

Method for Growth and Purification of Bacteriophage 643 on *Streptococcus lactis* ML3

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Received 15 December 1983/Accepted 25 April 1984

The yield of bacteriophage 643 was increased by infecting cultures of *Streptococcus lactis* ML3 in late-log phase growth, harvesting the infected cells, and suspending them in fresh, phosphate-buffered minimal medium. The cells lysed after this treatment and produced high titers of bacteriophage. The phage particles were dissociated from debris by 2 M NaCl and purified by differential and CsCl band centrifugation.

Many lytic bacteriophages specific for streptococci have been isolated from dairy factory whey samples as a consequence of problems arising through the infection of starter cultures during cheese manufacture. Various authors (5, 8, 11) have published studies of host range, morphology, and serology of streptococcal phage isolates, but little is known about the genetics, genome size, and genome structure of these viruses. This is due, in part, to difficulties encountered in preparing sufficient quantities of phage DNA.

The host organisms are nutritionally fastidious and require complex media. Normal phage maturation and lysis in infected cultures is inhibited by low pH conditions which arise as a result of lactic acid production by the culture, and as a consequence, final phage titers are generally low. Nyiendo and co-workers (8) reported a general method for growing lactic streptococcal phages in a complex medium buffered with Tris and obtained titers of up to 10^{13} PFU/ml of lysate for some strains. The phage particles were precipitated and concentrated from crude lysates with polyethylene glycol. In our hands, this procedure proved unsatisfactory for the production of high-titer lysates of the *Streptococcus lactis* phage 643 and the purification of bacteriophage from these lysates. We report here a method for the growth and purification of *S. lactis* phage 643 in the low-phosphate M16 medium of Lowrie and Pearce (6). Phage purified by this method was used for the preparation of DNA in milligram quantities that was used to demonstrate the unusual features of this DNA that we have described elsewhere (7; D. J. Lyttle and G. B. Peterson, Virology, in press).

Bacteriophage 643 and its host, *S. lactis* ML3, were obtained from the New Zealand Dairy Research Institute, Palmerston North. *S. lactis* ML3 was maintained on slopes of M16 agar (5) and was subcultured weekly. Phage stocks were prepared from single plaque isolates and stored as suspensions in 56/2 buffer (2).

S. lactis ML3 was grown in M16 (6) or M17 (11) medium in screw-capped Erlenmeyer flasks at 35°C with gentle shaking. Medium M17 differs from M16 by the addition of β -disodium glycerophosphate, which increases its buffering capacity (11). The absorbance of growing cultures was measured at 600 nm against a medium blank. Bacteriophage 643 plaque counts (PFU) were determined by the soft agar overlay method (1) after overnight incubation at 35°C. Both the hard agar and the soft agar overlay were supplemented with 5 mM calcium borogluconate. Bacterial colony counts of uninfected cultures were similarly determined by serially diluting the culture and plating the diluted cells in soft agar.

The ability of cultures of *S. lactis* to support phage growth in the two different media, M16 and M17, was investigated. Cultures were infected with identical amounts of phage early in log phase. The results are presented in Table 1.

It was found that lysed cultures of *S. lactis* ML3 that had been infected with phage 643 at low multiplicities of infection gave titers of 1×10^{10} to 2×10^{10} PFU/ml in M16 medium and 2×10^{10} to 4×10^{10} PFU/ml in M17 medium. Marginally higher phage yields were obtained if cultures grown in M17 medium were infected in early log phase (2.5 h), and yields declined in cultures which were infected late in log phase (3.5 h). A bacterial culture in M16 medium infected late in log phase did not lyse, and phage production was reduced by two orders of magnitude. On further investigation (see below) it was shown that cells harvested from such cultures lysed and yielded high titers of phage when suspended in fresh buffer. It was not possible to achieve titers greater than about 4×10^{10} PFU/ml from normal phage lysates, even if the multiplicity of infection was varied over an order of magnitude.

A culture (500 ml) of *S. lactis* ML3 was grown and infected with bacteriophage 643. The culture lysed 2 h after infection, and the cooled lysate was centrifuged at $8,000 \times g$ for 10 min. The distribution of viable phage in the various fractions derived from this culture is summarized in Table 2. Approximately two-thirds of the PFU from the original crude lysate sedimented at $8,000 \times g$ in this experiment. The purification of phage from such lysates, clarified by centrifugation at $6,000 \times g$, proved difficult due to the presence of bacterial cell wall debris which could not be separated from the phage by conventional purification procedures. Ethanol at pH 3.8 quantitatively precipitated all of the phage particles, but on resuspension this material appeared to be irreversibly aggregated. In a preliminary experiment it appeared that polyethylene glycol at 6% in the presence of 0.5 M NaCl precipitated the phage from crude lysates, but it was subsequently found that in this preparation, which had been centrifuged at $8,000 \times g$ before adding polyethylene glycol, ca. 40% of the total PFU remained in the 6% polyethylene glycol supernatant. A substantial proportion (60%) of the PFU in crude lysates were recovered by centrifugation alone, and treatment with polyethylene glycol at 6% resulted in precipitation of this particulate, bound phage fraction. No phage was precipitated by polyethylene glycol if this fraction was completely removed by centrifugation of crude lysates at $29,000 \times g$ instead of at $8,000 \times g$.

Several treatments designed to dissociate phage from the particulate fraction were investigated. It was found that the phage particles were quantitatively dissociated from the

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TABLE 1. Comparison of the growth of bacteriophage 643 on *S. lactis* ML3 in M16 and M17 media

Medium	Infection time (h)	Optical density (600 nm)	Nominal multiplicity of infection	Lysis time (h)	Final titer of culture (PFU/ml)
M17	2.5	0.450	0.005	4	2.6×10^{10}
M17	3.5	1.120	0.002	5.5	2.2×10^{10}
M16	2.5	0.435	0.005	4	1.0×10^{10}
M16	3.5	0.923	0.002		1.0×10^8

particulate debris upon treatment of lysates with 2 M NaCl, and the debris could then be removed by centrifugation at $29,000 \times g$. The phage particles were recovered from the $29,000 \times g$ supernatant by centrifuging at 34,000 rpm in a Spinco type 35 rotor.

It was observed that some cells harvested from an unlysed, infected culture of *S. lactis* that had been suspended in 52/2 (2) buffer lysed after overnight storage at 4°C and yielded phage at titers of $>10^{11}$ PFU/ml. This delayed lysis phenomenon did not occur in cultures grown in M17 medium. Figure 1 summarizes the information derived from observations of a large number of infected cultures grown in both media. The stage of growth at which cultures of *S. lactis* were susceptible to bacteriophage infection and lysis is superimposed on the experimentally determined growth curves in M16 and M17 media. Preparation of bacteriophage from infected cells that had been resuspended and allowed to lyse greatly increased yields. The stage of growth at which the culture was infected was critical for the success of the procedure; if the culture was infected too early, premature lysis of the cells occurred and gave reduced phage yields; if infection was delayed, the culture entered stationary phase and did not support phage growth.

The procedure finally adopted for the growth of phage 643 on *S. lactis* ML3 was as follows. An overnight culture of *S. lactis* ML3 was grown at 35°C in a gyratory shaking bath. Five milliliters of the overnight culture was inoculated into 500 ml of M16 medium and grown at 35°C with gentle agitation in a gyratory shaking bath for 3.5 to 4 h to an optical density at 600 nm of 0.6 to 0.7. The culture was made 5 mM with calcium borogluconate and infected with phage 643 at a nominal multiplicity of infection of 2×10^7 PFU/ml. The infected culture was incubated for a further 2 h at 35°C and harvested by centrifugation at $16,000 \times g$ for 10 min. The bacterial pellet was suspended in 56/2 buffer (75 ml) and stored overnight at 4°C to allow the cells to lyse.

DNase and RNase (each at 2 µg/ml) were added to the cell lysate, which was incubated at 37°C for 30 min. The lysate was adjusted to 2 M salt by adding solid NaCl, left on ice for 1 h, and centrifuged at $27,000 \times g$ for 30 min. The supernatant from this step was then transferred to polycarbonate bottles and centrifuged at 34,000 rpm for 150 min in a Spinco

TABLE 2. Purification of phage 643 from a crude lysate^a

Stage	Vol (ml)	Titer	Total PFU ($\times 10^{12}$)	% Recovery ^b
Crude lysate	475	1.34×10^{10}	6.37	100
$8,000 \times g$ pellet	20	1.98×10^{11}	3.96	62
$8,000 \times g$ supernatant	475	3.76×10^9	1.72	28

^a Viable phage before and after the initial purification steps.

^b Total recovery, 90%.

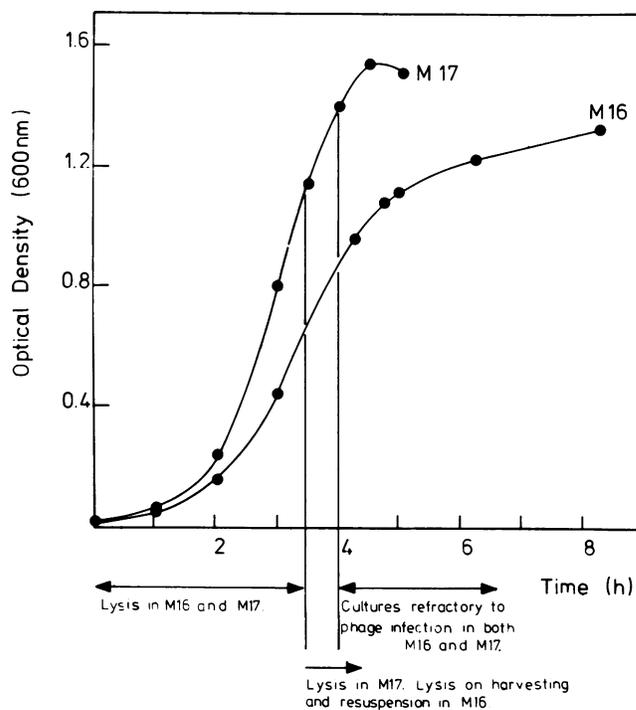


FIG. 1. Susceptibility of cultures of *S. lactis* ML3 growing in M16 and M17 media to lysis after infection with bacteriophage 643. The stage of growth at which cultures of *S. lactis* ML3 were susceptible to bacteriophage infection and lysis is superimposed on the experimentally determined growth curves in M16 and M17 media. The diagram summarizes the information derived from observation of a large number of infected cultures.

type 35 rotor. The pellet was suspended in a small volume (5 to 6 ml) of 56/2 buffer and stored at 4°C in this buffer. Table 2 presents the results of four separate preparations. The key step in the procedure, the premature harvesting of the unlysed cells followed by their suspension in 56/2 buffer, not only provided a convenient means of concentrating the bacteriophage preparation initially but resulted in increased yields of phage. Treatment of lysates with high concentrations of salt dissociated phage from cell debris, overcoming the problems of aggregation that proved troublesome in other procedures that were tested.

As a final purification step, the phage were purified on CsCl step gradients constructed by successively underlayering 1-ml volumes of CsCl solutions of densities 1.3, 1.4, 1.5, and 1.6 g/ml, respectively, in 5-ml cellulose nitrate tubes. One milliliter of partially purified phage suspension was layered onto each gradient, which was then centrifuged for 36 h at 35,000 rpm in a Spinco SW-65 rotor. Peak concentrations of viable phage and DNA, as measured by the diphenylamine colorimetric procedure (3), coincided with the major peak of 260 nm absorbance.

Fractions from the central portion and the trailing edge of a phage peak purified on a preparative CsCl gradient were pooled separately. The uncorrected sedimentation coefficients (12) for each sample were found to be identical within the range of experimental error (64S and 67S, respectively).

Electron micrographs of bacteriophage 643 were taken by Susan Burgess and S. Bullivant of the Department of Cell Biology, Auckland University. Samples of bacteriophage were diluted onto grids and stained with phosphotungstic

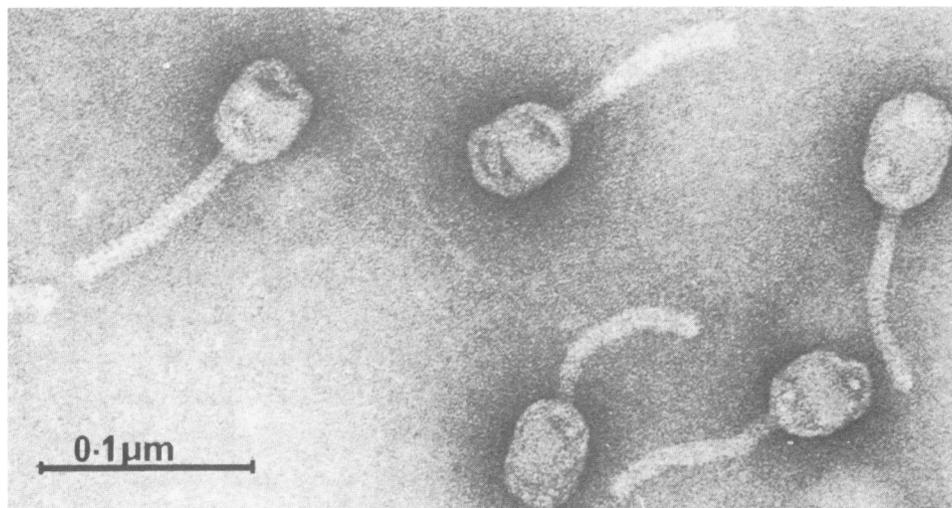


FIG. 2. Electron micrograph of bacteriophage 643 particles. Phage particles were diluted onto an electron microscope grid and negatively stained with phosphotungstic acid. The figure shows phage particles purified on CsCl step gradients.

acid. Phage dimensions were measured directly from the photographic prints. The phage particles were uniform in appearance (Fig. 2), showed prolate morphology, and had a striated tail with a channel running through the center which was seen more clearly in particles with empty heads. Some particles appeared to have a number of minute spikes terminating the tail, but the definition of these structures was equivocal. By virtue of its morphology, bacteriophage 643 was assigned to the streptococcal phage group d (10), and this has been independently confirmed by Heap and Jarvis (4). The head measurements made in the present study fall within the range for prolate phage quoted by the latter authors (values in parentheses), i.e., diameter, 35 nm (35 to 47 nm); and length, 53 nm (54 to 61 nm). The tail length of phage 643 measured in this study (98 nm) was, however, found to be shorter than the 130 nm recorded by Heap and Jarvis (4).

The delayed lysis procedure reported in this paper increased the yields of bacteriophage 643 from infected cultures and achieved a concomitant concentration step. Cultures of *S. lactis* ML3 infected in late-log phase growth and which were apparently refractory to lysis were found to lyse upon harvesting and suspension of the cells in a phosphate-buffered minimal medium (56/2 medium). Delayed lysis was successful only for cultures grown in M16 medium; cultures grown in the more strongly buffered M17 medium were found to support lytic phage growth when infected at a cell density that was suitable for M16 medium. The simplest explanation for this finding is that phage maturation and growth are arrested by high levels of acid in the culture medium, and if such a culture is harvested and resuspended in fresh medium, lysis will occur. Under normal conditions, bacteriophage 643 produces a potent lysozyme which could also serve to limit further multiplication of phage as a result of the premature lysis of uninfected cells.

Phage particles purified on CsCl gradients were homogeneous with respect to buoyant density and sedimentation coefficient and were used for the preparation of phage 643 DNA. It was essential to establish that the purified phage preparations did not contain multiple phage species since it was found that the DNA extracted from these preparations contained several molecular species (7; Lyttle and Petersen, in press).

We thank T. Thomas of the New Zealand Dairy Research Institute, Palmerston North, for cultures of the bacteriophages and their hosts used in this study, and S. Burgess and S. Bullivant of the Department of Cell Biology, University of Auckland, for preparing the electron micrographs of bacteriophage 643. We thank G. Hughes for her skilled technical assistance and Diana F. Hill for helpful discussions.

This study was assisted by grants (to G.B.P.) by the Medical Research Council of New Zealand and by the Medical Committee of the New Zealand Lottery Board of Control.

LITERATURE CITED

1. Adams, M. H. 1959. Bacteriophages, p. 27-43. Interscience Publishers Inc., New York.
2. Adelberg, E. A., and S. N. Burns. 1960. Genetic variation in the sex factor of *Escherichia coli*. *J. Bacteriol.* **79**:321-330.
3. Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**:315-323.
4. Heap, H. A., and A. W. Jarvis. 1980. A comparison of prolate and isometric headed lactic streptococcal bacteriophages. *N. Z. J. Dairy Sci. Technol.* **15**:75-81.
5. Jarvis, A. W. 1977. The serological differentiation of lactic streptococcal bacteriophage. *N. Z. J. Dairy Sci. Technol.* **12**:176-181.
6. 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.
7. Lyttle, D. J., and G. B. Petersen. 1979. Properties of bacteriophage 643 DNA: demonstration of a single strand gap in the duplex DNA molecule of the mature bacteriophage. *Proc. Univ. Otago Med. Sch.* **57**:61-62.
8. Nyiendo, J., R. J. Seidler, W. E. Sandine, and P. R. Elliker. 1974. Preparation and storage of high-titer lactic streptococcus bacteriophages. *Appl. Microbiol.* **27**:72-77.
9. Pearce, L. E. 1978. The effect of host-controlled modification on the replication rate of a lactic streptococcal bacteriophage. *N. Z. J. Dairy Sci. Technol.* **13**:166-171.
10. Terzaghi, B. E. 1976. Morphologies and host sensitivities of lactic streptococcal phages from cheese factories. *N. Z. J. Dairy Sci. Technol.* **11**:155-163.
11. Terzaghi, B. E., and W. E. Sandine. 1975. Improved medium for lactic streptococci and their bacteriophages. *Appl. Microbiol.* **29**:807-813.
12. Vinograd, J., R. Bruner, R. Kent, and J. Weigle. 1963. Band-centrifugation of macromolecules and viruses in self generating density gradients. *Proc. Natl. Acad. Sci. U.S.A.* **49**:902-909.