Bacteriophages of L-Glutamic Acid-Producing Bacteria

Part X. Size and Morphology of *Brevibacterium* Phages*

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Received September 1, 1967

Morphological properties of the P- and Ap-series phages described in previous papers were examined by means of electron microscopy, analytical centrifugation, CsCl density-gradient centrifugation and ultrafiltration.

In view of the buoyant density of phage particles, fourteen *Brevibacterium* phages were classified into four groups, *i.e.*, the phages under group I had buoyant density of 1.511 to 1.514 g cm⁻³, and those under groups II, III and IV, densities of 1.482, 1.492 and 1.508 g cm⁻³, respectively; and above grouping corresponded to that by the serological characteristics of the phages.

Electron microscopic observation by means of shadow-casting or negative staining recognized all the phages as tadpole-shaped; their particles having polyhedral head (40~70 m μ in diameter) and long tail (80~275 m μ in length).

In relation to particle sizes of the phages as estimated by all of above-mentioned methods, no significant differences were observed between the sizes calculated from ultrafiltration and those obtained directly from electron micrographs by negative staining.

It has been known that there is great variety in size and morphology among phages and that these characteristics are likely to be more valuable as taxonomic criteria for phages than they have been for bacteria.¹⁾ However, the morphological studies on the phages of L-glutamic acid-producing bacteria have been reported only by few workers.^{2,3)} One of the authors previously stated⁴⁾ that the phages which belong to serological group I among the

the phages of *Brevibacterium lactofermentum* No. 2256, namely, P61, P114, P401 and P465, had the shapes similar to tadpole, each with a long tail attached to a spherical head; and that electron microscopic observation by shadow-casting with metal estimated the sizes of the phage particles at 75~100 m μ for the heads and 180 $\times$ 20~260 $\times$ 15 m μ for the tails.

The present paper further describes the morphological properties of the four representative phage particles of *Br. lactofermentum*, in view of electron microscopy, ultrafiltration, analytical centrifugation and CsCl density-gradient centrifugation.

MATERIALS AND METHODS

Phages and bacteria. Four phages each representing one serological group⁵⁾ and two host strains were

* This work was presented at the Meeting of the Kansai Division of Agricultural Chemical Society of Japan, held at Kyoto, on September 24, 1966.

1) H. M. Adams, "Bacteriophages", Interscience Publishers, N. Y., 1959, p. 426.

2) T. Tsuchiya, T. Shiio, H. Okada, I. Kameyama and T. Tsunoda, *Nippon Nogeikagaku Kaishi*, **37**, 686 (1963).

3) S. Seto, T. Osawa and S. Yamamoto, *ibid.*, **39**, 41 (1965).

4) T. Oki, T. Matsui, K. Akahoshi and R. Harada, *ibid.*, **38**, 381 (1964); *idem.*, *Amino Acid and Nucleic Acid*, **9**, 59 (1964).

5) T. Oki, T. Matsui and A. Ozaki, *Agr. Biol. Chem.*, **31**, 861 (1967).

used; more specifically, the phages P465 (group I), P468 (group II) and Ap85III (group III) and their host strain YO2, and the phage P4 and its host strain 468-5SM^c of *Br. lactofermentum* No. 2256 were used. These host strains were used as indicators when titers of the phages were determined. Other twelve phages and their host strains⁵⁾ were also used in some part of this work. In determination of buoyant density of phage particles, the phages λ ch and ϕ 80c of *E. coli*, obtained from Dr. H. Ozeki, were used as references of density; their host strains were *E. coli* C600 and W3623, respectively.

Assay of phages. Assay was proceeded by the double-layer method as described in previous papers.^{6,7)}

Preparation of purified phages. High-titer phage lysates were prepared by the procedure previously described.⁸⁾ Such lysates contained phage titers of $5 \sim 10 \times 10^{10}$ PFU per ml. Fresh lysates were stored in a refrigerator for a week, during which period mucoid material was much eliminated by spontaneous precipitation. The lysates were filtrated with Millipore filter type AA. The phages were then concentrated from such lysates by differential centrifugation as previously described.⁹⁾ The phage pellet was slowly resuspended in a 0.01 M phosphate buffer (pH 7.4) containing 0.1 M NaCl. The phage suspension was further purified by CsCl density-gradient centrifugation using a Hitachi 40SP Swinging Roter, at 25,000 rev/min, for 20 hr; and the purified phage was dialyzed in a 0.01 M phosphate buffer (pH 7.4) containing 0.1 M NaCl, for removal of CsCl. Thus dialyzed pure phage suspension had an opalescent appearance and contained approximately $10^{11} \sim 10^{12}$ PFU per ml.

Preparation of specimens for electron microscopy. Above-mentioned pure phage suspension was adequately diluted either with distilled water or with 0.01 M phosphate buffer (pH 7.5). The specimens were prepared by shadow-casting with Pt-Pd alloy or by negative staining with phosphotungstic acid. Shadow-casting was performed as follows: The diluted phage suspension was deposited in the form of minute droplets on carbon-coated collodion-filmed

microscope grids, air-dried, then washed with distilled water once or twice, dried again, and finally shadow-casted with Pt-Pd alloy.

On the other hand, the technique as described by Brenner and Horne⁹⁾ was applied to the proceedings of negative staining. A 2% solution of phosphotungstic acid (PTA) was adjusted to pH 7.2 with normal KOH, and 1.0 ml of above PTA was added to the same amount of the phage suspension in water. The mixture was allowed to stand for 15 min at room temperature, and thereafter the solution was dropped directly on the carbon-coated grids. These specimens were examined with an Electron Microscope (Japan Electron Optics Laboratory Model JEM 7) at instrumental magnifications of 40,000 to 100,000 $\times$.

Ultrafiltration of phage particles. Commercially produced membranes, Millipore filters, greatly facilitate filtration procedures. A rough estimation of the size of phages can be readily done: the equipment described by Hsiung¹⁰⁾ was used for filtration of phage suspension. A 5-ml syringe was attached to a pair of Swinny hypodermic adapters connected one upon the other with a hypodermic needle. Such was autoclaved at 115°C for 15 min, and a Millipore filter AA type with large pore diameter was placed in the upper adapter and the test membrane, in the lower. The types of Millipore membranes used were: AA ($0.8 \pm 0.05 \mu$), DA ($0.65 \pm 0.03 \mu$), HA ($0.45 \pm 0.02 \mu$), PH ($0.30 \pm 0.02 \mu$), GS ($0.22 \pm 0.02 \mu$), VC ($100 \pm 8 \text{ m}\mu$), VM ($50 \pm 3 \text{ m}\mu$) and VF ($10 \pm 2 \text{ m}\mu$). They were sterilized by exposing to ultraviolet light for 30 min on each side. Phage titers in the suspension were determined before and after filtration, and the range of particle sizes of phages was determined according to the filtration end point.

Ultracentrifugal analysis of phage particles. Purified phage suspension in a 0.01 M phosphate buffer containing 0.1 M NaCl (pH 7.4), at a concentration of $5 \sim 10 \times 10^{10}$ PFU per ml, was analyzed at 12,590 to 14,290 rev/min with a Spinco Ultracentrifuge Model E equipped with ultraviolet optics. The sedimentation boundaries were photographed on Kodak commercial films at 4-minute intervals. These photographs were analyzed with a Spinco Analytorol. The sedimentation coefficient was corrected to the water basis at the temperature of 20°C and was expressed

6) T. Oki, T. Matsui, K. Akahoshi and R. Harada, *Nippon Nogeikagaku Kaishi*, **38**, 376 (1964).

7) T. Oki, T. Matsui, K. Akahoshi and R. Harada, *Amino Acid and Nucleic Acid*, **9**, 53 (1964).

8) T. Oki and K. Ogata, *Agr. Biol. Chem.*, **32**, 242 (1968).

9) S. Brenner and R. E. Horne, *Biochem. Biophys. Acta*, **34**, 103 (1959).

10) G. D. Hsiung, *Bacteriol. Rev.*, **29**, 481 (1965).

in the Svedberg units of sedimentation.

Preparative CsCl density-gradient centrifugation. The technique as described by Meselson *et al.*^{11,12} was applied. CsCl obtained from E. Merk AG was used to bring the density of the phage suspension to $1.50 \pm 0.01 \text{ g cm}^{-3}$. This was done by mixing 2.4 g of CsCl in a 3.0-ml phage suspension which contains the phage to be examined, of a final concentration of $1 \sim 5 \times 10^6$ PFU per ml, and the standard reference phages, λ ch and ϕ 80 c. When final adjustment of density was necessary, it was made by the addition of a small amount of water or CsCl. The density at 25°C was calculated as follows:

$$\rho^{25} = 10.8601 n_D^{25} - 13.4974^{13}$$

where n_D^{25} is designated as the index of refraction of the solution for sodium light at 25°C, determined in an Abbé refractometer. A 3.0-ml adjusted phage suspension was then poured into a lusteroid tube with about 0.5 g of paraffin, and such was centrifuged with a Hitachi 40SP Swinging Bucket Rotor at 25,000 rev/min. After 20 hr in operation, the equilibrium was closely kept, and the rotor was allowed to come to rest without braking. Then, the plastic tube was taken out, and the resultant phage suspension emerging from the bottom of the tube that was pierced with a fine needle was collected, three drops in each small tube. From the 3.0-ml sample suspension were yielded 150 drops which rendered successive density layers in the gradient. Each fraction was then analyzed by usual method for its phage titer and density.

RESULTS

Determination of the buoyant density of fourteen phages.

The phages were scarcely inactivated in a 45% CsCl solution (1.5 g cm^{-3}) below 10°C for 24 hr. The buoyant densities of the phages in the CsCl solution, as calculated from the position of the band, *i. e.*, the value of the density gradient and the buoyant density of the

TABLE I. DENSITY OF PHAGE PARTICLE.

Serological group	Phage	Density (g cm^{-3})
I	P61	1.513
	P114	1.513
	P401	1.511
	P465	1.511
	P468I	1.513
	P508	1.512
	P650	1.513
	P204	1.512
	Ap615	1.514
II	P468II	1.482
	Ap85II	1.481
	P468III	1.492
III	Ap85III	1.491
	P4	1.508
IV	λ ch	1.508
	ϕ 80 c	1.493

CsCl phage suspensions were centrifuged in a Hitachi 40SP swinging bucket rotor at 25,000 rev/min for 20 hr. Each value is an average of at least two determinations.

standard reference phages (*E. coli* λ ch: 1.508 and ϕ 80 c: 1.493 g cm^{-3}), were 1.481 to 1.514 g cm^{-3} as shown in Table I. The densities of the phages varied largely from one serological group to another, and the phages were classified accordingly into four groups which corresponded to the serological groupings. Therefore, when the four phages P465 (group I), P468II (group II), Ap85III (group III) and P4 (group IV) were centrifuged in the same one tube with the standard reference phage, the differences in density of these phages were distinctive, as graphically represented with four peaks in Fig. 1. The density of the group I phages was the highest and that of the group II, the lowest. With respect to serological group I, seven host range mutants⁵¹ derived from an original parental phage P61 were centrifuged simultaneously in one tube and thereafter assayed separately with a specific phage-resistant strain. The results are given in Table I, where the difference of of buoyant density was slightly shown.

11) J. Weigle and M. Meselson, *J. Mol. Biol.*, **1**, 379 (1959).

12) M. Meselson, F. Stahl and J. Vinograd, *Proc. Nat. Acad. Sci. Wash.*, **43**, 581 (1957).

13) J. B. Ifft, D. H. Voet and J. Vinograd, *J. Phys. Chem.*, **65**, 1138 (1961).

FIG. 1. Preparative CsCl Density-Gradient Centrifugation of *Brevibacterium* phages.

I: P465, II: P468II, III: Ap85III, IV: P4.

Electron microscopic observation of phage particles.

The electronmicrographs show only four morphologically different phages as prepared by negative staining. As seen in Figs. 2 to 5, there are but a little morphological differences among the groups. Any structural details of the heads could not be observed, but the heads appeared either hexagon or circle. They can be said therefore to be polyhedral-shaped. The tails were relatively long and sheath-like; the tail-fibers, as seen upon T-even

coli phages, could not be seen upon all these phages examined. The results obtained by platinum-paradium (Pt-Pd) shadow-casting were also similar to those of negative staining.

As the result of measuring the phages by the size of their heads and tails, the four phages were different morphologically. The results are shown in Table II, with the results previously reported.⁴¹ The phages under group I had elongated hexagonal heads, each about 60 m μ in diameter with a remarkably

FIG. 2. Electronmicrograph of Phage P465 by Negative Staining Method.

Embedded in alkaline phosphotungstic acid. Magnification: $\times 50,000$.

FIG. 3. Electronmicrograph of Phage P468II by Negative Staining Method.

Embedded in alkaline phosphotungstic acid. Magnification: $\times 40,000$.

FIG. 4. Electronmicrograph of Phage Ap85III by Negative Staining Method.

Embedded in alkaline phosphotungstic acid. Magnification: $\times 50,000$.

FIG. 5. Electronmicrograph of Phage P4 by Negative Staining Method.

Embedded in alkaline phosphotungstic acid. Magnification: $\times 50,000$.

long tail of about $300\text{ m}\mu$ in length. Those in group II were extremely small, with spherical heads, each about $40\text{ m}\mu$ in diameter with a relatively short tail of about $80\text{ m}\mu$ in length. And, the phages under groups III and IV were intermediate in size, situated between those of groups I and II.

Determination of sedimentation coefficient and filtration end point from ultrafiltration.

Ultracentrifugation was also used for measuring the size of the phage particles. The phages which were purified by CsCl density-gradient centrifugation gave the typical ultraviolet absorption spectrum as shown in Fig. 6. The ultracentrifugal patterns, as taken by means of the ultraviolet optics, showed an extremely sharp sedimenting boundary, which indicates a high degree of molecular homogeneity, as an example shown in Fig. 7. The sedimentation coefficients, $s_{20,w}$, of P465, P468II, Ap85III and P4, in a 0.1 M phosphate buffer (pH 7.4) containing 0.1 M NaCl, were respectively 347, 203, 314 and 569. Assuming spherical particles, the apparent diameters were calculated as below, from $s_{20,w}$, as based on the Stokes Law:

$$r^2 = 9 \eta s_{20,w} / 2(\rho v - \rho s)^{14)},$$

TABLE II. ESTIMATES OF PARTICLE SIZE OF *Brevibacterium* PHAGES BY ELECTRON MICROSCOPE

Serological group	Phage	Dimensions in $\text{m}\mu$			
		Negative staining with PTA		Shadow-casting with Pt-Pd	
		Head	Tail	Head	Tail
I	P465	50×60	275×10	90×95	310×15
	P61	—	—	90	210×15
	P114	—	—	75	260×15
	P401	—	—	80	250×15
	P465	—	—	100	280×20
II	P468II	40	80×10	55	95×15
III	Ap85III	50	203×15	70	240×18
IV	P4	65×70	150×18	75×85	165×20

Each value represents the average of twenty or more particles.

* Shadow-casting with chromium.

14) F. W. Putnam, *Science*, **111**, 481 (1950).

FIG. 6. UV Absorption Spectra of Purified Phage in 0.01 M Phosphate Buffer (pH 7.4) plus 0.1 M NaCl.

- A: P468II, 8.9×10^{10} PFU/ml.
 B: Ap85III, 5.9×10^{10} "
 C: P465 ($\times 3$), 4.0×10^{10} "
 D: P4 ($\times 2$), 4.1×10^{10} "

FIG. 7. Sedimentation Pattern of Purified Phage P468II.

Phage concentration: 8.9×10^{10} PFU/ml in 0.01 M phosphate buffer plus 0.1 M NaCl (pH 7.4).

The photographs were taken at the time intervals of 4 minutes after reaching 14,290 rev/min.

$s_{20,w} = 203$.

in which r designates radius; η , the viscosity of water at 20°C ($0.01013 \text{ g/cm} \cdot \text{sec}$); and ρ_v and ρ_s , the density of the phage particle and the solvent (1.0047 g/cm^3), respectively. As shown in Table III, apparent spherical diameters calculated from sedimentation were fairly small as compared with the dimensions obtained directly from electronmicrographs.

One of the simplest and oldest methods for estimating the size of phage particles is ultrafiltration. This is usually done by passing a

TABLE III. PARTICLE SIZE ESTIMATION OF *Brevibacterium* PHAGES BY ULTRACENTRIFUGATION AND ULTRAFILTRATION

Phage	Ultracentrifugation		Ultrafiltration* mμ
	$s_{20,w}$ 10 ⁻¹³ sec	d_{eq} mμ	
P465	347	34	66
P468II	203	28	36
Ap85III	314	34	50
P4	569	46	62

$s_{20,w}$: represents the average of two experiments.

d_{eq} : The apparent spherical diameter computed from the sedimentation coefficient.

*: Computed from filtration end point using Millipore filter.

phage suspension through a series of collodion membranes of graded porosity. The size of phage particles is related to the size of pores in the finest filter where infectious phage particles go through. Several limitations and complications had been encountered^{15,16}, and besides, we were out of graded membranes, as they were not supplied commercially since some years ago. However, we have found that the commercially produced Millipore

FIG. 8. Determination of Filtration End Point by Millipore Filter.

- ×: P465   ●: Ap85III
 ○: P468II   △: P4

15) W. J. Elford and C. H. Andrewes, *Brit. J. Exp. Pathol.*, **13**, 36 (1932).

16) W. J. Elford, J. R. Perdraw and W. Smith, *J. Pathol. Bacteriol.*, **36**, 49 (1933).

filters would greatly facilitate filtration procedures, and rough estimate of the size of phages could be made readily using a series of Millipore filters of various pore sizes. The results of ultrafiltration with the four phages are presented in Fig. 8. In this the average pore diameter of the membrane, which holds the phage so that there is no phage activity in the filtrate, is the filtration end point. By the use of the Elford's empirical correction factor^{17,18)}, these filtration end points were converted into the corresponding particles diameters as shown in Table III. These values were exactly met with the dimensions obtained from electronmicrographs.

DISCUSSION

The size or weight of phage particles can be calculated from density, electronmicrograph dimension, sedimentation and diffusion constants and ultrafiltration dimension; and it has always been employed as a parameter in classification of phages.

By means of CsCl density gradient, the fourteen phages were classified into four groups, which corresponded to their serological groupings. The buoyant density of these phages was about 1.5 g cm^{-3} as reported by Weigle and Meselson¹¹⁾ and Matsushiro.¹⁹⁾ This is close to the average of the buoyant density of DNA (1.7 g cm^{-3}) and of protein (1.3 g cm^{-3}), suggesting that the phages contain DNA and protein equal in volume.

With respect to the morphological characteristics of the phages of L-glutamic acid-producing bacteria, there are a few results of studies reported^{2,3)} on electron microscopic observation by means of shadow-casting, but detailed studies on size and shape of phage particles have not been published. By

electron microscopic observation, it was ascertained that all the *Brevibacterium* phage particles were characteristically tadpole-shaped with polyhedral heads and long tails like many other phages which had been discussed²⁰⁻²³⁾; and that the four phages each representing one serological group were different each other morphologically.

In relation to particle sizes of the four phages as estimated by various methods listed in Tables II and III, there were no significant differences between the sizes calculated from ultrafiltration and those obtained directly from electronmicrographs by negative staining. But, the results from shadow-casting with metals are larger than those from negative staining; and dimensions as calculated from the sedimentation coefficient are somewhat smaller as compared with those on the electron microscope and ultrafiltration data which were given of *coli* phage by Putnam.²⁴⁾

For example, the head of the phage, Ap85III, was $50 \text{ m}\mu$ in diameter by negative staining, but $70 \text{ m}\mu$ by metal shadowing. The phage P465 had apparent equivalent spherical diameter of $66 \text{ m}\mu$ according to ultrafiltration; of $34 \text{ m}\mu$ as calculated from the sedimentation coefficient; and of $50 \times 60 \text{ m}\mu$ or $90 \times 95 \text{ m}\mu$, according to the type of contrast employed on electron microscopic observation. Therefore, it should be noted that the equivalent spherical diameter of a tailed phage as calculated from $s_{20,w}$ is the diameter of a sphere of the same mass and volume as the actual nonspherical particle, so it does not correspond to the overall dimensions of the particle.

On the basis of the electronmicrograph dimensions and density as mentioned above,

17) W. J. Elford, "Handbuch der Virusforschung" ed. by R. Doerr and C. Hallauer, Springer, Wein, 1938, p. 126.

18) W. J. Elford and C. H. Andrewes, *Brit. J. Exp. Pathol.*, **13**, 446 (1932).

19) A. Matsushiro, K. Sato and S. Kida, *Virology*, **23**, 299 (1964).

20) H. M. Adams, "Bacteriophages", Interscience Publisher, N. Y., 1959, P. 40.

21) M. Hongo and A. Murata, *Agr. Biol. Chem.*, **30**, 292 (1966).

22) M. Terada, "Studies on Bacterial Viruses", Naya Co., Tokyo, 1956.

23) R. C. Williams and D. Feaser, *J. Bacteriol.*, **66**, 458 (1953).

24) F. W. Putnam, *J. Polymer Sci.*, **12**, 391 (1954).

the dry weight of particles of the phages, P465, P468II, Ap85III and P4, were calculated to be 1.71×10^{-16} , 0.50×10^{-16} , 0.98×10^{-16} and 2.48×10^{-16} g, respectively; and, therefore, the Avogadro particle weight (the weight of N particles) was the order of 1 to 2×10^8 g. The values of these phages agree with those of many other phages.^{20,24)}

Acknowledgement. The authors wish to express their sincere thanks to Dr. H. Ozeki of the National Institute of Health and to

Emeritus Prof. H. Katagiri, Prof. K. Onodera, Dr. T. Tochikura, Dr. H. Yamada and Dr. S. Toyama of Kyoto University for their kind guidances and suggestions during the course of this investigation. The authors are also indebted to Mr. M. Suzuki, President, and Dr. A. Ozaki, Director of the Central Research Laboratories, of Sanraku-Ocean Co., Ltd., for their constant encouragement throughout the course of this work, and to Mr. H. Kawasaki and Mr. S. Yasuda for their technical assistances.