Isolation and Characterization of Bacteriophages from Fermenting Sauerkraut†

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This paper presents the first report of bacteriophage isolated from commercial vegetable fermentations. Nine phages were isolated from two 90-ton commercial sauerkraut fermentations. These phages were active against fermentation isolates and selected Leuconostoc mesenteroides and Lactobacillus plantarum strains, including a starter culture. Phages were characterized as members of the Siphoviridae and Myoviridae families. All Leuconostoc phages reported previously, primarily of dairy origin, belonged to the Siphoviridae family.

Commercial cabbage fermentations in the United States typically are carried out by epiphytic lactic acid bacteria (LAB), without the benefit of an added starter culture (8). Four species of LAB have been identified as the primary species involved in sauerkraut fermentations: Leuconostoc mesenteroides, Lactobacillus brevis, Pediococcus pentosaceus, and Lactobacillus plantarum (15). Pederson and Albury (12, 13) determined that if the conditions of salt and temperature (2% NaCl and 18°C) are correct, a succession of these species will result in the consistent production of high-quality sauerkraut. For this reason, researchers have concluded that starter cultures are not needed for sauerkraut fermentations (14, 16).

Our interest in studying the microbial ecology of sauerkraut fermentations has developed from the current need of the vegetable fermentation industry to reduce waste chloride production. Low-salt fermentation procedures are currently being developed as a means to reduce the chloride waste for sauerkraut fermentations (Fleming et al., unpublished). These procedures may require the use of starter cultures because lower salt concentrations may not result in the production of high-quality sauerkraut (12).

Bacteriophages active against all major LAB genera have been isolated and characterized (1), including phages of dairy origin active against Lactobacillus, Lactococcus, and Streptococcus species. In contrast, phages infecting other species of lactic acid bacteria have received relatively little attention. To our knowledge, the phages active against Leuconostoc have been characterized as belonging to the Siphoviridae family (1). Phages active against Oenococcus oeni (formerly classified as Leuconostoc oenos) isolated from wine fermentations have also been classified as Siphoviridae (14).

The presence and ecology of bacteriophages in vegetable fermentations remain unexplored. Our objective was to identify bacteriophages in commercial cabbage fermentations, including one inoculated with a Leuconostoc mesenteroides starter culture.

Sauerkraut fermentations were carried out at a commercial processing plant and consisted of one inoculated and one uninoculated tank, both of 90-ton capacity. L. mesenteroides LA10 was used as a starter culture; cells were grown overnight at 30°C in 8 liters of MRS broth containing 1% NaCl. The culture (10^9 CFU/ml) was then diluted to 76 liters with tap water and sprayed (20 to 40 lb/in^2) onto the sliced, salted cabbage as it was being conveyed on a belt to the fermentation tank. This resulted in ~10^9 CFU/g of the culture being uniformly distributed in the fermentation tank. This fermentation was carried out with a final (equilibrated) NaCl concentration of 1.0 to 1.2%. The starter culture was used in an attempt to produce a normal fermentation under low-salt conditions.

An uninoculated tank, salted according to the normal industry procedure (equilibrated at 2.25% NaCl), was also sampled for phages. The fermentation temperature was not controlled, but the temperature in these commercial fermentations typically averages 18°C. Brine samples for the isolation of LAB and bacteriophage were obtained with a 1-cm-diameter, perforated stainless steel tube from a depth of ~60 cm from the tops of the fermentation tanks, about 60 cm from the sides, at the time intervals indicated below. Samples were transported on wet ice by overnight mail in 15-ml plastic screwcap tubes (430791; Corning Inc., Corning, N.Y.) from the commercial facility to our laboratory. Brine samples were refrigerated and processed within 24 h of arrival.

Selected LAB cultures were used as potential phage hosts: Pediococcus acidilactici LA74 (ATCC 33314), Lactobacillus confusus LA277 (NRRL B-1064), L. mesenteroides LA10 (C33), Leuconostoc sp. strain BI116 (4G12, unidentified species), L. mesenteroides LA112 (ATCC 10880), L. plantarum LA280 (KCCM 11322), and L. plantarum LA89. These cultures were obtained from the U.S. Department of Agriculture Agricultural Research Service (USDA-ARS) Food Science Research Unit culture collection (our lab, Raleigh, N.C.). In addition, LAB colonies were isolated directly from brine samples using spread plates with 0.1 ml of brine on MRS agar (Difco Laboratories, Detroit, Mich.) supplemented 0.02% sodium azide (MMRS, to prevent the growth of yeasts [9]). All...
cultures were incubated at 30°C during growth in the laboratory.

Identification of LAB isolates was carried out by a PCR method (6) based on the length of the 16S-23S rDNA intergenic transcribed spacer (ITS-PCR). Primers G1 and H1 (6) were used to amplify the spacer region. PCR products were separated on a 1.5-mm-thick 5% polyacrylamide gel using a vertical gel electrophoresis box (BRL). The PCR banding patterns shown in Fig. 1 indicated that the isolate from the control fermentation (LA296) was a Lactobacillus. All LAB isolates exhibited gas formation via heterofermentative metabolism during growth for 48 h at 30°C in 5 ml of MRS broth containing a Durham tube (Labsource, Chicago, Ill.).

Bacteriophages were isolated from the fermentation brine by centrifugation in 15-ml plastic screwcap tubes (Corning) at 3,000 × g for 10 min (Sorvall model RC5-B, with a SS-34 rotor; Sorvall Products, Newtown, Conn.) to remove suspended matter. Supernatant (0.1 ml) was added to 10 ml of MRS broth along with 0.1 ml of 1 M CaCl₂ (Sigma) and 0.5 ml of an early-log-phase culture of the host LAB, with an optical density of 0.3 to 0.4 (corresponding to approximately 10⁷ CFU/ml). After overnight incubation at 30°C, the samples were centrifuged (as above) and the supernatant (phage lysate) was filtered using 0.45-μm filters (Pall Corp., Ann Arbor, Mich.). The phage-containing filtrate (approximately 9 ml) was treated with 0.1 ml of chloroform (Sigma) and stored at 4°C. The phage lysate samples (0.1 ml) were mixed with 3 ml of soft agar (warmed to 50°C), 0.1 ml of 100 mM CaCl₂, and 0.1 ml of a mid-log culture of the cells and poured onto MRS agar plates. Plates were incubated overnight at 30°C for plaque assays (2).

Host strains for the initial phage isolation were identified by using the brine supernatant (described above) in a plaque assay with cycloheximide (50 μg/ml; Sigma) added to the soft agar in order to suppress yeast growth. All bacteriophage isolates were repurified using a single plaque from a plaque assay of the filtered lysate prior to further characterization.

Bacteriophages from the inoculated and uninoculated tanks are shown in Table 1. Phages active against the LA10 starter culture were present at greater than 10⁷ PFU/ml in the initial enrichment broths during the first 3 days after the start of the fermentation in the inoculated tank. Interestingly, phages active against the starter culture, L. mesenteroides LA10, were also isolated from the day 2 and 3 samples of the uninoculated fermentation, though at lower numbers after enrichment. Viable phages were isolated from the fermentation tanks over a range of pH values (from 5.6 to 3.5) for up to 21 days after the start of the fermentation, although most phages were recovered in the first 3 days, including the selected isolates Y4, Y5, Y10, R1, R2, and R3. Other phages selected for further characterization, phages Y11, Y12, and Y20, were isolated from day 7.

![FIG. 1. Strain identification by ITS-PCR products of LAB isolates from sauerkraut fermentation. Lane 1, L. plantarum LA280; lane 2, L. mesenteroides LA10; lane 3, Leuconostoc sp. strain BI116; lane 4, Leuconostoc sp. strain LA291; lane 5, Leuconostoc sp. strain LA292; lane 6, Leuconostoc sp. strain LA294; lane 7, Lactobacillus sp. strain LA296; lane 8, Leuconostoc sp. strain LA295; lane 9, Leuconostoc sp. strain LA293; M, molecular weight markers (100-bp DNA ladder).](http://aem.asm.org/)

<table>
<thead>
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<th>Time (days)</th>
<th>Tank pH</th>
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<th>Activity against selected host</th>
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- Elapsed time from the start of the fermentation.
- Inoculated with LA10.
- +++, above 300 PFU/plate; ++, 100 to 200 PFU/plate; +, under 100 PFU/plate; –, no plaques. LA74, Pediococcus acidilactici; LA277, Lactobacillus confusus; LA280, Lactobacillus plantarum; LA10, Leuconostoc mesenteroides; BI116, Leuconostoc species (unidentified).
The reported measurements represent the mean values determined for each group, followed by the standard deviation in parentheses. *Myoviridae* morphotypes are described in the text.
with EcoRI and KpnI gave distinct banding patterns on agarose gels for these five phages (data not shown). Prior to this work, all reported Leuconostoc phages have been identified as Siphoviridae, and phages R1 and R2 were characterized as Siphoviridae, group B of Bradley’s classification (3). Phage Y1 was characterized as Podoviridae, with a short or absent tail structure. This phage was isolated from kimchi, a Korean fermented cabbage product, in a previous study (16).

The burst size and latent period were determined for selected phages on the host used for primary isolation by a modification of the method of Ellis and Delbruck (7). Bacteriophages and the indicated mid-log host culture, at a multiplicity of infection between 0.05 and 0.1, were incubated for 10 min at 30°C. The phage-host suspension was diluted with 10 volumes of MRS, and cells were harvested by centrifugation and resuspended in 10 ml of fresh MRS for continued incubation. The latent period was determined as the time between challenge with phage and the initial rise in the initial phage titer). The latent period was determined as the time between challenge with phage and the initial rise in phage titer in the MRS broth.

The phage isolates exhibited a range of burst sizes from 11 ± 0 PFU/cell for phage Y5 to 74 ± 10 PFU/cell for phage Y20. The latent periods for these phages were 46 ± 1 min and 19 ± 2 min, respectively, for Y5 and Y20. The other bacteriophage isolates were within these ranges for burst size and latent period with the exception of phage Y4, active against the L. mesenteroides starter culture, which had a burst size of greater than 200 PFU/cell, and phage Y11, which had an eclipse phase of 1.5 h (data not shown).

Previous research with starter cultures for sauerkraut from our laboratory (5, 10) has shown that LAB starter cultures may significantly influence the microbial ecology of small-scale laboratory fermentations. In the commercial-scale fermentations, we found that bacteriophages active against the starter culture, L. mesenteroides LA10, were present in brine samples from the first day after inoculation. In this study, it was not possible to follow the growth or population changes of the unmarked starter culture in the inoculated commercial fermentation due to the naturally present Leuconostoc strains. Further research to determine the effects of bacteriophages on starter cultures in low-salt fermentations is suggested by this study.

To our knowledge, these experiments represent the first isolation and characterization of phages from a commercial vegetable fermentation. The diversity of phage species found in the two commercial sauerkraut fermentations indicates that phages may play a significant role in the ecology of this natural fermentation. Additional studies are forthcoming to fully characterize these phage species and the impact of these phages on the LAB present in vegetable fermentations.

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REFERENCES