

Characterization of *Lactobacillus bulgaricus* Bacteriophage ch2

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Bacteriophage ch2, a virulent bacteriophage of *Lactobacillus bulgaricus* CH2, was characterized according to its morphology, genome size, structural proteins, and growth kinetics. Electron micrographs revealed that bacteriophage ch2 has an icosahedral head of 50-nm diameter and a long tail of 170 nm. Its genome is linear and 35 kilobases in length, and its structural proteins consist of two major and eight minor proteins. One-step growth kinetics of bacteriophage ch2 under optimal conditions (45°C in MRS medium [Oxoid Ltd.]) showed that the latent time was 40 min, the rise period was 15 min, and the burst size was 130 bacteriophages per cell. To monitor the effects of bacteriophage infection on host growth and β -galactosidase production, the absorbance of the culture and the β -galactosidase activity were followed during the infection cycle. Before lysis the infected culture continued to grow and produce β -galactosidase at the same rate as the uninfected culture.

Bacteriophage contamination is an important problem confronted by all industries in which microorganisms are used in the manufacturing process. Dairy fermentation is one industry which is affected by this problem. Lactic acid bacteria are used in the production of numerous varieties of cheese and yogurt as well as other food products. Bacteriophage infection of the starter culture is a common occurrence. This problem has been partially contained by using bacteriophage-resistant cultures and mixed-strain starter cultures (15).

Although bacteriophage contamination is not a devastating problem in the dairy industry, the study of these bacteriophages and their interaction with lactic acid bacteria will lead to a further understanding of this group of microorganisms. Despite the economic importance of these microorganisms, detailed genetic analysis of most of them has not been pursued. This lack of knowledge of the genetics of lactic acid bacteria has impeded advances in strain improvement through modern techniques in genetic manipulation. Among the bacteriophages that have been found to infect lactic acid bacteria, those specific for lactic streptococci have been investigated most extensively. Much less information is available on the bacteriophages of the thermophilic starters such as lactobacilli.

The present study focused on the characterization of bacteriophage ch2, a virulent bacteriophage of *Lactobacillus bulgaricus* CH2, originally isolated from the whey of Swiss cheese (1). We studied the basic properties of this bacteriophage, including its morphology, DNA, structural proteins, burst size, and latent period, and the effect of bacteriophage infection on host growth and β -galactosidase production.

MATERIALS AND METHODS

Bacterial strain and bacteriophage. *L. bulgaricus* CH2 and its homospecific bacteriophage ch2 were obtained from W. Sandine (Oregon State University). Frozen stock cultures of *L. bulgaricus* CH2 were maintained at -70°C in MRS broth (Oxoid Ltd.) with 15% glycerol. For inoculum preparation, frozen cultures were subcultured twice in litmus milk, fol-

lowed by transfer into MRS broth. All cultures were incubated at 45°C.

Media. MRS medium and litmus milk (Difco Laboratories) were prepared according to the manufacturers' directions and supplemented with 2% agar for agar plates.

Propagation and purification of bacteriophage. Liquid stocks of bacteriophage ch2 were made by infecting early-exponential-phase *L. bulgaricus* CH2 cells grown in MRS medium at 45°C at a multiplicity of infection of approximately 0.01 bacteriophage per cell. The infected culture was incubated at 45°C for 3 h, which resulted in total lysis of the culture. Cell debris was removed by centrifugation at 5,000 $\times g$ for 10 min. The bacteriophages were precipitated at 4°C in the presence of polyethylene glycol 6000 (9%, wt/vol) and NaCl (0.5 M) (18). The precipitated bacteriophages were collected by centrifugation at 7,800 $\times g$ for 15 min and suspended in 100 mM Tris (pH 8.0)-10 mM MgCl₂. DNase I (Sigma Chemical Co.) and RNase A (Sigma) were added to a final concentration of 1 μ g/ml and incubated for 30 min at room temperature. The bacteriophages were subsequently purified by equilibrium centrifugation in CsCl (density, 1.45 g/cm³). After centrifugation at 120,000 $\times g$ for 18 h with a Beckman Vti 50 rotor and Beckman L5-75 ultracentrifuge, the band of bacteriophage particles was removed and dialyzed in 1 liter of buffer containing 10 mM NaCl, 50 mM Tris hydrochloride (pH 8.0), and 10 mM MgCl₂. The purified bacteriophages were stored at 4°C.

Bacteriophage titering. Bacteriophage titers were determined by a modified version of the overlay method of Adams (2). A 0.1-ml bacteriophage sample was added to 0.5 ml of early-exponential-phase *L. bulgaricus* CH2 culture in MRS with 10 mM CaCl₂. This mixture was incubated at 45°C for 5 min to allow for adsorption, 10 ml of MRS soft agar (0.85% agar) containing 10 mM CaCl₂ was added and poured onto an MRS agar plate, and the plate was incubated at 45°C aerobically. After 18 to 24 h, plaques were counted.

DNA isolation and characterization. Bacteriophage DNA was obtained by the extraction method for bacteriophage lambda DNA described in detail by Maniatis et al. (8). Restriction digests of bacteriophage ch2 DNA were performed according to the instructions of the enzyme manufacturer (International Biotechnologies, Inc.) and subjected to agarose gel electrophoresis. Electrophoresis was performed on a 1% agarose gel at 80 V for 1 h (8).

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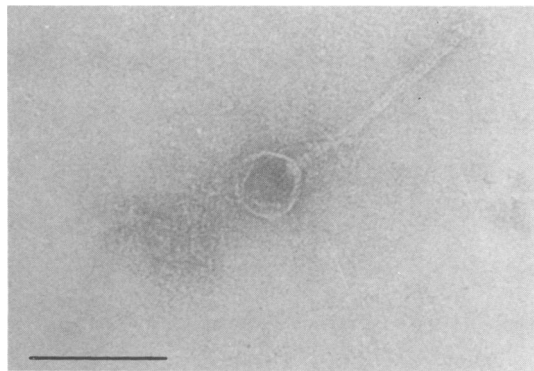


FIG. 1. Electron micrograph of bacteriophage ch2. Bar, 100 nm.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A 10- μ l sample containing 10^{12} PFU/ml was suspended in 40 μ l (final volume) of 80 mM Tris hydrochloride (pH 6.8)-10% glycerol-0.002% bromphenol blue-2% sodium dodecyl sulfate-100 mM dithiothreitol and incubated at 100°C for 10 min. The entire volume was loaded onto a 12.5% polyacrylamide-20% sodium dodecyl sulfate gel, electrophoresed at 150 V for 5 h, and stained with Coomassie brilliant blue (12). Molecular mass standards of 12,300, 18,400, 25,700, 43,000, 68,000, 97,400, and 200,000 daltons (obtained from Bethesda Research Laboratories, Inc., catalog no. 6001 LA) were used.

Electron microscopy. A bacteriophage sample (10^8 PFU/ml) was negatively stained with 2% uranyl acetate on carbon-coated grids. The bacteriophage DNA was spread on formamide and then rotary shadowed with platinum-palladium. Photographs were taken with a JEOL 100 B electron microscope at 80 kV.

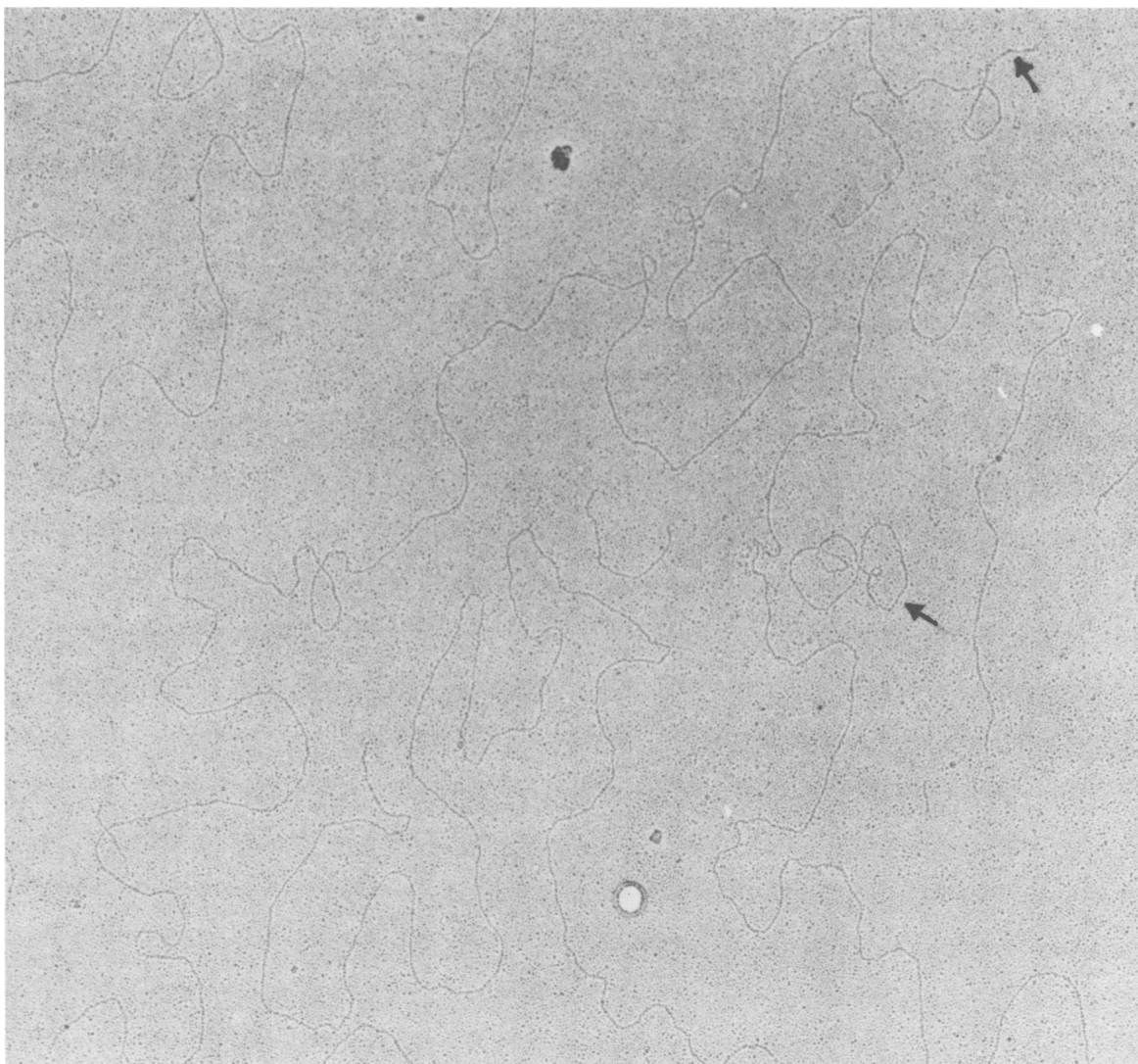


FIG. 2. Electron micrograph of linear bacteriophage ch2 DNA. Contour length measurement of the DNA was determined to be 40 kb. Circular ϕ X174 DNA was used as a size standard (5,386 base pairs). Arrows indicate end of linear bacteriophage ch2 DNA and circular ϕ X174 DNA.

One-step growth. *L. bulgaricus* CH2 cultures were grown in MRS broth until the optical density at 600 nm reached 0.085. To 0.9 ml of this culture, 0.1 ml of 1 M CaCl₂ and 0.1 ml of a bacteriophage ch2 stock (2×10^8 PFU/ml; multiplicity of infection, 5) were added, and 5 min was allowed for adsorption. After adsorption, the mixture was centrifuged ($6,000 \times g$, 5 min). The supernatant, containing unadsorbed bacteriophages, was discarded, and the pellet was suspended in 10 ml of prewarmed MRS broth. Serial dilutions were made, and samples from the 10^{-3} and 10^{-5} dilutions were titered at 3- to 5-min intervals.

Host growth and β -galactosidase production during bacteriophage infection. A 10-ml sample of early-log-phase *L. bulgaricus* CH2 culture was infected at an MOI of 5 bacteriophages per cell, and unadsorbed bacteriophages were removed as described previously. Growth of the culture and production of β -galactosidase were monitored at 10-min intervals during the infection process.

Growth of the culture was measured by the absorbance at 600 nm with a Bausch & Lomb Spectronic 20 spectrophotometer.

The β -galactosidase assay was adapted from that of Miller (9). A 200- μ l sample of culture was added to 2 ml of Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM β -mercaptoethanol). Two drops of toluene was added to permeabilize the cells; then 0.4 ml of *o*-nitrophenyl- β -D-galactopyranoside (4 mg/ml) was added, and the mixture was incubated at 37°C. Once the mixture turned yellow the reaction was stopped by the addition of 1 ml of 1 M Na₂CO₃.

RESULTS AND DISCUSSION

Morphology. The ultrastructure of bacteriophage ch2 was examined by electron microscopy after negative staining with uranyl acetate (Fig. 1). After examination of a number

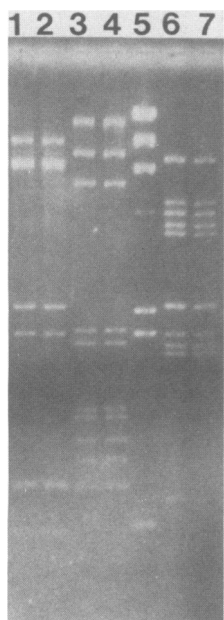


FIG. 3. Analysis of bacteriophage ch2 DNA digested with restriction endonuclease *Pst*I (lanes 1 and 2), *Eco*RI (lanes 3 and 4), and *Pvu*II (lanes 6 and 7). A *Hind*III digest of lambda DNA was used as the molecular weight standard (lane 5).

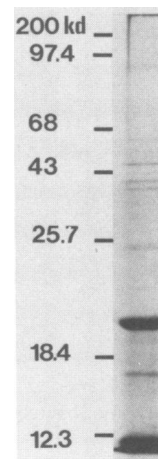


FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of bacteriophage ch2 structural proteins, kd, Kilodaltons.

of electron micrographs, the dimensions of its important features were determined. The head was hexagonal and 50 nm in diameter. The long narrow tail was 170 nm long and 10 nm wide and was flexible, noncontractile, and regularly striated. A collar appearing just below the head was 13 by 6 nm. A base plate (13 by 4 nm) at the end of the tail was also present. The morphology of this bacteriophage belongs to Bradley's classification group B (3).

The morphologies of lactobacillus bacteriophages are diverse (1, 13). At least one lactobacillus bacteriophage has been isolated for each of the morphological groups in Bradley's system; however, the majority of these bacteriophages can be assigned to Bradley's group B. The morphology of bacteriophage ch2 closely resembles those of *Lactobacillus casei* bacteriophages PL-1 (14) and J1 (13) and *L. lactis* bacteriophage ϕ LL55 (13).

Characterization of bacteriophage DNA. Formamide spreading of bacteriophage ch2 DNA revealed only linear DNA molecules. The contour length based on measurement of 15 DNA molecules was determined to be 37 ± 1.6 kilobases (kb), corresponding to a molecular mass of about 24 megadaltons (Fig. 2).

The restriction pattern of bacteriophage ch2 DNA generated by digestion with *Eco*RI, *Pst*I, and *Pvu*II is shown in Fig. 3. Addition of the molecular masses of the fragments gives the bacteriophage DNA a total size of about 35 kb. The size of bacteriophage ch2 DNA is similar to those reported for *L. casei* bacteriophages PL-1 (39 kb [16]), J-1 (37 kb [7]), and ϕ FSN (42 kb [6]).

Structural protein analysis. The structural proteins (Fig. 4) of bacteriophage ch2 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This analysis revealed two major bands of 19,500 and 12,200 Da and eight minor bands of 92,000, 56,000, 47,000, 45,000, 42,000, 39,500, 38,000, and 16,000 Da. The total molecular mass of bacteriophage structural proteins observed was 407,200 Da. With the conversion of 270 base pairs of DNA coding for a 10,000-Da protein (4), the total molecular mass of all the structural proteins corresponded to 11.0 kb of DNA, representing about 33% of the bacteriophage ch2 genome. By the same calculation, with data reported for *L. casei* bacteriophages ϕ FSW and PL-1, slightly larger protein size/genome ratios were found for ϕ FSW (36% [6]) and PL-1 (48% [14]).

Burst size and latent period. The infection cycle of bacteriophage ch2 in MRS was characterized by its one-step

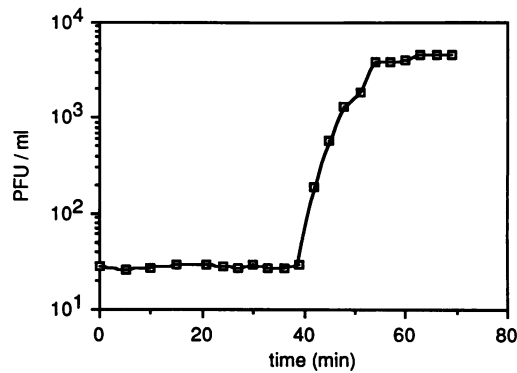


FIG. 5. One-step growth curve of bacteriophage ch2 showing its propagation in MRS broth at 45°C.

growth kinetics. Figure 5 graphically represents the increase in PFU per milliliter as a function of time during infection at 45°C. The events in bacteriophage development were determined: the latent time was 40 min, the rise period was 15 min, and the burst size was 130 bacteriophages per cell.

The burst size and latent time of a number of lactobacillus bacteriophages have been reported. De Klerk and Coetzee studied a number of lactobacillus bacteriophages and found that the burst sizes of 11 *Lactobacillus fermenti* bacteriophages ranged from 30 to 100 bacteriophages per cell (5) and that the burst sizes of four *L. casei* bacteriophages ranged from 20 to 33 bacteriophages per cell. Others reported that *L. casei* bacteriophages PL1 and LL55 have higher burst sizes: 200 and 80 bacteriophages per cell, respectively (11, 17). The burst size of ch2 is certainly within this range; however, the latent time of 40 min is much shorter compared with those reported for a number of lactobacillus bacteriophages (over 70 min [5, 11, 17]).

Effect of bacteriophage infection on host growth kinetics and β -galactosidase production. In the dairy industry, bacteriophage infection of lactic cultures is thought to cause slow acid development in milk (11, 17). Two rapid and simple methods were used to determine how bacteriophage ch2 infection affects host growth rate and protein synthesis during and after infection. The growth was followed by absorbance measurements, and β -galactosidase production was followed by an enzyme assay. The growth kinetics of two cultures, one infected and the other uninfected, are shown in Fig. 6. Before lysis, both cultures exhibited a

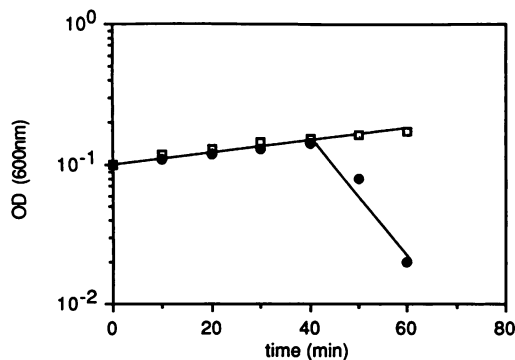


FIG. 6. Growth kinetics of an *L. bulgaricus* CH2 culture infected with bacteriophage ch2 (●) and an uninfected culture (□). OD, Optical density.

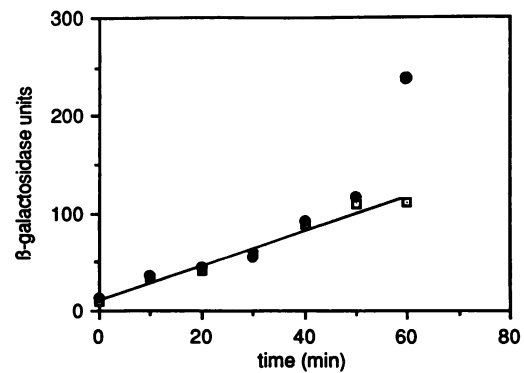


FIG. 7. β -Galactosidase activity of a *L. bulgaricus* CH2 culture infected with bacteriophage ch2 (●) and an uninfected culture (□).

similar exponential growth rate with a doubling time of about 60 min. There was no indication that host growth was inhibited by the bacteriophage infection.

β -Galactosidase is an easily assayable protein produced constitutively by *L. bulgaricus* CH2. The production of this host protein was followed to determine whether its synthesis was affected by bacteriophage ch2 infection. Figure 7 shows the increase of β -galactosidase activity with time in an infected culture and an uninfected culture. No difference between the infected culture and the uninfected culture was observed, suggesting that β -galactosidase synthesis and perhaps synthesis of other proteins may not be inhibited by bacteriophage ch2 infection. The increase in activity after 40 min was due to the onset of cell lysis, which released cytoplasmic material including β -galactosidase into the medium. The rate of β -galactosidase synthesis before lysis, however, was the same in the infected and uninfected cultures.

In the dairy fermentation industry, bacteriophage infections have been thought to inhibit acid production (11, 12, 17). Evidence presented here for bacteriophage ch2 suggests that the infected culture remains active during the infection process and that inhibition of acidification is the result of lysis of the culture. The growth rate of the host culture during the latent period did not show any change with respect to that of the uninfected control (Fig. 6). β -Galactosidase activity was not affected by bacteriophage infection and continued to increase like that of the uninfected culture (Fig. 7). Repression of host enzyme synthesis characterizes many virulent bacteriophage infections. In other bacteriophages, such as T7 and lambda, induction of β -galactosidase production was repressed upon infection (10); however, preliminary evidence presented here indicates that the integrity of β -galactosidase synthesis was not destroyed by bacteriophage ch2 infection.

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