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Bacteriophage Active Against the Lactic Acid Beverage-Producing Bacterium *Lactobacillus casei*

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A virulent bacteriophage which causes a decrease in acid production during fermentation of a lactic acid beverage named Yakult with *Lactobacillus casei* was isolated from the abnormal fermentation tank and named PL-1. *L. casei* S strain was the exclusive host cell among 18 lactic acid bacteria tested. The plaque was round with an average diameter of about 0.5 mm. It exhibited serological cross-reaction with previously isolated J1 phage. Under an electron microscope, the phage had a spermatozoon shape, with an icosahedral head (63 nm) and a long tail (12.5 by 275 nm) with about 55 striae. The free phage particles were stable at *p*H 5 to 8. The phage was quite sensitive to ultraviolet irradiation or to heating (60 C, 5 min), and the host was more sensitive than the phage to these treatments. Many kinds of antimicrobial chemicals were also phagocidal. Calcium ion (5 mM) was specifically essential for the phage growth cycle. A one-step growth experiment under optimum conditions (37 C and *p*H 6.0) showed that the eclipse period was about 75 min, that the latent period was 100 min after the phage infection, and that the average burst size was about 200. The possibility of arresting phage development in lactic acid fermentation is discussed.

The prevention of bacteriophage infections is one of the important problems confronting industries which use microorganisms in their productive processes. In dairy industries such as the manufacture of cheeses or fermented milk products, in which several kinds of lactic acid bacteria are employed, bacteriophages are thought to cause, in a large measure, the slow acid development in milk inoculated with a lactic culture. Among the bacteriophages lytic to lactic acid bacteria, streptococcal phages (2) have been investigated most extensively for the purpose of preventing phage contamination, particularly in cheese manufacture, one of the largest users of bacterial cultures. However, there is much less information on the bacteriophages of lactobacilli (12) than there is concerning streptococcal phages. However, some kinds of phages lytic to the following lactobacilli have been found in various sources, such as saliva, dung, sewage, or bacterial cultures: *L. arabinosus* (8), *L. brevis* (8), *L. bifidus* (21), *L. casei* (4, 11, 17), *L. fermenti* (3, 4, 11), *L. helveticus* (9, 11), *L. lactis* (9), and *L. salivarius* (11).

Some investigators have been engaged in the production of a lactic acid beverage named Yakult, one of the commercial live *Lactobacillus* preparations, employing a special strain of *L. casei*. As this strain was once attacked by bacteriophage J1 (6), a strain resistant to the phage was selected for use. However, a decrease in lactic acid production as was previously observed was detected in the course of fermentation, and another bacteriophage designated as PL-1 (K. Jin-nai et al, Annu. Meeting Nishi-Nippon Branch Agr. Chem. Soc. Jap., Saga, 1967) was isolated from the abnormal cultures. Although another phage-resistant strain has now been selected in the factory, it is still very likely that the recurrence of phage attack on newly isolated phage-resistant strain will occur. Therefore, we decided to define the biological properties of PL-1 phage in the hopes of finding measures of suppressing phage development in the factory.

MATERIALS AND METHODS

Bacteria and cultures. *L. casei* Shirota strain (designated as S strain) was used as the indicator of plaque counting and for propagation of the phages. To test the host range of phage, lactobacilli, streptococci, and *Leuconostoc* cultures were used. Streptomycin-resistant S strain was supplied by A. Murata.

MR medium (1.0% polypeptone, 1.0% glucose, 1.0% sodium acetate, 0.3% yeast extract, 0.3% beef extract, 0.15% $CaCl_2$, 0.1% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot H_2O$, 0.0001% $FeSO_4 \cdot 7H_2O$, *p*H 6.0) devised by Murata (14) was used for the most part. Basal and overlayer media for the double-layer method contained, respectively, 1.0 and 0.7% agar in medium. Where indicated, TM medium (1.0% polypeptone, 1.0% glucose, 1.0% sodium acetate, 0.3% yeast extract, 0.5% beef extract, 30% tomato juice filtered with a filter paper, *p*H 6.6) was used.

The stock cultures were stabbed in agar medium supplemented with 1% $CaCO_3$. For preparing the indicator cultures, 5% (volume) of the overnight cultures was incubated in a fresh liquid medium at 37 C for 3 to 4 hr until the optical density of the cultures was about 0.25 (cell numbers, 3×10^8/ml).

For estimation of bacterial growth, colony counting was used for live cells, and turbidity measurement in a Hitachi photoelectric colorimeter (EPO-B) at 660 nm was used for cell mass.

Phages and the assay of phage titers. Phage PL-1 obtained here and J1 isolated by Hino (6) were used. The phage titers were assayed as described below by the double-layer method of Adams (1). A 0.1-ml amount of the phage suspensions was dropped on a basal layer (20 ml) solidified in a petri dish. The mixture of indicator strain (0.1 ml) and melted soft agar medium (3 ml) kept in a water bath at 50 C was overlaid onto the phage sample. The double layer plate thus prepared was incubated for about 18 hr at 37 C, and the plaques were counted and recorded as plaque-forming units (PFU) per milliliter.

Purification of phage particles. Fresh phage lysates having phage titers of over 10^{10} PFU/ml were stored in a refrigerator for 1 week to precipitate spontaneously such admixtures as mucoidal materials and purified as described in Fig. 1. The centrifuges used were a Martin Christ preparative ultracentrifuge (Omega) and a Tominaga refrigerated centrifuge (90-UV).

Preparation of antiphage sera. Purified phage suspensions (5×10^{11}/ml) were injected intraperitoneally into 2.7-kg male rabbits three times a week for 4 weeks, starting with 0.5-ml injections and gradually increasing to 2 ml per dose (a total of 17 ml). The bleeding was performed 7 days after the last injection by the usual procedures. After removal of host cell antibodies, all sera were heated at 56 C for 30 min and stored at −20 C without preservatives (K = 500 − 1,000).

Preparation of specimens for electron microscopy. Purified phage suspensions were adequately diluted with deionized water, and an equal volume of 2% phosphotungstic acid neutralized with 1 N KOH was added to these phage dilutions. After standing for 15 min at room temperature, the mixture was deposited in the form of a minute droplet on carbon-coated celloidine-filmed microscope grids and air-dried. The specimens thus obtained were examined with a Japan-Electron electron microscope (JEM 7).

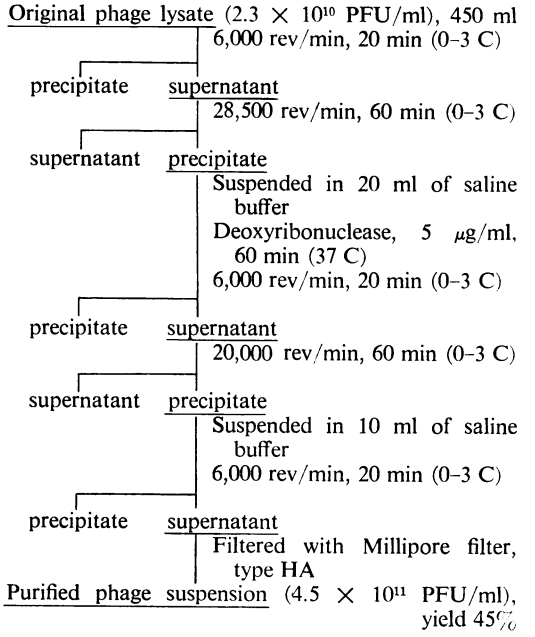

FIG. 1. *Purification of phage particles. Saline buffer consisted of 10^{-2} M phosphate buffer (pH 7.2), 0.8% NaCl, and 10^{-3} M $MgSO_4$. Deoxyribonuclease was from Sigma Chemical Co. (crude, from beef pancreas).*

RESULTS

Isolation and confirmation of bacteriophage. In a normal lactic acid fermentation with rejuvenated young cultures of *L. casei* S in a main tank medium (*p*H 6.5) containing mainly skim milk (15%) and *Chlorella* extracts (0.4%), titration acidity of cultures reached a maximum within 3 or 4 days at 37 C, with a *p*H of about 3.6. On one occasion, however, an abnormal fermentation was observed in which the titration acidity never approached the normal maximum and the cell numbers remained below 10^7/ml for a long time. To clarify the cause of this abnormal fermentation, experiments were conducted to detect microbial contaminants and bacteriophages. One kind of plaque-forming principle could be detected by planting the culture filtrates with S strain as an indicator. The lytic principle picked up from a single plaque was then shown to multiply hereditarily, to pass through a membrane filter (Millipore Corp., type HA), and to be inactivated by heating at 100 C for 10 min. The lytic principle was, therefore, certified to be one of the bacteriophages. The size of plaque was somewhat smaller than that of J1. It was clear and round with a diameter of 0.3 to 0.7 mm.

Host-range specificity. Specificity of the phage for several lactic acid bacteria was examined by the following two methods. (i) A 0.1-ml amount of the samples (10^4 PFU/ml) was plated with each test bacterium and, after overnight incubation at 37 C, was examined for plaque formation. (ii) The test bacteria were infected with the samples (10^8 PFU/ml) in liquid medium and, after overnight incubation at 37 C, were examined for lysis. The phage was practically species-specific, attacking only original *L. casei* S strain and the J1 phage-resistant strain. The following lactic acid bacteria were quite insensitive to the phage: *L. casei* IFO 3425, *L. casei* IFO 3914, *L. acidophilus* IFO 3831, *L. brevis* IFO 3960, *L. brevis* ATCC 8287, *L. buchneri* IFO 3961, *L. delbrueckii* IFO 3202, *L. fermenti* IFO 3954, *L. helveticus* IFO 3219, *L. plantarum* IFO 3074, *L. thermophilus* IFO 3863, *Streptococcus faecalis* ATCC 8043, *S. lactis* IFO 3434, *S. thermophilus* IFO 3535, *Leuconostoc mesenteroides* var. *Sake* IFO 3832.

From the results thus obtained, the abnormal fermentation was considered to have been caused by another lytic principle different from J1 phage. We have designated the phage as PL-1.

Serological cross-neutralization tests. The cross-neutralization reactions were performed to demonstrate the serological relationship between PL-1 and J1 phage. A 0.1-ml amount of each phage dilution (1.2×10^7 PFU/ml) was added to 0.9 ml of its homologous or heterologous anti-phage serum dilutions at 37 C, and, at 5-min intervals, unneutralized phages were assayed. The inactivation curve of PL-1 and J1 phage by its homologous or heterologous antiserum (Fig. 2) agreed almost with the first-order equation. The ratio of serological inactivation rate constant of respective antisera against both the phages calculated after 15 min of incubation was as follows. In the case of anti-PL-1 serum, J1/PL-1 = 1.05; for anti-J1 serum, PL-1/J1 = 0.73. These data indicate that the phages are closely related to each other, belonging to a same serological group. The phage PL-1 is, therefore, likely to be one of the host-range mutants derived from the parental phage J1.

Shape of PL-1 phage. The electron microphotograph of PL-1 phage (Fig. 3) obtained by negative staining showed that the phage was spermatozoon-shaped with a long tail, and that there were few morphological differences between PL-1 and J1 phage. The head appeared to be a regular icosahedron with a diameter of about 63 nm, although its structural details could not be observed. The tail was relatively long, being about 12.5 nm in width and 275 nm in length. The fine structures of the tail end, as seen on T-even *Escherichia coli* phages, could not be seen. However, about 55 lateral striae were observed along the tail on all phages examined.

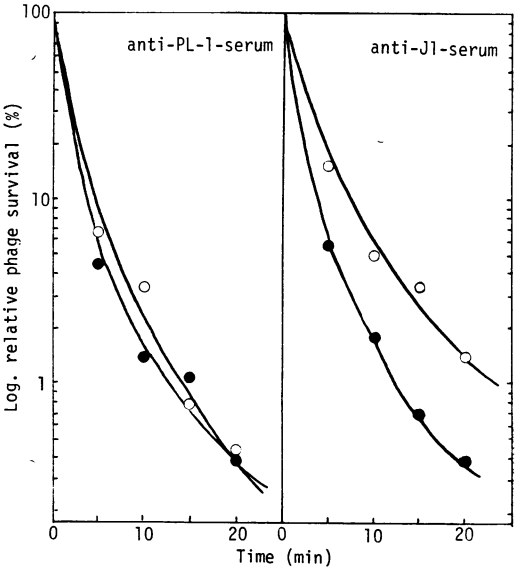

FIG. 2. *Neutralization of PL-1 and J1 phage by homologous or heterologous antiserum. Each phage (initial titer, 1.2×10^6/ml) was exposed to antiserum (diluted over 1:500) at 37 C for 20 min. Symbols:* ○, *PL-1;* ●, *J1.*

Isolation of the phage-resistant cells and lysogenicity. The host cells were incubated with large amounts of PL-1 phage on solid media for about 1 week at 37 C to allow the development of resistant cells. Phage-resistant cells were usually obtainable in only one contact of phage. About 30 colonies picked up at random were purified by repeating the plating single colony and ascertained to be phage-resistant. The culture filtrates of all of the resistant cells formed no plaques on the host cells, showing that these resistant cells were all nonlysogenic. Accordingly, PL-1 is considered to be a phage of virulent type.

Stability of the phage. PL-1 phage lysates showed little decrease in titers even after 6 months of storage in a refrigerator. However, as shown in Table 1, the phage was fairly unstable when diluted with deionized water or with some kinds of salt solutions such as physiological saline or phosphate buffer. The addition of divalent cations such as Mg^{2+} or Mn^{2+} (10^{-3} M) to the buffers protected the phage from inactivation.

The effect of antimicrobial chemicals on PL-1 phage is shown in Table 2. All disinfectants and antiseptics tested were cidal to free phage particles at concentrations which affected bacterial growth.

Thermal stability of PL-1 phage and its host S strain was examined in TM medium (*p*H 6.8) after incubation at temperatures from 20 to 80 C

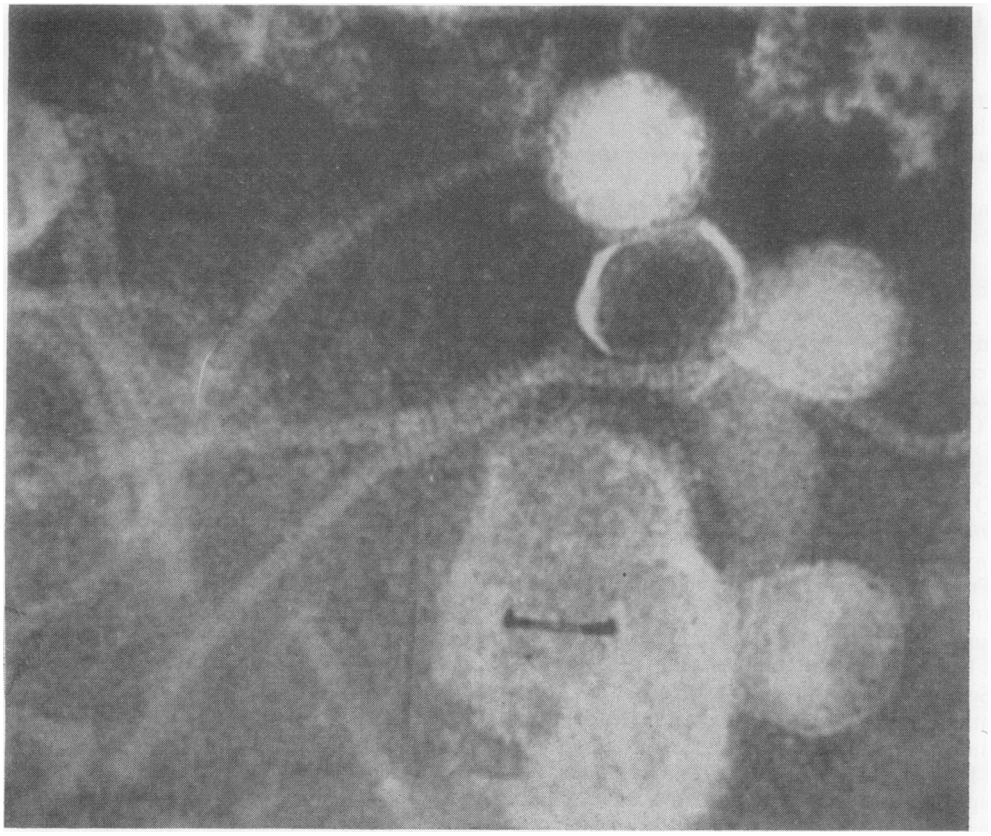

FIG. 3. *Electron photomicrograph of PL-1 phage by negative-staining method.*

TABLE 1. *Stability of PL-1 phage in salt solutions*[a]

Salt solution	Survival
	%
Control (0 hr of incubation)	100
Deionized water	10
0.85% Saline	34
0.85% Saline + $MgSO_4$ (10^{-3} M)	43
0.85% Saline + $CaCl_2$ (10^{-3} M)	34
0.067 M Phosphate buffer (*p*H 7.2)	67
0.01 M Phosphate buffer (*p*H 7.2)	87
0.01 M Phosphate buffer + $MgSO_4$ (10^{-3} M)	103
0.01 M Phosphate buffer + $CaCl_2$ (10^{-3} M)	75
0.01 M Phosphate buffer + $MnCl_2$ (10^{-3} M)	100
0.001 M Phosphate buffer (*p*H 7.2)	81
0.01 M Tris[b] buffer (*p*H 7.4)	98

[a] Initial phage titer, $10^{8.5}$ PFU/ml; at 37 C for 60 min.

[b] Tris(hydroxymethyl)aminomethane.

for 5 min. The phage particles were stable below 50 C but less stable above this point and almost completely inactivated at 60 C (Fig. 4a). The host cell was more heat-labile than PL-1 phage and even at 45 C was inactivated to a small extent. The time course of thermal inactivation at 55 C (Fig. 4b) showed that the kinetics of inactivation followed the formula of the first-order reaction in both cases, and that the host cell was more sensitive than PL-1 phage to the thermal treatment. The values for thermal inactivation rate constant (K) were calculated to be 4.6×10^{-2}/min and 3.3×10^{-1}/min for PL-1 and S strain, respectively.

*p*H stability of the phage was examined in MR medium at *p*H values ranging from 2 to 11, adjusted with lactic acid and NaOH, after incubation for 30 min at 37 C. PL-1 phage was stable between *p*H 5 and 8 and was inactivated at below 4 and above 10. The *p*H range in which the phage was stable almost agreed with that for the stability of the host S strain.

Both PL-1 and the host were diluted to contain

TABLE 2. *Effects of antimicrobial chemicals on PL-1 phage*[a]

Chemicals	Concn (%)	Survival (%)
Control		100
Disinfectants		
Propanol	10	100
Propanol	20	0
Ethanol	30	0
Methanol	40	3
Acetic acid	0.0001	8
H_2O_2	0.0001	10
H_3BO_2	0.1	38
LiCl	1	30
NaClO	0.1[b]	89
NaClO	1[b]	0
Detergents		
Benzethonium chloride	0.0001	18
Benzethonium chloride	0.001	0
Benzalkonium chloride	0.0001	51
P3-Z (commercial cleanser)	0.001	21
Neogen (commercial cleanser)	0.01	4
Antiseptics		
Sodium sorbate	0.01	100
Sodium sorbate	0.1	54
Sodium sorbate	1	0
Sodium dehydroacetate	5	15
Sodium benzoate	5	60
Sodium propionate	15	84

[a] Initial phage titer, 10^4 PFU/ml; at 37 C for 60 min.

[b] Expressed as micrograms per milliliter.

10^4 PFU/ml with 10^{-2} M phosphate buffer (*p*H 6.8) plus 10^{-3} M $MgSO_4$. These suspensions, placed in a petri dish (90 mm in diameter and 1 mm in the depth of the solution), were held 40 cm from a germicidal lamp (Toshiba, GL-15, 15 w at 253.7 nm) and irradiated with a gentle shaking for a period of time under dim light to avoid photoreactivation. The time courses of their inactivation by ultraviolet light showed that the kinetics of inactivation followed the first-order reaction, and that the host cell was also more sensitive than PL-1 to ultraviolet irradiation. Their reaction constants per second were 4.4×10^{-2} and 1.4×10^{-1} for PL-1 phage and the host cell, respectively.

Lytic characteristics of the phage-infected cultures and calcium ion requirement. Considering that some bacteriophages (5, 10, 16, 18, 19, 20) including J1 require divalent cations such as Ca^{2+}, Mg^{2+}, or Mn^{2+} in their multiplication cycles at concentrations over those needed for the growth of their host cells, the effect of calcium ion concentrations on the multiplication of PL-1 phage and the host cells was at first investigated. The

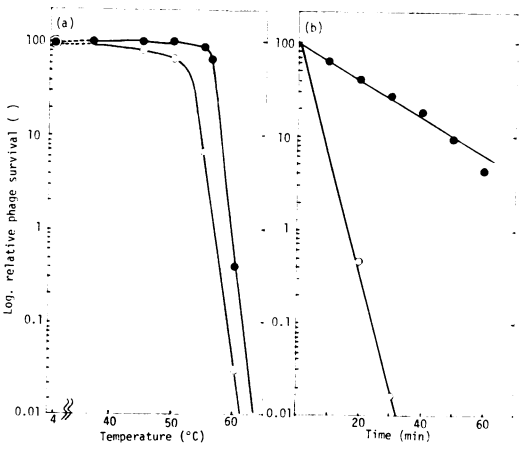

FIG. 4. *(a) Thermal stability of PL-1 phage and the host. (b) Thermal inactivation curve of PL-1 phage and the host. Initial phage titer, 1.7×10^4 PFU/ml; host, 4.2×10^4 cells/ml. Symbols: ●, phage PL-1; ○, host S strain.*

host cells (final, 6.2×10^7/ml) were infected with PL-1 (final, 1.2×10^7 PFU/ml) in MR medium exclusive of metal salts except for $CaCl_2$ being added at various concentrations (0.1 to 5.0 mM), and incubated at 37 C. At intervals for 9 hr, the turbidity of the cultures was measured. The growth rate of the phage-infected cells in the absence of added $CaCl_2$ was the same as that of control (the phage-uninfected cells), although 0.1 to 0.2 mM equivalent total calcium could be detected in the medium by an atomic absorption analysis.

On the other hand, in the presence of added $CaCl_2$, the higher the concentrations of calcium ion in the medium, the sooner the phage-infected cells lysed and released their progeny phages. The concentrations of calcium ion sufficient to permit the culture to achieve the typical lysis in the shortest time were about 5 mM. Calcium ion at higher concentrations did not accelerate lysis further. Both the stability of free phage particles and the growth of the host cells were not affected even when 50 mM calcium ion was present.

The other divalent cations such as Mg^{2+}, Mn^{2+}, or Ba^{2+} were less effective than Ca^{2+} in lysing the phage-infected cultures (Fig. 5b). Manganese ion accelerated the growth of host cells. A definite effect of the other metal salts such as $ZnSO_4$, $SnCl_2$, $BeSO_4$, or $CoSO_4$ was not observed. Furthermore, such metal salts as $NiSO_4$, $CuCl_2$, or $Cd(NO_3)_2$ inhibited the growth of the phage-uninfected bacteria.

One-step growth experiment. To clarify the growth characteristics of the phage, a one-step

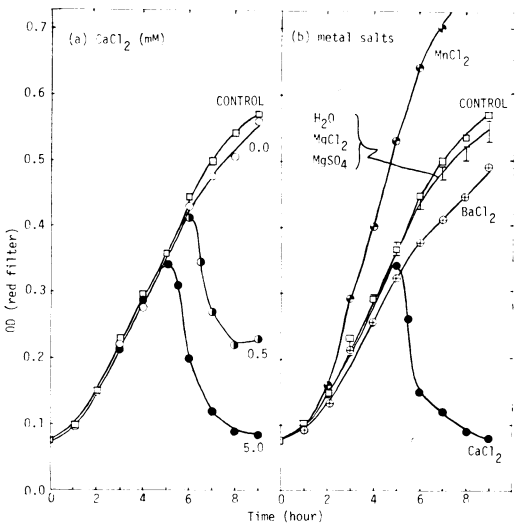

FIG. 5. *Effect of divalent cations on the turbidity of PL-1 phage-infected cultures of S strain. (a) Calcium ion concentrations. (b) Kinds of divalent cations. MR medium exclusive of minerals (pH 6.0) at 37 C. Control: culture not infected with phage.*

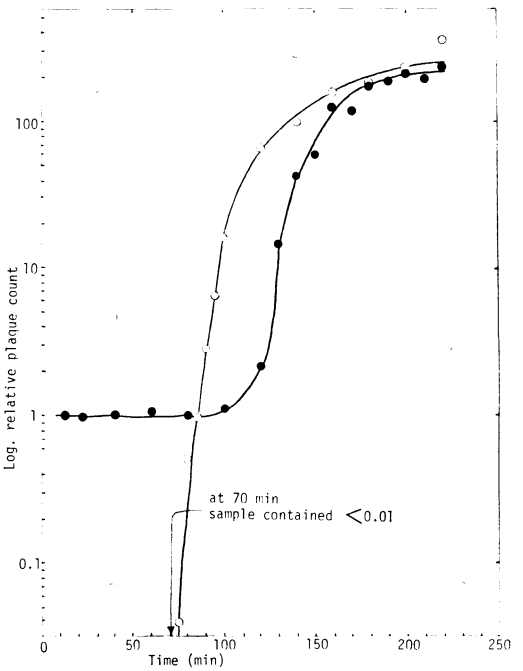

FIG. 6. *One-step growth curve of PL-1 phage. At 37 C, MR medium (pH 6.0) had a multiplicity of infection of 0.07. Premature lysis was produced at intervals by treating the sample with 2 mg of streptomycin per ml for 60 min to induce lysis. Symbols: ●, extracellular phage; ○, intracellular phage.*

growth experiment was done next. A 0.9-ml amount of the rejuvenated cultures of S strain was infected with 0.1 ml of PL-1 phage (multiplicity of infection = 0.07) for 5 min at 37 C. Then, 0.1 ml of the mixture was added to 0.9 ml of diluted antiphage serum and incubated for 5 min at 37 C to neutralize 99% of the unadsorbed free phage particles. After 5,000-fold dilution of this mixture into nutrient medium to stop any further action of the antiphage serum, the diluted culture was incubated further at 37 C. At regular time intervals, 0.1-ml portions were sampled and assayed for the extracellular appearance of mature phage particles in the culture.

Intracellular phages were assayed by the method of Murata (15). Additional 0.9-ml samples were removed at intervals from the growth tube, quickly added to 0.1 ml of streptomycin (2 mg/ml), and incubated for 60 min at 37 C to lyse the host cells. Appropriate dilutions were then plated by the double-layer technique by using a streptomycin-resistant S strain as an indicator. After incubating overnight at 37 C, the plaques were counted.

The first intracellular appearance of PL-1 phage occurred at approximately 75 min (eclipse period) after the phage infection (Fig. 6). The latent period was extended to approximately 100 min, with extracellular phage release continuing logarithmically up to about 170 min to reach its peak. The average burst size was about 200. Both the burst size and the latent period were influenced by the *p*H values of the medium, and the optimum *p*H was 6.0. No phage growth was observed at *p*H 7.6 even after 5 hr of incubation.

DISCUSSION

In microbial fermentations, it is important to find any effective method for suppressing phage contamination. Since the multiplication of phage depends exclusively on the metabolism of the host cell, finding antiphage substances with high selective toxicity has been considered to be very difficult. To prevent phage infections, phage-resistant mutants have been selected as a usual countermove in many industries which use bacteria. However, this method is not always successful. In some cases the inhibition of phage growth by chemical agents without inhibiting the growth and fermentation of host bacteria used has been reported to be successful. For example, Murata and Hongo (13) proposed a method of preventing intracellular phage growth in a butanol fermentation by using *Clostridium saccharoperbutylacetonicum* (a new strain similar to *C. butylicum*), in their study, strains resistant to tetracyclines, chloramphenicol, streptomycin, or fradiomycin were used, and these antibiotics were added to

the medium. The inhibitory action of ammonium oxalate on the multiplication of phages active against *S. lactis* and *S. cremoris* in milk was reported by Kadis and Babel (7). Even if such effective chemicals were discovered, however, they would not always be practical in food fermentations because the added chemicals should be harmless to human body.

In manufacturing lactic acid beverage, abnormal fermentations originate both from microbial contaminants and bacteriophage infections. In the fermentation, the infection by phages is more serious than that by microbes. In addition to J1, phage PL-1 active against both the J1 phage-resistant strain and the original *L. casei* S strain has now been isolated in the bacterial culture. Serologically PL-1 and J1 phage are considered to be the same. However, they are different from each other in many ways, such as host range and plaque and growth characteristics. These results suggest that in the future other phages will also be found. PL-1 and J1 phage require more calcium ion than the concentrations that are needed for the growth of host bacteria. Therefore, the elimination of calcium ion with specific chelators, as reported by many investigators, is assumed to be an effective check to phage propagation. However, such chelators must be harmless to the human body. Investigations of these problems are now in progress.

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