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Analysis of *Lactobacillus* Phages and Bacteriocins in American Dairy Products and Characterization of a Phage Isolated from Yogurt

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Yogurt and acidophilus milk that contain *Lactobacillus acidophilus* could promote human health because *L. acidophilus* can inhibit enteric and food-borne microbial pathogens. To evaluate the stability of dairy *L. acidophilus* cultures, we studied whether some dairy lactobacilli could be inhibited by phages or bacteriocins released by other dairy lactobacilli. From 20 yogurts and two acidophilus milks purchased at local food markets, 38 *Lactobacillus* strains were isolated. Eight *Lactobacillus* type strains were used as controls. With mitomycin induction and agar spot assay, phages and bacteriocins were isolated from these strains and their activities were analyzed. *Lactobacillus* strains from 11 yogurts released phages, while the strains from most of the remaining products released bacteriocins. One phage, designated ϕy8, was characterized. It was spontaneously released from its host strain, *L. acidophilus* Y8, at a rate of about 10^4/ml. This phage lysed nine other dairy *Lactobacillus* strains tested. It had a burst size of 100, an elongated prolate head of 39 by 130 nm, a long, flexible but noncontractile tail of 300 nm, and a 54.3-kb linear double-stranded DNA. DNA fingerprinting analysis indicated that *L. acidophilus* phages of nine yogurts in this study belonged to the same type as ϕy8. Although they may be sensitive to bacteriocins, all lysogens resisted further phage attacks, whereas most nonlysogens were sensitive to both phages and bacteriocins. Therefore, *Lactobacillus* cultures of some American yogurts and acidophilus milks may be unstable or unsafe because they can either be inhibited by phages or bacteriocins or release them to inhibit lactobacilli of other dairy products.

Dairy lactobacilli are widely used to process yogurt and acidophilus milk. Whereas lactobacilli serve as starter cultures to ferment milk into yogurt, these bacteria are simply additives in acidophilus milk, with the specific aim of increasing its presumptive health value (9, 18). Because viable lactobacilli can inhibit food-borne and enteric pathogenic microorganisms by producing lactic acid and other antimicrobial substances (3, 5, 10, 23), yogurt and acidophilus milk have been considered to be healthy probiotic diets. Since Metchnikov suggested the positive role of dairy lactobacilli in human health nearly a century ago (24), various brands of yogurt have been ingested as prophylaxis or as treatment for common intestinal infections, such as diarrhea (3, 27), and even for vaginal bacterial (11) and yeast (6, 13) infections. Therefore, in addition to being nutritious and delicious foods, yogurt and acidophilus milk may also promote human health by inhibiting common microbial pathogens.

The starter culture of a traditional yogurt normally includes a combination of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, which collaborate synergistically to ferment milk into yogurt. However, unlike *Lactobacillus acidophilus*, *L. delbrueckii* subsp. *bulgaricus* is not a species indigenous to humans and cannot colonize the intestine to promote human health (9). Therefore, to improve a yogurt's health value, the traditional *L. delbrueckii* subsp. *bulgaricus* culture in many yogurt products has been replaced by or combined with *L. acidophilus*, the species most commonly found in the intestine and the vagina of humans (6, 9–13, 16, 27). In European countries, yogurt made in this manner is specifically referred to as bio-yogurt or biogurt, while most yogurts in today's American food market have been made or incorporated with *L. acidophilus* since the 1970s (12). The yogurt products specifically labelled with "made with *Lactobacillus acidophilus*" include Dannon, Yoplait, and Colombo, which are all major brands of yogurt sold in the American food market.

Although the effectiveness of ingesting yogurt to treat common infections has been questioned (14), a particular brand of yogurt was successful in treating vaginal yeast infection in women during the years 1989 to 1991 (6, 13). This yogurt had a special *L. acidophilus* strain that could colonize the vagina, presumably through the fecal-urogenital passage, to inhibit yeast infection. The success of this yogurt could have been a major breakthrough in the probiotic food industry because yeast infections occur so commonly among women (17). Unfortunately, the yogurt currently no longer has the same anti-yeast *L. acidophilus* strain, so it is useless in treating yeast infections in women. Therefore, the stability of the dairy *L. acidophilus* cultures is important for maintaining not only the quality and flavor but also the health value of the probiotic food product.

It is impossible now to find the exact cause for the disappearance of the previous *L. acidophilus* strain in this particular brand of yogurt because its starter culture has been changed. Since it would be unthinkable for the manufacturer of the starter culture to delete such a profitable strain, its disappearance might have been a result of a natural cause, such as a lytic phage outbreak. Therefore, it would be of interest to identify whether the current *L. acidophilus* strain from the same brand of yogurt releases a phage that is lytic to other *L. acidophilus* strains. If it does, it would support the explanation that the virulent phage released by the successor strain might have eradicated its predecessor.

Since the first phage that attacks dairy lactobacilli was isolated 60 years ago from sewage water in New York City (19),

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TABLE 1. *Lactobacillus* dairy products analyzed in this study

Product	Manufacturer	Location
Acidophilus milk		
Anderson Erickson[a]	Anderson Erickson Dairy Co.	Des Moines, Iowa
Fairmont-Zarda[a]	Fairmont-Zarda Div. Roberts Dairy Co.	Omaha, Nebr.
Yogurt		
Alta Dena[a]	Alta Dena Certified Dairy, Inc.	Noustry, Calif.
Always Save	Associated Wholesale Grocers, Inc.	Kansas City, Kans.
Anderson Erickson	Anderson Erickson Dairy Co.	Des Moines, Iowa
Belfonte	Belfonte Ice Cream Co.	Kansas City, Mo.
Best Choice Life	Associated Wholesale Grocers, Inc.	Kansas City, Kans.
Breyers	Kraft General Foods, Inc.	Glenview, Ill.
Cascadefresh[a]	Cascadefresh, Inc.	Seattle, Wash.
Colombo[a]	Colombo, Inc.	Minneapolis, Minn.
Dannon[a]	Dannon	Jacksonville, Fla.
Dillons	Dillon's Store Div. of Dillon Co., Inc.	Hutchinson, Kans.
Fairmont-Zarda	Fairmont-Zarda Dairy Co.	Kansas City, Mo.
Great Value[a]	Wal*Mart Stores, Inc.	Bentonville, Ark.
Horizon Organic[a]	Natural Horizon, Inc.	Boulder, Colo.
Mountain High[a]	Mountain High, Inc.	Englewood, Colo.
Schnucks Lite	Schnuck Market, Inc.	St. Louis, Mo.
TCBY	Polytainers, Inc.	Little Rock, Ark.
Weight Watcher[a]	Weight Watcher International, Inc.	Pittsburgh, Pa.
Wells' Blue Bunny	Wells' Dairy, Inc.	LeMars, Iowa
Yonson	Favorite Foods, Inc.	Fullerton, Calif.
Yoplait[a]	Yoplait USA, Inc.	Minneapolis, Minn.

[a] Claimed to be made with live *L. acidophilus* culture.

many *Lactobacillus* phages have been isolated from traditional yogurt starter cultures, namely, from *L. delbrueckii* subsp. *bulgaricus* strains (4, 21), and from other fermented food starter cultures (25, 28). However, few data are available about phages and bacteriocins of *L. acidophilus* cultures in the yogurts and acidophilus milks sold in today's United States food market. To improve the stability of dairy *Lactobacillus* starter cultures, it is important to test whether some dairy *Lactobacillus* cultures release virulent phages or potent bacteriocins that may attack other dairy *Lactobacillus* strains. In this article, we present data about phage and bacteriocin activities of various dairy *Lactobacillus* cultures in yogurts and acidophilus milks sold in the American food market and the isolation and characterization of a phage from the particular brand of yogurt which was previously used to treat women's yeast infections.

MATERIALS AND METHODS

Bacterial strains, isolation, and culture condition. Two acidophilus milks and 20 yogurts were purchased at local grocery stores. The brand names, the manufacturers, and their locations are listed in Table 1. To isolate *Lactobacillus* strains from these products, a loopful of each product was first streaked on the selective *Lactobacillus* Rogosa SL agar (pH 5.2; Difco, Detroit, Mich.) and incubated in a candle jar at 37°C for 48 h. Each isolate from the Rogosa SL agar was further confirmed to be a *Lactobacillus* sp. by its rod cell morphology, gram-positive stain, and catalase-negative reaction. *Lactobacillus* species of these isolates were identified by comparing their sugar fermentation patterns with the scheme described in *Bergey's Manual* (16). After *Lactobacillus* strains were isolated from these dairy products, the *Lactobacillus* MRS medium (Difco) was used for routine bacterial culturing unless otherwise indicated. All isolated *Lactobacillus* cultures were maintained at −70°C in MRS broth with 10% glycerol. The control *Lactobacillus* type strains and the designations (C1 to C8) used in this study are listed in Table 2.

Phage and bacteriocin induction. Both phages and bacteriocins of these strains were induced by mitomycin (2). Briefly, 0.1 ml of overnight *Lactobacillus* culture in MRS broth supplemented with 10 mM $CaCl_2$ (MRS-C) was transferred into 10 ml of prewarmed fresh MRS-C broth. After about 3 h, the culture was divided equally into two test tubes. One tube was used as a control, and the other had mitomycin (Sigma Chemical Co., St. Louis, Mo.) added at a final concentration of 0.2 μg/ml. The induction of *Lactobacillus* phages or bacteriocins was indicated by a clear lysis of the turbid culture 5 to 7 h after the addition of mitomycin. The lysates were centrifuged and filtered to remove unlysed cells and maintained at 4°C with a drop of chloroform. Phages were differentiated from bacteriocins by the following procedures: (i) phage plaque assay, (ii) phage DNA isolation with the Qiagen (Chatsworth, Calif.) kit, (iii) DNA hybridization (22) of *Lactobacillus* chromosomal DNA with a nonradioactive biotin-labelled phage DNA probe (Life Technologies, Gaithersburg, Md.), and (iv) phage observation under an electron microscope. If no phages were identified from mitomycin-induced culture lysates, the inhibitory effect on indicator strains was then analyzed for its sensitivity to heat, proteolytic enzymes (trypsin, pepsin, and protease; Sigma), catalase (Sigma), and neutralization of pH to identify the presence of bacteriocin and to rule out the possibility of inhibition due to hydrogen peroxide and lactic acid.

Phage infection and bacteriocin inhibition assay. The agar spot assay for phage infection or bacteriocin inhibition was performed as follows: (i) 200 μl of each dairy *Lactobacillus* culture at the early exponential growth phase in MRS-C broth (optical density at 600 nm, 0.2 to 0.3) was mixed with 4 ml of MRS-C soft agar (0.6% agar; 48°C) and poured onto an MRS-C agar plate, (ii) 1 μl of each lysate was dropped onto the solidified soft agar, and (iii) the plates were incubated for 24 h at 37°C. A phage infection or bacteriocin inhibition was indicated by a clear lysis zone in the soft agar layer.

Phage propagation and titration. The phage ϕy8 isolated from *L. acidophilus* Y8 from a particular brand of yogurt by the mitomycin induction was propagated and the titer was determined with the indicator strain *Lactobacillus delbrueckii* subsp. *lactis* ATCC 15808 (C8). The phage lysate (0.1 ml) with series dilutions was added to 1 ml of mid-log-phase *L. delbrueckii* subsp. *lactis* ATCC 15808 culture in MRS-C medium and incubated at 37°C for 15 min to allow phage adsorption. Four milliliters of MRS-C soft agar at 48°C was mixed with the phage-infected cells before being poured onto an MRS agar plate. Plaques were counted after 24 h of incubation at 37°C.

For large-scale phage production, 2 liters of prewarmed MRS-C broth was inoculated with 10 ml of overnight ATCC 15808 culture. The cells were grown at

TABLE 2. Control *Lactobacillus* type strains used in this study

Strain[a]	Designation code
Lactobacillus delbrueckii subsp. *bulgaricus* NCDO 1489	C1
Lactobacillus delbrueckii subsp. *bulgaricus* ATCC 11842	C2
Lactobacillus delbrueckii subsp. *bulgaricus* ATCC 27558	C3
Lactobacillus delbrueckii subsp. *delbrueckii* ATCC 9649	C4
Lactobacillus helveticus NCDO 87	C5
Lactobacillus helveticus NCDO 2395	C6
Lactobacillus helveticus ATCC 15009	C7
Lactobacillus delbrueckii subsp. *lactis* ATCC 15808	C8

[a] ATCC, American Type Culture Collection; NCDO, National Collection of Dairy Organisms (Reading, England).

TABLE 3. Sensitivity of *Lactobacillus* strains to phages and bacteriocins of other dairy products

Strain source[a]	Sensitivity of strain to:																				
	Phage source											Bacteriocin source[b]									
	Y1	Y2	Y5	Y8	Y10	Y11	Y12a	Y13	Y15	Y16	Y19	A1	A2	Y3	Y6	Y9	Y12b	Y14	Y18	Y20	C1	C6
Lysogens																						
Y1	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+	+	+	−	−	+	−
Y2	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	+	−	−	−	+	−
Y5	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	+	−	−	−	+	−
Y8	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	+	−	−	−	+	−
Y10	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	+	−	−	−	+	−
Y11	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	+	−	−	−	+	−
Y12a	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	+	−	−	−	+	−
Y13	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	+	−	−	−	+	−
Y15	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	+	−	−	−	−	−
Y16	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	+	−	−	−	+	−
Y19	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	+	−	−	−	+	−
Nonlysogens																						
A1	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	+
A2	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	+
Y4	−	−	−	+	−	+	−	−	−	−	−	+	+	−	−	+	+	+	+	+	+	+
Y6	+	+	+	+	−	+	+	−	−	−	−	+	+	+	−	−	+	−	−	−	+	+
Y7	+	+	+	+	−	+	+	+	−	+	−	+	+	−	−	−	+	−	−	−	+	+
Y9	+	+	+	+	+	+	+	−	−	+	−	+	+	−	−	−	+	−	−	−	−	+
Y12b	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+
Y14	+	−	−	+	−	+	+	−	−	−	−	+	+	+	−	−	+	−	−	−	+	+
Y18	+	+	+	+	+	+	+	+	−	+	−	+	+	−	−	−	+	−	−	−	+	+
Y20	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−	+	+
Controls																						
C4	+	+	+	+	+	+	+	+	−	+	−	+	+	−	+	+	−	−	−	−	−	+
C8	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	−	+	−	−	−	−	+

[a] Strains Y3, Y17, C1, C2, C3, C5, C6, and C7 resisted all phages but were sensitive to bacteriocins A1, A2, and Y12b.
[b] Bacteriocin of C2 attacked only Y18; bacteriocin of C8 attacked only Y20.

37°C to an optical density at 600 nm of 0.2 to 0.3 and infected with the phage φy8 at a multiplicity of infection of 0.01. The infected culture was further incubated until lysis occurred. Chloroform (3 ml) was added to the lysate to ensure complete lysis. After removing bacterial cell debris and chloroform by centrifugation, the phage particles were precipitated at 4°C with 10% (wt/vol) polyethylene glycol 8000 and 0.5 M NaCl, collected by centrifugation at 10,000 × *g* for 15 min, and resuspended in phage buffer (100 mM Tris, 10 mM $MgCl_2$ [pH 8.0]). The high-titer (10^{11} PFU/ml) phage lysate was subsequently purified by differential centrifugation (2).

Phage DNA analysis. A high-titer phage lysate (10^{11} PFU/ml) was used for DNA isolation with the Qiagen lambda phage DNA isolation kit as described in the manufacturer's instructions. The purified phage DNA was subsequently analyzed with restriction enzyme digestion (Promega, Madison, Wis.). The digested phage DNA samples were subjected to gel electrophoresis on an 0.8% agarose gel at 40 V for 3 h (22). The gel was stained with ethidium bromide and photographed under a UV light.

One-step growth curve. *L. delbrueckii* subsp. *lactis* ATCC 15808 was grown at 37°C in MRS-C broth until the mid-exponential phase. To 1 ml of this culture, 0.1 ml of φy8 lysate (10^8 PFU/ml; multiplicity of infection, 0.1) was added and mixed. Fifteen minutes were allowed for adsorption at 37°C. After adsorption, 0.5 ml of the phage-infected cell culture was added to 4.5 ml of prewarmed MRS-C medium, and the mixture was incubated for 3 h at 37°C. One-tenth milliliter of culture was then removed at 15-min intervals, and a series of dilutions was made. Each diluted culture was added to 4 ml of melted soft MRS-C agar at 48°C and rapidly mixed, and the mixture was poured onto an MRS plate and incubated at 37°C. Plaques were numerated after 24 h of incubation.

Spontaneous phage induction. One milliliter of mid-log-phase culture of *L. acidophilus* Y8 grown in MRS-C at 37°C was centrifuged to remove bacterial cells. The supernatant, which contained spontaneously released phage particles, was filtered through a sterile 0.2-μm-pore-size filter before it was used to infect the indicator strain *L. delbrueckii* subsp. *lactis* ATCC 15808 by the soft agar overlay method described above. Plaques were counted after 24 h of incubation at 37°C. The burst rate of *L. acidophilus* Y8 due to spontaneous induction of the prophage φy8 was determined by the number of total spontaneously released phages being divided by its burst size and the density of the host cells in the culture.

Electron microscopy. One drop of purified phage φy8 in 0.1 M ammonium acetate (pH 7.0) was spotted on grids with a carbon-coated Formvar film (Ladd Research Industries, Inc., Burlington, Vt.). After drying for 1 min, the sample was negatively stained with 2% uranyl acetate (pH 4.2). Electron microscopy was performed at 80 kV with a CM12 transmission electron microscope (Philips Electronic Instruments, Inc., Mahwah, N.J.).

RESULTS

Isolation and identification of *Lactobacillus* strains. Among 22 dairy products, 38 *Lactobacillus* strains were isolated. On the basis of their sugar fermentation patterns (16), sensitivity patterns to a collection of dairy *Lactobacillus* phages and bac-

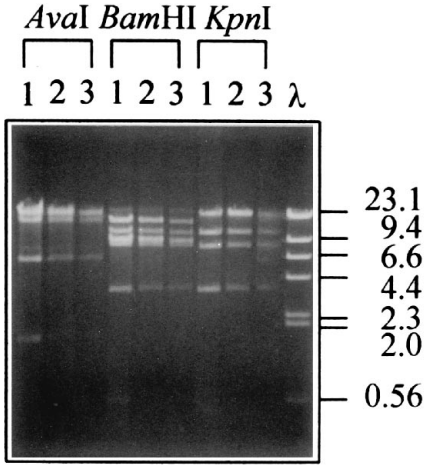

FIG. 1. Phage DNA fingerprinting analysis. Lanes: 1, phage DNA from φy13; 2, phage DNA from φy1; 3, phage DNA from φy8; λ, λ phage DNA digested with *Hin*dIII as molecular size standards (in kilobase pairs).

TABLE 4. Restriction fragment of ϕy8 phage genomic DNA

Restriction fragment	Fragment sizes (kbp)[a]	Total size (kbp)
*Ava*I	27.6, 18.3, 6.7, 1.6	54.2
*Bam*HI	18.6, 12.9, 9.7, 8.6, 3.6, 0.6	54.0
*Kpn*I	28.8, 13.6, 8.3, 3.7	54.4
*Sac*I	30.1, 17.8, 6.6	54.5
*Sal*I	45.0, (5.1), 4.2, 3.3, 1.8	54.3
*Xba*I	20.1, 17.8, 13.7, (2.7), 1.5, 1.2	54.3

[a] Values in parentheses indicate that a fragment of this size was dissociated by heat treatment.

teriocins, plasmid profiles, and phage content, the isolates were tentatively identified as the following species: *L. acidophilus* (23 strains in two groups, namely, group A, 10 strains with a 6-kb cryptic plasmid, and group B, 13 strains without a plasmid), *L. delbrueckii* subsp. *bulgaricus* (13 strains), and *L. delbrueckii* subsp. *lactis* (2 strains). The strains isolated from the two acidophilus milks (Table 1) were designated A1 and A2, while the strains isolated from the 20 yogurts were designated Y1 to Y20. To protect the reputations of these commercial dairy products, their brand names are not specifically correlated with the designated codes.

Phage and bacteriocin activities. With the agar spot assay, a collection of 46 mitomycin-induced lysates (8 from control type strains) was used to cross-react with the same 46 *Lactobacillus* strains. By phage plaque assay and by DNA hybridization with the labelled DNA of phage ϕy8 isolated from a particular brand of yogurt, 11 yogurt strains were identified as containing phages. These strains were Y1, Y2, Y5, Y8, Y10, Y11, Y12a, Y13, Y15, Y16, and Y19. Except for Y15, which was identified as *L. delbrueckii* subsp. *lactis*, all of the remaining lysogens belonged to *L. acidophilus* group A. The identification of most of these phages was performed by the direct plaque assay with the indicator strain *L. delbrueckii* subsp. *lactis* ATCC 15808. Fourteen strains displayed bacteriocin-like activities. Among them, bacteriocins of A1, A2, and Y12b remained active after boiling at 100°C for 10 min. All of the bacteriocins were sensitive to proteolytic enzymes, including trypsin, pepsin, and protease. Neither treatment with catalase nor adjustment of pH to neutral affected their activity, ruling out possible antibacterial effects due to H_2O_2 and lactic acid. The host ranges of these phages and antibiotic spectra of these bacteriocins are shown in Table 3. Clearly, all of the lysogens resisted further phage infections, indicating the presence of the prophage-encoded superinfection immunity (15).

Plasmid profile and phage DNA fingerprinting. Because the plasmid profile of each bacterium is one of the genetic characteristics used to identify its bacterial strain and species, all of the *Lactobacillus* isolates in our collection were screened for the presence of plasmids (20). A cryptic plasmid of about 6 kb was isolated from all 10 lysogens that belonged to the same *L. acidophilus* species group (group A). Among all strains that contained the plasmid, only Y14 did not display phage activity or we did not have an appropriate indicator strain. DNA fingerprinting analysis and DNA hybridization of these phages indicated that 9 of the 11 phages were genetically identical, although some of them had slightly different host ranges (Table 3). A DNA fingerprinting analysis of three of these identical phages is shown in Fig. 1. The two different phages were ϕy12a and ϕy15.

Analysis of ϕy8 genomic DNA. Because a particular brand of yogurt which was used to treat yeast infection in women has changed its starter *L. acidophilus* strain probably as a result of phage attack, we focused our efforts on studying its current strain, *L. acidophilus* Y8, and its phage, ϕy8. The size of the phage ϕy8 genome, 54.3 ± 0.3 kb, was estimated by averaging the sums of the lengths of restriction fragments generated by each restriction enzyme (Table 4). The restriction map of the ϕy8 genome was constructed for enzymes *Ava*I, *Bam*HI, *Kpn*I, *Sac*I, *Sal*I, and *Xba*I and is shown in Fig. 2. Digestion with four additional restriction enzymes, *Bgl*II, *Cla*I, *Eco*RI, and *Sph*I, was also performed to calculate the genome size. Additionally, the phage genome was digested by combinations of two enzymes or by partial digestion of a single enzyme. Some specific fragments were isolated from the agarose gel for digestion with a second enzyme and for hybridization with other restriction fragments to determine the order of the restriction fragments and the end fragments of the phage genome. The agarose gel electrophoresis, which allows discrimination among covalently closed supercoiled circular, nick-relaxed circular, and linear DNA molecules, showed only one distinct band of undigested phage ϕy8 DNA (data not shown). This indicated that the phage genome was a double-stranded linear DNA. The cohesive ends (*cos* sites) were determined by the presence of heat-dissociable DNA fragments. The restriction enzyme-digested phage DNA samples were divided into two parts: one part was held in ice, while the other part was heated to 70°C for 10 min. The two parts were then subjected to agarose gel electrophoresis. A 5.1-kb *Sal*I fragment was dissociated into two fragments (3.3 and 1.8 kb), and a 2.7-kb *Xba*I fragment was also dissociated into two fragments (1.5 and 1.2 kb) (Table 4). This result helped locate the cohesive ends in the phage DNA.

Physiology and morphology of ϕy8. The infection cycle of phage ϕy8 was characterized by its one-step growth curve kinetics (Fig. 3). Data obtained from two independent experiments indicated a latent period of 30 min and a rise period of 30 to 40 min. The burst size was about 100 phages per cell. Sensitivities of ϕy8 to heat and chloroform were tested with the crude, bacterium-free broth lysate. The heat sensitivity of ϕy8 was determined at 40 to 70°C. The same phage lysate was extracted twice with equal volumes of chloroform and separated by centrifugation. The heat- and chloroform-treated phage lysate was diluted and used to infect the indicator strain ATCC 15808. Results were expressed as the percent survival of the phage. Heat treatment of ϕy8 at 40 and 50°C for at least 30 min did not have a significant effect on its infectivity. Only 20% of the phages survived at 60°C for 10 min, while none survived at 70°C for 5 min. However, ϕy8 was able to tolerate chloroform without loss of its infectivity. The stability of ϕy8 in the presence of chloroform suggested that it did not contain a significant amount of structural lipids (2). The amount of spontaneously released ϕy8 phages in the supernatant of a mid-exponential-phase *L. acidophilus* culture was about 10^4/ml. By dividing the burst size of ϕy8 and the cell density (10^8 cells per ml), the burst rate of *L. acidophilus* Y8 due to spontaneous

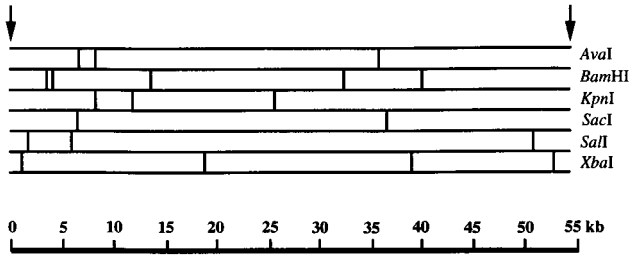

FIG. 2. Restriction map of ϕy8 phage genome. The *cos* sites are indicated by arrows.

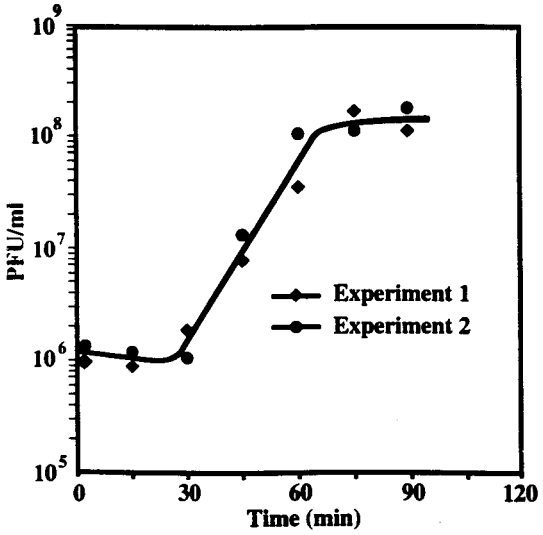

FIG. 3. One-step growth curve of φy8.

induction of the prophage φy8 was determined to be 10^{-6}. Other lysogens also displayed a similar burst rate as a result of spontaneous prophage induction (data not shown). Although growth of the host strain at either 37 or 43°C did not have a significant effect on the rate of its spontaneous induction of φy8, at 43°C the cells released phages earlier. However, the highest induction rate under both conditions was found at the mid-log growth phase.

The plaque of φy8 with *L. delbrueckii* subsp. *lactis* ATCC 15808 as an indicator strain was large, clear, and round with a diameter of 1 to 2 mm. Electron microscopy of φy8 (Fig. 4) revealed an elongated prolate head (39 by 130 nm) and a long (300-nm), flexible, but noncontractile tail. On the basis of this morphology, φy8 belongs taxonomically to the *Siphoviridae* family (8), and in Ackermann's morphological classification, it belongs to group B3 (1).

DISCUSSION

Yogurt and acidophilus milk that contain a large number of live *L. acidophilus* cells may be important probiotic foods because *L. acidophilus* can inhibit enteric and food-borne microbial pathogens and presumably promote human health (5, 6, 9–13, 16, 27). However, as evidenced by the loss of the previous antiyeast *L. acidophilus* strain in a particular brand of yogurt (6, 13), the ability to ensure the stability of ideal *L. acidophilus* cultures in these presumptive probiotic products is very important. Therefore, we studied whether some *Lactobacillus* strains from yogurts and acidophilus milks sold in the American food market could be inhibited by phages or bacteriocins released by other dairy lactobacilli. Among 20 yogurt products, 11 were found to contain lysogens that spontaneously released a large number of virulent phages that could attack *Lactobacillus* starter cultures of other yogurts. This indicated that phages could be a factor which inhibits yogurt starter cultures.

The *L. acidophilus* strain, Y8, isolated from the current particular brand of yogurt was found to be a lysogen that spontaneously released a phage, φy8, with a titer about 10^4 PFU/ml in a mid-exponential-phase culture (about 10^8 cells per ml). Because the number of viable *L. acidophilus* cells in this yogurt is more than 10^8/ml (13), the amount of phages released in yogurt can be more than 10^4/ml. The phage φy8 was lytic to nine other dairy strains, and no lysogens could be isolated among the φy8-attacked sensitive *Lactobacillus* cultures. It is unknown why the temperate phage φy8 was lytic to other dairy *Lactobacillus* strains. Because all lysogens except *L. delbrueckii* subsp. *lactis* Y15 belonged to *L. acidophilus* species group A, while the nonlysogens belonged to either *L. acidophilus* species group B or *L. delbrueckii* subsp. *bulgaricus*, certain host background differences, such as the compatibility between a phage and its integration site in the host chromosome (4, 21), may determine whether a phage-sensitive bacterium can be lysogenized by a particular phage. However, even though the current starter culture of the particular brand of yogurt was identified as releasing a virulent phage, it is now impossible to confirm whether this phage was responsible for the loss of its previous yeast-inhibiting *L. acidophilus* strain because the original strain was unavailable for testing. Nonetheless, the fact that this yogurt as well as 10 other ones spontaneously releases a large number of virulent phages creates a great concern about the safety and stability of the *L. acidophilus* cultures of these yogurts.

The morphology of the yogurt phage φy8 is unusual, namely, a combination of an elongated prolate head and a long tail (Fig. 4). Although two similar prolate-headed phages, JCL 1032 (7) and 0235 (26), have been isolated from *L. delbrueckii* subsp. *lactis*, φy8 has an even longer head, tail, and genomic DNA (Table 5). Furthermore, φy8 is isolated from a different *Lactobacillus* species, *L. acidophilus*. Therefore, φy8 is most likely a new phage. This phage also represents a major type of phages that affect American yogurts because among 11 phages isolated, 9 belonged to the same type as φy8 by DNA fingerprinting analysis.

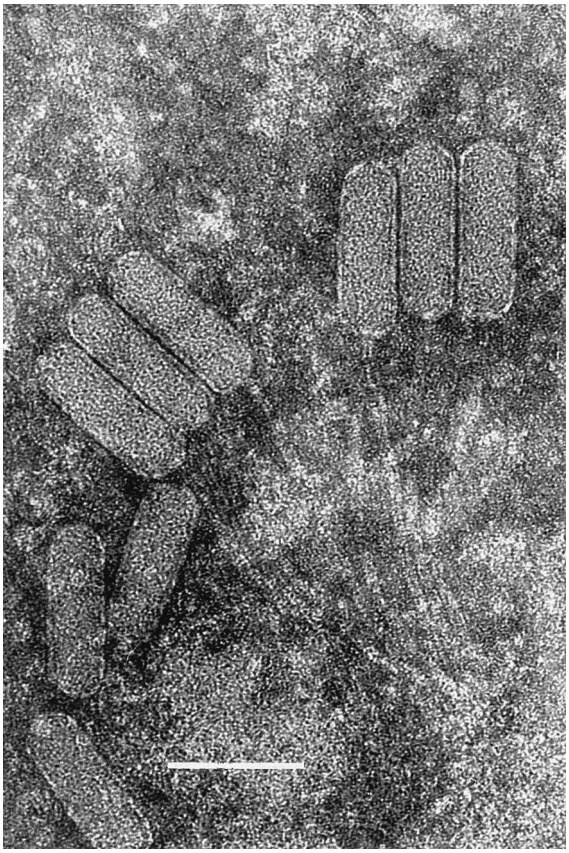

FIG. 4. Electron micrograph of negatively stained phage φy8. Bar, 100 nm.

TABLE 5. Comparison of three prolate-headed *Lactobacillus* phages identified to date

Phage	Host strain	Genome size (kb)	Head length × width (nm)	Tail length (nm)	Cross bar[a]	Reference
JCL 1032	*L. delbrueckii* subsp. *lactis*	45.8	120 × 40	270	Yes	7
0235	*L. delbrueckii* subsp. *lactis* CNRZ235	Unknown	125 × 50	230	No	26
ϕy8	*L. acidophilus* Y8	54.3	130 × 39	300	No	This study

[a] Cross bars along the tail.

Bacteriocin-producing dairy lactobacilli can prevent contamination by other nonsignificant *Lactobacillus* strains and can inhibit pathogenic microorganisms (10, 23). Therefore, these lactobacilli have been considered advantageous both in maintaining the purity of dairy cultures and in promoting health in humans. As a result, bacteriocin-producing strains have been selected by certain dairy manufacturing plants to be used in their products (27). However, we observed that bacteriocin-producing *Lactobacillus* strains might be sensitive to phage attacks. As shown in Table 3, nonlysogenic bacteriocin producers were sensitive to both phages and other bacteriocins, whereas lysogens were sensitive only to bacteriocins because of their immunity to infection by the same type of phages. It is unknown why none of the bacteriocin producers formed lysogens. Because phages and bacteriocins are remarkably similar in their induction mechanisms, their morphologies, and their lytic processes (2), the bacteriocin producers might be incompatible with phages and thus unable to survive phage attacks by forming lysogens. Therefore, a bacteriocin-producing strain in a dairy starter culture could be unstable because it can be eliminated by a phage.

While both phages and bacteriocins may inhibit sensitive dairy lactobacilli, phages normally have a narrower host range than bacteriocins. However, a lytic phage may cause greater damage to dairy starter cultures. Once a sensitive *Lactobacillus* culture encounters a virulent phage, the phage can be rapidly reproduced in the culture, releasing millions of new phages that can soon eliminate the entire sensitive strain. Conversely, an added or contaminated bacteriocin-producing *Lactobacillus* strain may cause only a limited or a slow, adverse effect on the preexistent starter strain because bacteriocins cannot be reproduced by target cells and can kill target cells only upon direct contact. Therefore, phage-releasing lysogens can be more virulent than bacteriocin producers in attacking other dairy *Lactobacillus* strains.

The risk of a *Lactobacillus* starter culture being attacked by phages may be evaluated by referring to the data presented in Table 3. First, if a dairy starter culture is already a lysogen, it may be immune from further infection by the same type of phages, but the culture itself may be a source of an infective phage and thus be hazardous. Second, if a culture is a bacteriocin producer but a nonlysogen, this culture does not release phages, but it may be sensitive to phage attacks. Therefore, to completely prevent phage attacks in a yogurt *L. acidophilus* starter culture and to ensure the safety of a yogurt product, it is important to isolate or develop ideal dairy *L. acidophilus* strains that are both phage free and phage resistant. Further studies about the newly characterized *L. acidophilus* phage ϕy8 and its interaction with host strains may help explain the phage virulence and the phage resistance mechanisms of *L. acidophilus*.

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