APPLIED MICROBIOLOGY, Jan. 1974, p. 72-77

Vol. 27, No. 1
Printed in U.S.A.

Preparation and Storage of High-Titer Lactic Streptococcus Bacteriophages[1]

J. NYIENDO, RAMON J. SEIDLER, W. E. SANDINE, AND P. R. ELLIKER
Department of Microbiology, Oregon State University, Corvallis, Oregon 97331

Received for publication 20 September 1973

Various techniques were employed for preparation of high-titer bacteriophage lysates of *Streptococcus lactis*, *S. cremoris*, and *S. diacetilactis* strains. Infection of a 4-h host culture in litmus milk at 30 C yielded the highest titers (2×10^9 to 4×10^{11} plaque-forming units/ml) for most phages. Host infection in lactose-containing broth produced similar virus numbers only when 0.1 M tris(hydroxymethyl)aminomethane buffer stabilized the pH. The pH at the time of infection as well as the inoculum phage titer were critical in obtaining high titers. Optimum conditions for infection in broth were coupled with a polyethylene glycol concentration procedure to routinely produce milligram quantities of phage from 1 liter of lysate. Neutralization of whey lysates, as a means of storage, offered no survival advantage over unneutralized samples. Storage of phage lysates in a 15% glycerol whey solution at −22 C yielded a high rate of survival in most cases, even with repeated freezing and thawing, over a period of 24 months.

Lactic streptococci are of critical importance to the dairy fermentation industry because these bacteria supply the lactic acid for curd production and their metabolic products impart characteristic and desirable flavors. Bacteriophage infection of these starter cultures results in insufficient acid production and usually a failure of the fermentation. The economic and public health consequences of these failures are well known (6). Various approaches have been utilized in an attempt to minimize bacteriophage infection during dairy fermentations. The use of culture rotation (4, 6, 15), mixed strain starter cultures (6, 15, 21), and a phage inhibitory medium (12) are currently in general use. These techniques never completely prevent failures, and constant precautions to prevent culture infections are advised.

The use of mixed strains and starter rotation relies on the utilization of lactic streptococci that are resistant to a diversity of bacteriophage. Periodic examination of virus resistance patterns of starter cultures would be advisable. However, convenient techniques for the isolation and maintenance of bacteriophage stocks, which would be useful to industry, presently are not available.

Previous studies of lactic streptococcus viruses have dealt with electrolyte requirements and the influence of culture medium on the efficiency of plating (2, 3, 5, 9, 19, 20). Other studies dealing with bacteriophage propagation have not dealt specifically with the development of high titers and have not been concerned with extended survival times of the viruses (10, 11, 13, 18, 22).

In the present study, two procedures are described for routinely obtaining bacteriophage titers in excess of 10^{10} plaque-forming units (PFU)/ml. A simple storage procedure also is described which has proven suitable for maintaining these high titers during a 2-year study period.

MATERIALS AND METHODS

Bacteriophages and hosts. Seventeen bacterial cultures and their homologous bacteriophage were obtained from Barbara Keogh, C.S.I.R.O., Melbourne, Australia. The host organisms used were *Streptococcus diacetilactis* DRC1, DRC2, and DRC3; *S. lactis* C2, C10, and H1, and *S. cremoris* EB4, EB7, EB9, ML1, HP, E8, C1, C3, C11, C13, and R6.

Media. The phages were propagated at 30 C in either lactic broth (7) or sterile Matrix Mother Culture Medium (Galloway West, Fond du Lac, Wis.) having 11% milk solids and 1.5% litmus. Stock cultures of host organisms were maintained by weekly transfer of a 1% inoculum in 10 ml of the sterile litmus milk with incubation at 30 C for 14 to 18 h. Culture lysates were assayed for plaque-forming units by the double layer plate method of Adams (1) by using lactic broth supplemented with Bactoagar (1.5% bottom layer; 0.8% top layer), and seeded with about 10^7 host cells.

Propagation of phages in milk. Host cultures selected for these experiments were EB4, EB7, EB9,

[1] Technical paper no. 3472, Oregon Agricultural Experiment Station.

ML1, E8, C2, DRC1, DRC3, C1, C3, C11, and C13. For each host, eight tubes of litmus milk (10 ml each) were inoculated with 0.1 ml taken from a 24-h litmus milk culture and then incubated at 30 C. Duplicate tubes were infected with 10^5 to 10^6 phage particles, at each of four incubation times: 0.5 h, 2 h, 4 h, and 6 h. The infected cultures were incubated overnight at 30 C, and the appearance of each tube was recorded. Sterile lactic acid was added to a final concentration of 1.0% (vol/vol) to aid in whey separation. The contents were centrifuged (15 min at 10,000 × *g*), filtered through a membrane nitrocellulose filter (Millipore Corp.), 0.45 μm, to remove any bacterial cells or other debris, and titered for plaque-forming units per milliliter.

Influence of buffer on phage growth in lactic broth. Experiments were initiated to study the influence of tris(hydroxymethyl)aminomethane (Tris)-buffered lactic broth on phage propagation. Control experiments indicated that 0.1 M Tris (pH 7.1) did not affect the growth of the host and was effective in maintaining the pH above 6.5 through 3.5 h of growth. A comparative study was made of five phage-host systems propagated in Tris-neutralized or unneutralized lactic broth. Duplicate flasks of each host were infected on the shaker at 34 C with homologous phage; one flask contained Tris buffer at a concentration of 0.1 M, pH 7.1, whereas the second flask of each host remained unbuffered. A 10-ml portion of an overnight culture containing 10^9 colony-forming units/ml was inoculated into 50 ml of the broth. Flasks were inoculated with homologous phage at zero time. All flasks were titered after 6 h.

Effect of polyethylene glycol (PEG) on concentration of phage lysates. Phage lysates were prepared on the shaker in lactic broth made 0.1 M with

TABLE 1. *Final titers and appearance of litmus milk cultures after staggered phage infections*

Phage	Time phage added (h)	Appearance of 18-h infected host			Phage titer (PFU/ml × 10^8)	Phage	Time phage added (h)	Appearance of 18-h infected host			Phage titer (PFU/ml × 10^8)
		Acid	Reduction	Coagulation				Acid	Reduction	Coagulation	
eb4	0.5[a]	+	++++[b]	++++	3.6	c10 (2)	0.5	−	−	−	79.0
	2	+	++++	++++	5.3		2	−	+	−	340.0
	4	+	++++	++++	2.5		4	−	++	−	>600.0
	6	+	++++	++++	—		6	+	++++	++++	<0.001
eb7	0.5	−	+	−	23.0	drc1	0.5	−	−	−	170.0
	2	−	++	−	150.0		2	−	−	−	460.0
	4	+	+++	−	320.0		4	−	++	−	3800.0
	6	+	+++	++++	<0.0001		6	+	++++	++++	1400.0
eb9	0.5	−	−	−	13.0	drc3	0.5	+	++++	++++	91.0
	2	−	−	−	18.0		2	+	++++	++++	320.0
	4	−	++	−	30.0		4	+	++++	++++	2200.0
	6	+	++++	++++	0.11		6	+	++++	++++	2000.0
ml1	0.5	−	−	−	57.0	c1	0.5	+	++++	−	1.0
	2	−	−	−	150.0		2	+	++++	−	77.0
	4	−	++	−	980.0		4	+	++++	−	1100.0
	6	+	++++	++++	21.0		6	+	++++	++++	5.0
e8	0.5	+	++++	++	32.0	c3	0.5	+	++++	++++	6.0
	2	+	++++	++	88.0		2	+	++++	++++	30.0
	4	+	++++	++	290.0		4	+	++++	++++	100.0
	6	+	++++	++++	90.0		6	+	++++	++++	1.0
c2	0.5	+	++++	++	33.0	c11	0.5	+	++++	++++	13.0
	2	+	++++	++++	340.0		2	+	++++	++++	29.0
	4	+	++++	++++	0.35		4	+	++++	++++	94.0
	6	+	++++	++++	0.0029		6	+	++++	++++	15.0
c10 (1)	0.5	−	−	−	79.0	c13	0.5	+	++++	++++	16.0
	2	−	+	−	330.0		2	+	++++	++++	53.0
	4	−	++	−	>1000.0		4	+	++++	++++	230.0
	6	+	++++	++++	<0.001		6	+	++++	++++	410.0

[a] Duplicate tubes with 10 ml of litmus milk were inoculated with 1% (wt/vol) of the host culture, then infected with 10^5 to 10^6 PFU/ml homologous phage at the indicated times. The appearance of the milk and phage titers were observed after 18 h at 30 C. Results with each phage are the average of two experiments.

[b] "+" indicates intensity of reaction; "−" indicates no reaction.

Tris-hydrochloride buffer as described in the preceding section. Concentration of the lysates was achieved in an aqueous PEG-NaCl two-phase system. With one exception, the protocol of Yamamoto et al. (23) was followed. The influence of NaCl concentration on phage recovery was studied in the early stages. NaCl was added to some of the lysates in 1 M concentration and to the others in 0.5 M concentration. The 1 M concentration proved more effective. PEG (Carbowax 4000) was added to the supernatant at 10% (wt/vol) immediately after centrifuging in the presence of NaCl. The flasks were stored at 4 C overnight, centrifuged for 20 min at 10,000 × *g* and decanted. The pellet was suspended in 1/33 of the original volume of lactic broth (1.5 ml), and the phage suspension was titered.

Storage by freezing in glycerol. Litmus milk whey lysates containing 15% (vol/vol) added glycerol were frozen at −22 C. They were thawed and refrozen as needed. The lysates were retitered periodically.

RESULTS AND DISCUSSION

Bacteriophage titers in litmus milk. Preliminary experiments demonstrated that the lactic bacteriophage were quite unstable when stored as low-titer lysates (less than 10^4 PFU/ml). For example, when such lysates were maintained in natural or neutralized (0.1 M Tris, pH 7) whey, 50% of the bacteriophage strains could not be recovered after only 7 days at 2 C. For this reason it was necessary to develop a convenient system to achieve and maintain lysates with high titers.

TABLE 2. *Comparison of final phage titers in lactic broth and Tris-neutralized lactic broth*[a]

Phage-host system	Phage inoculum	Final titers and pH[b]			
		Without Tris		With Tris[c]	
		PFU/ml	pH	PFU/ml	pH
c2	6.0×10^4	8.7×10^7	4.7	4.0×10^{11}	6.0
eb7	1.0×10^5	4.9×10^8	4.5	4.2×10^{10}	6.1
c10(1)	2.3×10^5	3.0×10^6		6.0×10^{10}	
drc1	6.0×10^6	7.7×10^{10}	6.2	3.0×10^{11}	6.7
drc3	8.0×10^6	1.2×10^{10}	5.9	5.5×10^{11}	6.5

[a] Hosts were infected upon inoculation into the broth at 34 C.

[b] The final plaque-forming units per milliliter and pH were recorded after 6 h of incubation.

[c] 0.1 M Tris; initial pH with Tris, 7.1; without Tris, 6.6.

Staggered infection in litmus milk was used to explore conditions for obtaining maximum titers, and it was found (Table 1) that the time of bacteriophage addition was critical. By using a 1% (vol/vol) host inoculum and 30 C incubation, highest titers were achieved, with only three exceptions (eb4, c2, and c13), by infecting the culture at 4 h. When infection was delayed until 6 h, there was a dramatic drop in the final titer attained for most bacteriophages examined. At optimum infection times, the final titers were generally 2 to 3 logs higher than that previously reported for lactic bacteriophages propagated in milk (8, 11, 17, 18, 19). After 18 h of incubation, many litmus milk cultures containing the highest bacteriophage titers were incompletely reduced, with little or no acid and with slight or no coagulation. On the other hand, host cultures infected at 6 h (low final bacteriophage titers) were completely reduced and firmly coagulated. Thus, the degree of successful bacteriophage propagation could be readily monitored by inspection of the tube contents.

Preparation of high titers from lactic broth cultures. Production of milligram quantities of bacteriophage was necessary for the nucleic acid characterization studies now under way in our laboratory. To our knowledge, only the preliminary report of Lowrie deals with the

TABLE 3. *Influence of NaCl and the concentrating effect of PEG on titers of phage lysates*[a]

Phage-host system	Titer of lactic broth lysate after addition of		After overnight in 1 M NaCl	Titer of PEG concentrated lysate from		Titer of supernatant
	1.0 M NaCl	0.5 M NaCl		1.0 M NaCl	0.5 M NaCl	
eb7	1.1×10^9		1.3×10^8	1.1×10^{10}		2.2×10^7
ml1	1.1×10^{10}		6.7×10^8	8.3×10^{10}		2.6×10^7
drc2			7.0×10^7	2.4×10^{10}		1.1×10^7
drc1	4.3×10^9			3.3×10^{10}		
drc3	9.5×10^{10}			1.0×10^{12}		
c2 (w)		1.2×10^{10}			1.0×10^{11}	
c10 (1)		6.0×10^{10}			5.4×10^{11}	
c10 (2)		1.6×10^{10}			3.0×10^{11}	
h1	2.1×10^{10}			7.2×10^{12}		
p2	9.0×10^9			3.7×10^{12}		
r6	6.8×10^{10}			3.1×10^{12}		

[a] Host cells and phage were inoculated simultaneously as described in Materials and Methods into 50 ml or 1 liter of lactic broth containing 0.1 M Tris buffer, pH 7.1, at 34 C. PEG concentration was initiated after 6 h of phage propagation. Measurements are in plaque-forming units per milliliter.

TABLE 4. *Comparison of phage titers after repeated freezing[a] and thawing of a 15% glycerol whey lysate*

Phage		Initial phage titer[b] × 10^7	No. times thawed and refrozen	Length of storage (months)	Final phage titer[b] × 10^7	Survival (%)
eb4	Trial 1	730	6	7.5	740	101.2
	Trial 2	250	9	10	280	112.0
		250	18	29	270	108.0
eb7	Trial 1	190	6	7.5	150	78.9
	Trial 2	3,200	13	11	2,700	84.4
		3,200	>20	29	2,470	77.0
ml1		100	8	7.5	92	92.0
e8	Trial 1	380	3	7.5	4	1.1
	Trial 2	2,900	8	10	700	24.1
	Trial 3	440	3	9	330	75.0
		440	12	27	320	72.7
hp		1,100	12	10	1,100	100.0
		1,100	>20	29	1,020	92.8
c2	cw(w)	700	7	7.5	440	62.9
		700	16	24	430	61.5
c2 (w)		90	4	7.5	91	101.0
c10 (1)	Trial 1	1,500	6	7.5	570	38.0
	Trial 2	10,000	8	11	3,000	30.0
		10,000	>20	29	2,900	29.0
c10 (2)	Trial 1	1,900	6	7.5	460	24.2
	Trial 2	8,200	10	11	1,100	13.4
	Trial 3	0.000023	5	12	0.0000081	35.2
drc1	Trial 1	2,000	8	7.5	820	4.1
		40,000	8	11	1,700	4.3
		40,000	>20	29	1,200	3.0
drc2	Trial 1	180	9	7.5	203	112.8
	Trial 2	2,900	10	10	2,400	82.8
		2,900	>20	29	2,400	82.8
c1	Trial 1	2,600	6	7.5	1,000	38.5
	Trial 2	11,000	12	11	2,500	22.7
		11,000	>20	29	1,800	16.4
c3	Trial 1	110	9	7.5	114	103.7
	Trial 2	1,000	12	11	930	93.0
		1,000	>20	29	930	93.0
	Trial 3	300	12	11	300	100.0
c11	Trial 1	380	10	7.5	420	110.4
	Trial 2	940	13	11	810	86.3
		940	>20	29	790	84.0
c13	Trial 1	3,100	10	7.5	1,400	45.2
	Trial 2	4,100	3	11	2,700	66.0
	Trial 3	2,300	16	11	900	39.1
		2,300	>20	29	890	38.7

[a] Lysates were frozen at −22 C and thawed in a 37 C water bath.
[b] PFU/ml.

preparation of a large volume of high-titer lactic bacteriophage (16). In that report, dealing only with phage ml3, the host was concentrated 10-fold just prior to infection in fresh, double-strength medium containing 0.005 M calcium borogluconate. Several modifications of this procedure did not yield satisfactory results in our hands with several other phage-host systems. We, therefore, have developed a reliable system for achieving titers of 10^{10} to 10^{13} PFU/ml by using buffered lactic broth coupled with the PEG-NaCl concentration technique.

The use of 0.1 M Tris was found to maintain the pH near 7.0 without any adverse effects on bacterial growth. Table 2 compares the results of phage infection of homologous hosts in unbuffered or Tris-buffered lactic broth. In each instance, a lower final titer in the absence of added buffer was observed. More significant was the much higher final titers found with c2, c10(1), and eb7 in Tris-buffered as compared with unneutralized lactic broth. These fast acid-producers apparently overwhelm the phage particles with acid and thereby prevent any significant increase in titers. There exists a serious problem then, when a low-titer phage preparation is used for inoculum. In contrast, infection of even fast acid-producers with a few milliliters from a low phage titer lysate in 0.1 M Tris-buffered lactic broth allows maintenance of the pH above 6.0 through several cycles of infection. Thus, final titers greater than 10^{10} to 10^{11} PFU/ml are obtained.

The effect of pH on production of phage particles has been documented (8, 14, 19). The current study is in agreement with earlier observations but offers a simple, routine procedure for overcoming the pH problem for preparation of large volumes of lactic bacteriophages in high titer.

Concentration of phage lysates. Yamamoto et al. (23) recently reported that PEG-NaCl solutions would allow rapid bacteriophage sedimentation by low-speed centrifugation and that large scale virus purification could be obtained with its application. As shown in Table 3, the average lysate was concentrated 10-fold by using 0.5 M NaCl. With 1 M NaCl, the average concentration was 10-fold, but occasionally reached as high as 100-fold. When using 1 M NaCl, more than 99.9% of the phage were removed from the lysate. Final titers of 1 to 30 $\times$ 10^{10} PFU/ml were routinely achieved. One point of interest was that the lysates suffered up to a 100-fold decrease in titer after standing overnight in 1 M NaCl, before centrifugation or addition of PEG. This indicated that the procedure should not be interrupted before the PEG is added to the salt solution. The use of PEG otherwise proved to be a fast, economical, and efficient technique to obtain large amounts of high-titer lactic phage lysate. In fact, 1 liter of lysate, prepared and concentrated as described above, yielded 2 to 4 mg of DNA. Without PEG, the volume of lysate required to obtain this same yield would be 10 to 50 liters. Preparation of broth lysates in the absence of Tris buffer, particularly from fast-acid producers, would be impractical, requiring from 100 to 10,000 liters of lysate to obtain milligram yields of phage.

Storage of lysates in 15% glycerol. Freezing in 15% glycerol was investigated as a means of storage for bacteriophage stocks in whey lysates. This method had been examined by Henning (11) who suggested that phages stored at -20 C in sterile 10% nonfat milk with 15% added glycerol could be recovered after freezing and thawing. Unfortunately, no data were provided to substantiate the usefulness of this menstrum. We have found that, although the percentage of survival varied with each phage, storage of viruses in a 15% glycerol-whey mixture was successful in maintaining stock phage preparations for 2 years or more, even with repeated freezing and thawing (Table 4). Low- as well as high-titer phage suspensions were stored in this manner, and the results indicate that phage lysates may be stored indefinitely in this manner.

The procedures described in this study also have been applied to an additional 18 bacteriophages isolated from commercial cheddar cheese whey samples. The results obtained with these phages substantiated the results obtained with the laboratory phage strains reported in this paper.

ACKNOWLEDGMENTS

This investigation was supported in part by a grant from Microlife Technics, Sarasota, Fla. U.S. Public Health Service grant EF-69 from the Food and Drug Administration, and by Training grant no. 2 T01 GM00704-11 from the National Institute of General Medical Sciences.

We gratefully acknowledge the technical assistance of Michele LaChance during the course of this work.

LITERATURE CITED

1. Adams, N. H. 1959. Bacteriophages. Interscience Publishers, Inc., New York.
2. Cherry, W. B., and D. W. Watson. 1949. The *Streptococcus lactis* host-virus system. I. Factors influencing quantitative measurement of the virus. J. Bacteriol. **58**:601-610.
3. Cherry, W. B., and D. W. Watson. 1949. The *Streptococcus lactis* host-virus system. II. Characteristics of virus growth and the effect of electrolytes on virus adsorption. J. Bacteriol. **58**:611-620.
4. Collins, E. B. 1962. Behavior and use of lactic streptococci and their bacteriophages. J. Dairy Sci. **45**:552-558.
5. Collins, E. B., F. E. Nelson, and C. E. Parmelee. 1949.

Nutrition of the lactic group of streptococci and its relation to bacteriophage multiplication. J. Dairy Sci. **32**:701-702.
6. Elliker, P. R. 1950. The problem of bacteriophage in the dairy industry, p. 24-29. Proc. 11th Annu. Biol. Colloq., Oregon State Univ.
7. Elliker, P. R., A. W. Anderson, and G. Hannesson. 1956. An agar culture medium for lactic acid streptococci and lactobacilli. J. Dairy Sci. **39**:1611-1612.
8. Erskine, J. M. 1970. Adsorption of lactic streptococcal bacteriophage by milk proteins. N. Z. J. Sci. Technol. **5**:861-864.
9. Graham, D. M., and N. N. Potter. 1953. Growth characteristics of the lactic streptococcus bacteriophage. Appl. Microbiol. **1**:138-142.
10. Hadley, P., and E. Dabney. 1926. The bacteriophage relationship between *E. coli*, *S. faecalis*, and *S. lacticus*. Proc. Soc. Exp. Biol. Med. **24**:13-18.
11. Henning, D. R., D. Kienle, W. E. Sandine, and P. R. Elliker. 1964. Factors affecting isolation and survival of bacteriophages for lactic streptococci. J. Dairy Sci. **47**:977-980.
12. Henning, D. R., W. E. Sandine, P. R. Elliker, and H. Hays. 1965. Studies with a bacteriophage inhibitory medium. J. Milk Food Technol. **28**:273-277.
13. Hunter, G. J. E. 1943. Bacteriophages for *Streptococcus cremoris* phage development at different temperatures. J. Dairy Res. **13**:136-145.
14. Krueger, A. P., and J. Fong. 1937. The relationship between bacterial growth and phage production. J. Gen. Physiol. **21**:137-150.
15. Lawrence, R. C., and L. E. Pearce. 1972. Cheese starters under control. Dairy Ind. **7**:73-78.
16. Lowrie, R. J., and L. E. Pearce. 1970. Preparation of high titer bacteriophage. Proc. 18th Int. Dairy Congr. **1E**:134.
17. Lowrie, R. J., and L. E. Pearce. 1971. The plating efficiency of bacteriophages of lactic streptococci. N. Z. J. Dairy Sci. Technol. **6**:166-171.
18. Lowrie, R. J., and L. E. Pearce. 1971. Use of indicator strains for the assay of bacteriophages of lactic streptococci. N. Z. J. Dairy Sci. Technol. **6**:192.
19. Overcast, W. W., F. E. Nelson, and C. E. Parmelee. 1950. Influence of pH on proliferation of lactic streptococcus bacteriophage. J. Bacteriol. **61**:87-95.
20. Potter, Norman N., and F. E. Nelson. 1953. Role of calcium and related ions in proliferation of lactic streptococcus bacteriophage. Iowa Agricultural Experiment Station, Ames, Iowa **66**:508-516.
21. Sandine, W. E., P. R. Elliker, Lois K. Allen, and W. E. Brown. 1962. Symposium on lactic starter cultures. J. Dairy Sci. **45**:1262-1266.
22. Whitehead, H. R., and G. A. Cox. 1935. The occurrence of bacteriophage in cultures of lactic streptococci. N. Z. J. Dairy Sci. Technol. **16**:319-320.
23. Yamamoto, K., B. Alberts, R. Benzinger, L. Lawhorne, and G. Treiber. 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. Virology **40**:734-744.