Bacteriophages of L-Glutamic Acid-Producing Bacteria

Part IX. Properties of Deoxyribonucleic Acids from *Brevibacterium* Phages and Their Host

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Deoxyribonucleic acids were isolated from *Brevibacterium lactofermentum* No. 2256 and its four representative phages belonging to different serological groups, *i.e.*, P465 (group I), P468 II (group II), Ap85 III (group III) and P4 (group IV), by phenol extraction.

DNA's isolated from the four phages contained only usual four bases, *i.e.*, guanine, adenine, cytosine and thymine. The G-C content of the phage DNA was determined by thermal denaturation method (T_m); the values of P465, P468 II, Ap85 III and P4 were 54.0, 54.6, 56.6 and 55.3%, respectively. Sedimentation coefficient was measured by ultracentrifugal analysis using ultraviolet optics; $s_{20,w}$ of the phage DNA's were 15.5 to 31.8 s.

Unusual bases were not detected also in the host bacterial DNA. The G-C content of the bacterial DNA determined by paperchromatography was 55.1% which came very close to the G-C content derived from the T_m .

In spite of the fact that a large number of investigation of the nucleic acids of various phages have so far been made, nothing has been reported on the nucleic acids of phages of the L-glutamic acid-producing bacteria. Takayama *et al.*¹⁾ reported that the base compositions of DNA's from representative strains of L-glutamic acid-producing microorganisms were measured and that most of them showed 56% guanine-cytosine content (G-C %).

The present study describes the properties of the DNA's from the four representative *Brevibacterium* phages and their host strain of *Br. lactofermentum* No. 2256, in respect to isolation, sedimentation coefficient and base composition.

MATERIALS AND METHODS

Phages and bacterium. The four phages each

representing a serological group, *i.e.* P465 (group I), P468 II (group II), Ap85 III (group III) and P4 (group IV), and their host strain, *Br. lactofermentum* No. 2256—Y 02, were used.²⁾

Concentration and purification of phages from crude lysates. High titer phage lysates were prepared as follows; To 300 ml of G-medium²⁾ in a 3-litre shaking flask 10 ml of the overnight culture broth of the host strain was inoculated, which was shaken for about 4 hr at 30°C. When the number of cells therein reached 1.2×10^8 per ml, phage was added to give a final concentration of $5 \sim 10 \times 10^8$ plaque-forming units (PFU) per ml, and it was incubated with shaking until the maximum lysis occurred (measured by the absorbancy of the culture at 610 m μ), which was usually obtained within 15~20 hr. This lysate was stored in a refrigerator for 7 days and then centrifuged to remove bacterial cells and debris. This supernatant fraction was termed crude lysate. Such lysates contained phage titers of $8 \times 10^8 \sim 5 \times 10^{10}$

1) K. Takayama, S. Abe and S. Kinoshita, *Nippon Nogeikagaku Kaishi*, **39**, 342 (1965).

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

PFU/ml. Further purification was carried out in a cold room at 4°C.

The crude lysate was then concentrated and purified by the enzyme treatment and differential centrifugation as indicated in Fig. 1. A Hitachi 40SP Centrifuge for ultracentrifugation and a Kubota KR-6P Centrifuge for a low-speed centrifugation were used. Ribonuclease (RNase) I and deoxyribonuclease (DNase), the enzymes used for enzyme treatment, were the product of Sigma Chemical Company; and trypsin, of Worthington Biochemical Corp.

The purified final phage suspension as obtained above had a milky, opalescent appearance and contained approximately $10^{12} \sim 10^{13}$ PFU per ml.

Isolation of phage DNA. Phage DNA was separated from protein by the phenol method of Kaiser and Hogness.³⁾ All operations were carried out at cold-room temperature of about 4°C. The purified phage suspension and phenol freshly saturated with standard saline-citrate buffer (0.15 M NaCl plus 0.015 M Na-citrate, pH 7.0: hereafter referred to SSC), equal in volume, were mixed in a glass-stoppered tube and shaken by hand for 5 min. Two phases of above mixture were further separated by centrifugation, and the aqueous layer was recovered. This process

was repeated twice more, at each time fresh SSC-saturated phenol being mixed with the recovered aqueous layer. The three resulting phenolic layer residues were extracted successfully with a small volume of SSC. The two resulting aqueous fractions were combined and dialyzed against SSC, until phenol was thoroughly removed. The clear and viscous solution obtained contained approximately 300 to 500 μg of DNA per ml. The DNA was stored in the solution at 5°C. For the use as reference, the DNA's of *E. coli* T7 and T4 were prepared by a procedure similar to above.

Bacterial cultures. G-Medium used contained 10 g of glucose, 8 g of urea, 1 g of KH_2PO_4 , 0.4 g of MgSO_4 , 4 g of corn steep liquor, 2 mg of Fe^{++} , 2 mg of Mn^{++} , 2 μg of biotin and 100 μg of thiamine-HCl, per litre. Fully grown cells on nutrient agar at 30°C for 24 hr were harvested and then suspended in above G-medium to a concentration of around 10^9 cells per ml. One milliliter of this suspension was inoculated into 20 ml of the culture medium in a 500-ml shaking flask. Culturing was carried out at 31°C on a reciprocal shaker, during which sterilized 50% urea solution was added when necessary to maintain the culture medium slightly alkaline. The cells at an early stage of the stationary phase were harvested by centrifugation and washed once with saline-EDTA. About 8 g of packed cells were usual-

3) A. D. Kaiser and D. S. Hogness, *J. Mol. Biol.*, **2**, 392 (1960).

FIG. 2. Isolation of Bacterial DNA

a) 0.15 M NaCl-0.1 M EDTA (pH 8).

b) Sodium lauryl sulfate

c) 4 vols. of phenol plus 1 vol. of 0.1 M Tris-HCl buffer —1% SLS —0.1% NaCl

d) SSC, 0.15 M NaCl plus 0.015 M Na-citrate

e) 9 vols. of 0.015 M NaCl —0.0015 M citrate (pH 7) plus 1 vol. of 3 M acetate —0.001 M EDTA.

ly obtained from 1 litre of culture broth.

Isolation of bacterial DNA. On the cell wall lysis of the L-glutamic acid-producing bacteria, Takayama *et al.*¹⁾, Shibukawa *et al.*⁴⁾ and Hirose *et al.*⁵⁾ have reported that the cells are resistant to

lysozyme, trypsin and sonic oscillation. The sonic oscillation with sodium lauryl sulfate (SLS) was the most effective when tested by preliminary experiments. DNA was extracted from the cell sonicate with phenol by the procedures of Marmur,⁶⁾ Takayama *et al.*¹⁾ and Saito and Miura,⁷⁾ as described in detail in Fig. 2. These procedures can be outlined

4) M. Shibukawa, S. Okabe, A. Miura and T. Ohsawa, *Amino Acid and Nucleic Acid*, **14**, 35 (1966).

5) Y. Hirose, J. Nakamura, H. Okada, K. Kinoshita, I. Kameyama and T. Shiio, *Nippon Nogekagaku Kaishi*, **41**, 19 (1967).

6) J. Marmur, *J. Mol. Biol.*, **3**, 208 (1961).

7) H. Saito and K. Miura, *Biochem. Biophys. Acta*, **72**, 619 (1963).

as follows: first, the cells are disrupted; and the cell debris and protein are removed by denaturation and centrifugation, and then RNA, by RNase I (Sigma Chemical Co.); and DNA is obtained by selective precipitation with isopropanol. Approximately 8 to 10 mg of DNA was obtained from 10 g of wet packed cells.

Determination of sedimentation coefficient and molecular weight by analytical centrifugation. The sedimentation coefficient, $s_{20,w}$, of DNA which was dissolved in the standard saline-citrate was determined by a Spinco Ultracentrifuge Model E, using ultraviolet optics, at a concentration of 20 μ g/ml and 59,780 rev/min in speed. The temperature was maintained at 15°C throughout the experiments. Sedimentation boundaries were photographed on Kodak commercial films at the interval of 4 min. The photographs were analyzed with a Spinco Analytorol, equipped with automicroanalyzer attachment. The molecular weight of the sample was calculated using the relationship established by Doty, McGill and Rice⁸⁾:

$$s_{20,w} = 0.063 M_w^{0.37}$$

Determination of base composition of DNA from its thermal denaturation temperature. The thermal denaturation temperature, T_m , was determined by the methods of Marmur and Doty^{9,10)} and Doty *et al.*¹¹⁾ The DNA concentration was approximately 20 μ g per ml in SSC, and DNA was contained in a 3-ml glass-stoppered quartz cuvette having a 1-cm light path. The samples were placed in the chamber of a Shimadzu Multipurpose Spectrophotometer Model MPS-50, where the temperature could be raised by circulating hot water through around the chamber. The temperature in the cuvette was raised at a rate of around 1°C per min, which was measured by a Toshiba Thermistor Thermometer TT-2 D. Setting the optical density at 25°C as 1, the ratio (relative absorbance) was plotted against the temperature of the solution. The base composition was calculated according to the Marmur and Doty's equation¹⁰⁾:

$$\text{Per cent G-C} = (T_m - 69.3)/0.41$$

As reference phage DNA, the DNA's *E. coli* T 4 and T 7 of were used.

Analytical procedures. Phosphorus was determined by the Nakamura's method.¹²⁾ DNA, RNA and protein were determined by the indole method,^{13,14)} the orcinol method¹⁵⁾ and Folin's method,¹⁶⁾ respectively.

The base compositions of DNA of the host bacteria were determined also by descending paperchromatography with the hydrolyzates by perchloric acid.¹⁷⁾ Approximately 5 mg of DNA was hydrolyzed with 0.2 ml of 70 per cent perchloric acid at 100°C for 2 hr. The above was chromatographed on sheets of Toyoroshi No. 51 A filter paper with the isopropanol-HCl-H₂O mixture as the solvent system described by Wyatt.¹⁸⁾ The bases were eluted with 0.1 N HCl from the paper-strips and were quantitated according to the Bendich's method.

RESULTS AND DISCUSSION

Characterization of Phage DNA

The four representative phages purified by means of enzyme treatment and differential centrifugation were analyzed for DNA and RNA by the indole and orcinol reactions, respectively. Consequently, the four *Brevibacterium* phages were found to contain only DNA as nucleic acid (RNA less than 1%).

Four phage DNA's isolated by the phenol method contained 9.0% of phosphorus and less than 2% of phage protein which remained in the aqueous layer after phenol extraction. The ultraviolet absorption spectrum of phage DNA's drew the curve as shown in Fig. 3, for an example, which coincided with

12) M. Nakamura, *Nippon Nageikagaku Kaishi*, **24**, 1 (1950).

13) Z. Dische, "The Nucleic Acids", Vol. I, ed. by E. Chargaff and J. N. Davidson, Academic Press, Inc., N. Y., 1955, p. 285.

14) G. Ceriotti, *J. Biol. Chem.*, **198**, 297 (1952).

15) G. Ceriotti, *ibid.*, **214**, 59 (1955).

16) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *ibid.*, **193**, 265 (1951).

17) A. Marshak and H. J. Vogel, *J. Biol. Chem.*, **189**, 597 (1951).

18) G. R. Wyatt, *Biochem. J.*, **40**, 584 (1951).

19) A. Bendich, "Methods in Enzymology," Vol. III, ed. by S. P. Colowick and N. O. Kaplan, Academic Press, Inc., N. Y., 1957, p. 715.

8) P. Doty, B. B. McGill and S. A. Rice, *Proc. Nat. Acad. Sci., Wash.*, **44**, 432 (1958).

9) J. Marmur and P. Doty, *Nature*, **183**, 1427 (1959).

10) J. Marmur and P. Doty, *J. Mol. Biol.*, **5**, 109 (1962).

11) P. Doty, H. Boedtker, J. R. Fresco, R. Haselkorn and M. Litt, *Proc. Nat. Acad. Sci., Wash.*, **45**, 482 (1959).

FIG. 3. The Ultraviolet Absorption Spectra of Phage P 465 DNA.

The DNA was dissolved in 0.15 M NaCl and 0.015 M Na-citrate (pH 7.0)

$\epsilon(P)$ = molar absorptancy index relative to phosphorus.

TABLE I. CHARACTERIZATION OF PHAGE DNA'S

Phage DNA	Wavelength (mμ)		260 230	260 280	$\epsilon(P)$ (260 mμ)	Hyperchromicity by DNase (%)
	max.	min.				
P 465	258	233	2.4	1.7	6800	29
P 468 II	258	234	2.2	1.8	6760	34
Ap 85 III	258	235	2.2	1.5	6730	36
P 4	259	232	2.1	2.0	6900	33

Explanations are the same as those in Fig. 3.

Treatment with pancreatic DNase: DNA of 20 μg/ml was incubated with 5 μg/ml DNase in Mg-saline (0.9% NaCl containing 0.005 M MgSO₄) at 30°C.

that of solutions of the native DNA.²⁰⁾ The absorption maximum was, at 259 mμ, $\epsilon(P)$ = 6730 to 6900; and the minimum, at 234 mμ, $\epsilon(P)$ = 2950 to 3180. When phage DNA is treated with pancreatic DNase, the molar absorptancy index relative to phosphorus at 260 mμ, $\epsilon(P)$, increases 1.29- to 1.36-fold at pH 7 in Mg-saline (0.9% NaCl containing 0.005 M MgSO₄) as shown in Table I.

An example of the ultracentrifugal pattern was taken, as in Fig. 4, by using ultraviolet optics (approximately 20 μg of phage DNA per ml). An exceedingly sharp sedimenting boundary shows that there is no unsediment-

FIG. 4. Ultracentrifuge Sedimentation Pattern of Phage P 468 II DNA in 0.15 M NaCl plus 0.015 M Na-citrate.

Using ultraviolet optics, pictures were taken every 4 min at 59,760 rev/min.

First exposure at left.

DNA concentration = 20 μg/ml.

Sedimentation coefficient, $s_{20,w}$ = 22.5.

TABLE II. SEDIMENTATION COEFFICIENT AND MOLECULAR WEIGHT OF DNA FROM PHAGES

Phages	$s_{20,w}$ (Svedberg units)	Molecular weight $\times 10^6$
P 465	15.5	2.90
P 468 II	22.5	7.91
Ap 85 III	31.8	20.1
P 4	25.8	11.4

$s_{20,w}$ was measured at a DNA concentration of approximately 20 μg/ml in 0.15 M NaCl plus 0.015 M Na-citrate (pH 7.0) at 12 to 15°C.

Molecular weight was computed from sedimentation coefficient by the Doty's equation: $s_{20,w} = 0.063 Mw^{0.37}$

able material and that the DNA preparations by this method hold a high degree of purity. Table II lists sedimentation coefficient, $s_{20,w}$, extrapolated to zero concentration and molecular weight calculated on the basis of the Doty's equation.^{8,1)} $s_{20,w}$ of P 465, P 468 II, Ap 85 III and P 4 were 15.5, 22.5, 31.8 and 25.8 s, respectively. Molecular weights computed from these data were 2.90×10^6 to 20.1×10^6 . These values almost agree with the molecular weight of native DNA from various phages as mentioned by Eigner and Doty.²¹⁾ In addition to these data, the thermal denaturation curves

20) G. H. Beaven, E. R. Holiday and E. A. Johnson, "The Nucleic Acids," Vol. I, ed. by E. Chargaff and J. N. Davidson, Academic Press Inc., N. Y., 1955, p. 493.

21) J. Eigner and P. Doty, *J. Mol. Biol.*, **12**, 549 (1965).

as will be described later indicate that the DNA samples isolated are predominantly in the native double-stranded configuration.

Determination of Base Composition of Phage DNA from Its T_m

The usual four bases, *i.e.*, guanine, adenine, cytosine and thymine, were detected from the four phage DNA's by paperchromatography. The mole per cent G-C was evaluated from Doty's equation, using their thermal denaturation temperature (T_m values) determined in SSC. Fig. 5 presents typical

FIG. 5. Thermal Denaturation Curves of Phage DNA's.

The optical density at each temperature was divided by the value at 25°C and the ratio was plotted as relative absorbance at 260 $m\mu$.

- ×: P 465
- : P 468 II
- : Ap 85 III
- △: P 4

thermal denaturation curves: all the curves of the four phage DNA's were remarkably sharp, and the magnitude of the hyperchromicity was 42 to 48 per cent. The T_m values, together with the mole per cent G-C values, are listed in Table III. The T_m values of DNA's from the phages T 4 and T 7 used as reference agreed with those as referred in a

TABLE III. MOLE PER CENT G-C, T_m AND BUOYANT DENSITY OF DNA'S FROM PHAGES

Phages	T_m (°C)	Mole per cent G-C		Buoyant density in g cm ⁻³	
		From T_m (a)	Literature	From (a)(b)	Literature
<i>E. coli</i> T 4	84.1	36.1	36.0 ⁽¹¹⁾	1.695	1.698 ⁽²³⁾
T 7	89.3	48.8	48.0	1.707	1.710
P 465	91.4	54.0	—	1.713	—
P 468 II	91.7	54.6	—	1.714	—
Ap 85 III	92.5	56.6	—	1.716	—
P 4	92.0	55.3	—	1.714	—

Each value represents the average of two or more independent experiments.

T_m was determined using SSC as the solvent.

(a) Computed by Doty's equation from T_m .

(b) Computed via Schildkraut's equation using data (a).

literature.⁽¹¹⁾

The G-C contents of DNA's of *Brevibacterium* phages are 54~56.6 per cent, which are higher values among those of other phages, such as T-odd and -even phages, S 20 V phage of *S. marcescens*⁽¹⁰⁾ and *Clostridium* phages.⁽²²⁾ No considerable differences in the G-C content of the DNA were observed among the four phages. Therefore, the similarity of the G-C content among them indicates that the four phages are closely related.

If the buoyant density of phage DNA is calculated from the mole per cent G-C value according to the equation, as:

$$\rho = 0.098 (\text{G-C}) + 1.660 \text{ g cm}^{-3(23)}$$

(ρ =buoyant density in g cm⁻³; (G-C)=mole per cent guanine plus cytosine), it is 1.713 to 1.716 as shown in Table III, at the right column.

Characterization and Base Composition of Bacterial DNA

DNA isolated from the host strain, YO2, by the phenol method contained 8.9% of phosphorus. Content of impurities such as

22) M. Hongo, H. Ono, S. Ogata and A. Murata, *Agr. Biol. Chem.*, **30**, 982 (1966).

23) C. L. Schildkraut, J. Marmur and P. Doty, *J. Mol. Biol.*, **4**, 430 (1962).

RNA or protein was less than 2%. It gave typical ultraviolet absorption spectrum: the absorption maximum at 260 m μ was $\epsilon(P)$ =6750; and the minimum at 234 m μ , $\epsilon(P)$ =3070. An ultracentrifugal pattern taken by ultraviolet optics (approximately 20 μ g of DNA per ml) showed an exceedingly sharp sedimenting boundary as those of phages. From this pattern, sedimentation coefficient, $s_{20,w}$, of the host bacterial DNA was calculated to be 22.5. The thermal denaturation curve was sharp, and the magnitude of the hyperchromicity was 34%. The T_m values, as well as per cent G-C values, for host strain, are given in Table IV. The G-C contents were agreeable

bases (guanine, cytosine, adenine and thymine). The DNA base compositions determined by paperchromatography are shown in Table IV. The molar ratios of adenine to thymine and guanine to cytosine are both close to 1, indicating that this DNA is double-stranded. The G-C content, 55.1%, obtained by chemical determination was close to that, 54.1%, derived from the T_m .

With respect to the DNA base composition of L-glutamic acid-producing bacteria, Takayama *et al.*¹¹ reported that the strains of a group which had higher activities in glutamic acid production showed the G-C content of 56% by chemical procedure. The values of G-C content of the four phages are 54 to 56.6% as mentioned above. Accordingly, there is little difference in the DNA base compositions between DNA's of the host bacteria and of the phages.

TABLE IV. DNA BASE COMPOSITION OF *Br. lactofermentum* No. 2256

Method	Exp.	Mole per cent				T_m (°C)	Per cent G-C
		gua- nine	ade- nine	cyto- sine	thym- ine		
Chemical analysis	1	28.4	22.0	27.1	22.4	—	55.5
	2	27.9	22.3	27.1	22.6	—	55.0
	3	28.0	22.6	26.7	22.7	—	54.7
Melting temp.	1					91.2	53.4
	2					91.7	54.6
	3					91.5	54.2

with those of the phage DNA's and were slightly higher than the value (53.7%) reported for a strain of *Br. lactofermentum* by Hirose *et al.*⁵¹

By chemical analysis of the DNA from the host strain, the four bases were detected on the chromatogram, having the R_f values which correspond to the values of authentic

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