DOWN primers were used in PCR amplification. Lane M, 100-bp DNA ladder; lane c1, insert from phage clone 1; lane c2, insert from phage clone 2; lane c3, insert from phage clone 3; lane c4, insert from phage clone 4; lane c5, insert from phage clone 5; lane c6, insert from phage clone 6; lane c7, insert from phage clone 7; lane (+), positive control insert; lane (-), negative control insert; lane P, insert from packaging control.

Table 1. Selected open reading frames in ΦJL-1 genome

ORF[a]	ORF[b]	Predicted star position	Predicted stop position[c]	Length in nt	ORF homology[d]	Organism matched	Accession no.
16	398	26572	27765	1236	lys	*Oenococcus oenos* phage 10MC	AF049087
18	147	26127	26567	441	P163	*Oenococcus oenos* phage 10MC	AF049087
19	249	25177	25923	747			
20	60	24725	24904	180			
21	749	22077	24323	2247	host specificity	*Lactococcus lactis* phage bIL170	AF009630
22	738	19871	22084	2214	ORF977	*Lactobacillus johnsonii* prophage Lj965	AF195900
23	441	18531	19853	1323	tail component protein	*Lactobacillus casei* phage A2	AJ251790
25	1133	15095	18493	3399	tape measure protein	*Lactococcus lactis* phage TP901-1	AF304433
27	140	14669	15088	420			
28	139	14367	14783	417			
29	199	13746	14342	597	gene 17.1	*Bacillus subtilis* phage SPP1	X97918
30	117	13380	13730	351	hypothetical protein		
31	125	13006	13380	375	head to tail joining	*Lactococcus lactis* phage TP901-1	AF304433
32	94	12732	13013	282	hypothetical protein		
33	113	12391	12729	339	DNA packaging	*Lactococcus lactis* phage TP901-1	AF304433
34	64b	12128	12319	192			
35	286	11190	12047	858	hypothetical protein	*Streptococcus pneumoniae* phage MM1	AJ302074
36	184	10616	11167	552	scaffold protein	*Streptococcus thermophilus* phage Sfi11	AF158600
37	273	9691	10509	819	putative protein	*Streptococcus pyogenes* prophage M1 GAS	AE006544
38	506	8223	9740	1518	portal protein	*Streptococcus thermophilus* phage Sfi11	AF158600
39	440	6890	8209	1320	putative terminase, large subunit	*Streptococcus pyogenes* prophage M1 GAS	AE006544
40	148	6481	6924	444	terminase, small subunit	*Bacillus subtilis* PBSX prophage	Z99110

[a]ORFs are designated along the genome.
[b]ORFs are designated according to the number of amino acids of the corresponding coded protein.
[c]The stop position does not include the stop codon.
[d]Empty cells indicate no blast hits found.

Table 2. PCR primers used for amplifying the ORFs of interest[a]

Primer	Sequence (5' to 3')
18-up	CGTTGGAATTCCATGAATAACATTTCAGAATTAAT
19-up	CGTTGGAATTCTTTGGTTGATTCAACTAATATT
20-up	CGTTGGAATTCATTGAGCGACGCTGTTATTAC
21-up	CGTTGGAATTCGATGGCAATTAGAACTTATGA
22-up	CGTTGGAATTCAATGACGGTGTTCAAAGATAT
23-up	CGTTGGAATTCTATGGCGGGCTATTTTTATAT
25-up	CGTTGGAATTCTATGGCACAAGTAGCAGCTA
27-up	CGTTGGAATTCATTGGACAGACTTAGTGGAAA
28-up	CGTTGGAATTCCATGAAGATTGGTAATACCGA
29-up	CGTTGGAATTCCATGGTAGCAGTTAATAACGG
30-up	CGTTGGAATTCCATGACATTATCAGAATGGTA
32-up	CGTTGGAATTCAATGAGATATAACGATCGAGT
33-up	CGTTGGAATTCAATGGCATTACTAGATTCGAT
34-up	TTTTGGAATTCCGTGTCAGCCGTGCCATC
18-down	GATTTGAAGCTTTTATTCCTCCGTTGCTGGTTTG
19-down	GATCGGAAGCTTTTATAAACTTTCTAAGAACTTGG
20-down	GATCGGAAGCTTTTATTGCTTGCTTTTCTCCTCCT
21-down	GATCGGAAGCTTTTAGTTGTCAGTAACCGAAACGT
22-down	GATCGGAAGCTTTTAATTGCCATCTACATTACCAC
23-down	GATCGGAAGCTTTTACGCGTATAAGTCTTGATAAT
25-down	GATCGGAAGCTTTTAACTCATAAAAAACTTACTGCT
27-down	GATCGGAAGCTTTTATTCACCTCCGTGATTAAACA
28-down	GATCGGAAGCTTTTAGGAGAGACTCTTCTTCATTT
29-down	GATTTGAAGCTTTTATGCTCCAGTTGTTCCGGTTT
30-down	GATCGGAAGCTTTTAAATTGTGAACGTGACTAAAAA
32-down	GATCGGAAGCTTTTATTTGCCACAGATTAACACCG
33-down	GATCGGAAGCTTTTATATGAACCTCATTCCAGCAC
34-down	GATTTGAAGCTTTTAAGCTGCGGTAACAGTAACG

[a]The restriction sites (in blue) and start codons (in red) are underlined.

Table 3. Phage titer in packaging reaction and packaging efficiency

Display phage	Phage titer pfu/ml	Packaging efficiency pfu/μg
18	1.5×10^8	9.0×10^7
19	1.7×10^8	1.0×10^8
20	1.4×10^8	8.4×10^7
21	4.9×10^6	2.9×10^6
22	3.2×10^5	1.9×10^5
23	1.5×10^6	9.0×10^5
25	8.0×10^7	4.8×10^7
27	1.4×10^8	8.4×10^7
28	1.0×10^8	6.0×10^7
29	1.1×10^8	6.6×10^7
30	9.0×10^7	5.4×10^7
32	9.0×10^7	5.4×10^7
33	8.0×10^7	4.8×10^7
34	1.3×10^8	7.8×10^7
(+) control insert	2.4×10^8	1.4×10^8
(-) control insert	8.7×10^4	5.2×10^4
Packaging control	2.1×10^9	1.3×10^9

Table 4. Phage titer before and after panning in MRS/Ca medium using individual display phage, phage from packaging control or phage mix plus packaging control phage

Display phage	Phage titer (pfu/ml)	
	before panning	after panning
18	1.7×10^7	5.8×10^3
19	1.2×10^7	8.7×10^3
20	1.6×10^7	5.6×10^3
21	1.4×10^7	3.8×10^3
22	1.3×10^7	9.2×10^3
23	6.8×10^6	4.3×10^3
25	1.3×10^7	4.4×10^3
27	1.5×10^7	7.3×10^3
28	1.6×10^7	8.8×10^3
29	1.3×10^7	6.2×10^3
30	1.6×10^7	4.3×10^3
32	1.5×10^7	2.0×10^3
33	9.7×10^6	1.9×10^3
34	1.2×10^7	3.0×10^3
Packaging control	2.0×10^7	9.6×10^3
Mix + packaging control	1.4×10^8	5.9×10^4

Appendix 1

Complete genome sequence of *Lactobacillus plantarum* bacteriophage ΦJL-1

Note: The complete genome sequence of phage ΦJL-1 presented here is in GenBank format.

```
LOCUS       PhiJL-1   36674 bp    DNA   linear  PHG       19-SEP-2002

DEFINITION  Lactobacillus plantarum bacteriophage phiJL-1, complete genome

ACCESSION   xxxxxx

VERSION     xxxxxx

KEYWORDS    GAMOLA annotation; helicase; DNA polymerase; DNA primase; lysin; major
            head protein; major tail protein; head to tail joining; DNA packaging;
            scaffold protein; portal protein; terminase; DNA binding; HNH homing
            endonuclease.

SOURCE      Lactobacillus plantarum bacteriophage phiJL-1
  ORGANISM  Lactobacillus plantarum bacteriophage phiJL-1
            Viruses; dsDNA viruses; Caudovirales; Siphoviridae.

REFERENCE   1 (bases 1 to 36674)
  AUTHORS   Lu, Z., Breidt, F., Fleming, H.P., Altermann, E., and Klaenhammer, T.R.
  TITLE     Isolation and Characterization of a Lactobacillus plantarum
            bacteriophage, phiJL-1, from a cucumber fermentation
  JOURNAL   International Journal of Food Microbiology, in press
  MEDLINE   in press

REFERENCE   2 (bases 1 to 36674)
  AUTHORS   Lu, Z., Altermann, E., Breidt, F., Predki, P., Fleming, H.P., and
            Klaenhammer, T.R.
  TITLE     Sequence analysis of Lactobacillus plantarum bacteriophage phiJL-1
  JOURNAL   Gene, in review
  MEDLINE   in review

FEATURES             Location/Qualifiers

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