Bacteriophages of L-Glutamic Acid-Producing Bacteria

Part VI. Serological Characteristics of the Twenty-one *Brevibacterium* Phages*

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Several new phages were obtained from the abnormally fermented broths and from the air in L-glutamic acid fermentation factory using *Br. lactofermentum*. These twenty-one phages were classified into five serological groups on the basis of cross-neutralization tests with homologous and heterologous antisera. Group I contained ten phages, i.e., P61, P114, P401, P465, P468I, P508, P650, P204, Ap615 and L2. Group II contained five phages, i.e., P468II, Ap85II, Ap62, Ap72 and S1. Group III contained P468III, Ap85III, Ap93 and Ap518, and groups IV and V one phage each, P4 and L1, respectively.

In view of serological similarities and of differences in the host specificity, the phages of group I are considered as host range mutants derived from a plaque type mutant, P114, of original phage P61.

INTRODUCTION

In L-glutamic acid fermentations the phage infections are quite serious. Since the authors, in 1964,¹⁻³⁾ reported on the phages P61, P114, P401 and P465 active against *Brevibacterium lactofermentum* No. 2256, several new phages as shown in Fig. 1 have been isolated from the abnormal broth cultures or from the air in the L-glutamic acid fermentation factory.

Classification of phages into serological groups on the basis of inactivation rates in homologous and heterologous antiphage sera offers a reliable method to determine whether there exists a close correlation among them.⁴⁾

Hongo et al.⁵⁾ reported in details with respect to the serology of the phages of acetone-butanol-producing bacterium, *Cl. saccharoperbutylacetonicum*. On the phages of L-glutamic acid-producing bacteria, there is only one report in which the phages of *Mic. ammoniaphilum* were divided into three serological groups.⁶⁾

The present paper describes the serological classification of twenty-one phages and the degree of relatedness among the phages within the same group.

MATERIALS AND METHODS

Bacteria and Phages. Subcultures designated as YO2 and 468-5, derived from the parent culture of *Br. lactofermentum* No. 2256 (ATCC 13869), were used as the indicator of phage count and for propagation of the phage.

The sources of the phages were abnormally ferment-

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1) T. Oki, R. Harada, K. Akahoshi and T. Matsui, *J. Agr. Chem. Soc. Japan* **38**, 192 (1964).

2) T. Oki, T. Matsui, R. Harada, K. Akahoshi and Y. Maeda, *ibid.*, **38**, 198 (1964).

3) T. Oki, T. Matsui, K. Akahoshi and R. Harada, *ibid.*, **38**, 376 (1964).

4) M. H. Adams, "Bacteriophages" Interscience Publishers, N. Y. p. 97, 1959.

5) M. Hongo, T. Aono and A. Murata, *This Journal*, **30**, 399 (1966).

6) S. Soto, T. Ohosawa and S. Yamamoto, *J. Agr. Chem. Soc. Japan*, **39**, 41 (1965).

Year	Bacterial strain	Phage isolated	
		from Fermentor (P-series)	from the Air (Ap-series)
1960	<i>Br. lactofermentum</i> No. 2256		?
1961	—YO2	sluggish	?
1962	—YO2 —YO2 —PF4 (YO2/61) —W413(YO2/114/401) —W413(")	P61 P114 P401 P465 P468I, II, III	?
1963	—W401(YO2/61/114/401/465) —W401(")	P508 P650	?
1964	—W401(")	P204	
1965	—YO2		Ap518, Ap62, Ap615, Ap72, Ap85, Ap95
1966	—468-5 SM ^r	P4	?

FIG. 1. Isolation of P- and Ap-series Phages.

ed broths (designated as P-series) and air in the fermentation plant (designated as Ap-series). Phages L1, L2 and S1 were supplied kindly from Ajinomoto Co., Inc. Whereas the phages P61 and P114 were isolated from the abnormally fermented broth of YO2, all the other phages were isolated from the lysed broth of each phage-resistant strain as shown in Fig. 1.

The phages were isolated as follows. To isolate P-series phages, lysed culture broths in 100-kl fermentor were centrifuged to remove bacteria and suitable dilutions of the supernatant were plated with host bacteria by the Adams' double layer method.⁷⁾ On the Ap-series phages existing in the air, agar plates spread with host bacteria were exposed to the air in fermentation factory for 30 minutes. Then, they were incubated overnight at 30°C and examined for plaques in the film of bacterial growth. Plaques obtained on agar plate were purified by two successive single-plaque isolations. A plaque scrapped from

the agar surface and 1 ml of overnight culture host strain were added to 20 ml of the medium in 500-ml shaking flask. The broth was shake-cultured at 30°C for 20 hours. Resulting lysate was centrifuged for 15 minutes at 6,000 r.p.m. (3,800 × and the supernatant was filtered through a membrane filter (Millipore filter, type PH or DA). Phage stocks with titers of 10⁹~10¹¹ plaque-forming units (PFU) per ml were routinely obtained. All the phages were stable for one year in cold (4°C).

Assay of Phage Titers. The plaque-forming unit of phage suspension was assayed by the Adams' double layer method as described below. Approximately 30 ml of basal layer medium was solidified in petri dishes (20 by 90 mm), and was dried 5 hours in an incubator at 37°C. Tubes containing 2 ml of overlay medium were kept melted in a 45°C water bath. 0.1 ml to 0.5 ml of the phage suspension diluted with nutrient broth or with dilution buffer (0.01 M Tris buffer containing 5 g of NaCl, 0.05 g of MgSO₄·7H₂O, 0.05 g of CaCl₂·2H₂O, 0.1 g of casein in 1,000 ml, pH 7.4) was added

7) M. H. Adams, "Bacteriophages", Interscience Publishers, N. Y., p. 450, 1959.

0.1 ml of the diluted broth of indicator strain. The broth of the indicator strain was previously diluted to give the optical density of 0.4 (10 mm cuvette) at 610 m μ (cell number: $3\sim6\times10^8$ per ml) in nutrient broth. Two milliliters of soft agar kept melted in a 45°C water bath was added to the above mixture. The agar mixture was overlayed on the basal layer. The double-layered plate thus prepared was incubated for about 20 hours at 30°C. The plaques were counted and recorded as plaque-forming units per ml (PFU/ml).

Media. The G medium used for growing bacteria and phages contained 100 g of glucose, 1 g of KH₂PO₄, 0.5 g of MgSO₄·7H₂O, 4 g of corn steep liquor, 8 g of urea, 2 mg of Fe⁺⁺, 2 mg of Mn⁺⁺, 2 μ g of biotin and 100 μ g of thiamine-HCl in 1000 ml of tap water. The pH of the medium was 7.6 after autoclaving for 15 minutes at 115°C. Basal and over layer media for double layer method consisted of 10 g of Polypepton, 10 g of meat extract and 5 g

of NaCl per 1000 ml, and contained, respectively, 1.5 and 0.5 per cent of agar in medium.

Preparation of Antiphage Sera. Antisera were prepared as usual by repeated injection of a phage stock solution into rabbits. The phage lysates were centrifuged twice at 6,000 r.p.m. ($3,8000\times g$) for 15 minutes at 0°C and these supernatant was filtered through a Millipore filter pH. The titers of phage stock solutions were 1 to 40×10^9 PFU per ml. An antigen was injected intravenously three times a week for 3 weeks, starting with 0.5 ml injections and gradually increasing to 6 ml per dose (a total of 30 ml). The bleeding was performed two weeks after the last injection according to the usual procedures. After removing host cell antibodies by absorption with host bacterial suspension, all sera were stored at 0°C without preservative, and were heated at 56°C for 30 minutes before use.

Cross-neutralization Tests. The cross-neutralization tests were performed by adding 0.5 ml of the

TABLE I. CROSS-NEUTRALIZATION BY ANTIPHAGE SERA AGAINST TWENTY-ONE PHAGES

Phages	Groups of antiserum														
	I							II			III		IV	V	
	61	114	401	465	508	650	204	468II	85II	S1	468III	85III	P4	L1	C
P61	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
P114	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
P401	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
P465	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
P468I	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
P508	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
P650	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
P204	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Ap615	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
L2	+	+	+	+	+	+	+	-	-	-	-	-	-	+	-
P468II	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
S1	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
Ap62	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
Ap72	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
Ap85II	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
Ap468III	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
Ap518	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
Ap85III	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
Ap93	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
P4	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
L1	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-

+: Neutralization -: No neutralization C: Normal serum

diluted stock phages, containing 5×10^8 PFU per ml, to 0.5 ml of the diluted antiphage serum (1:10) at 30°C. All dilutions were made in G medium. After 10 minutes, 0.1 ml of the samples of the phage-serum mixture was added to 9.9 ml of dilution buffer, and then plaques were counted by double layer method to determine whether most or all of the phages had been neutralized. A comparison was made between the controls and immune sera. Medium control containing G medium instead of antiserum, the other control contained normal rabbit serum at the same dilution as the immune sera.

Assay of Antiphage Sera. The inactivation rates of the phages by their homologous and heterologous antisera were determined by the following procedures. The phage stocks were diluted to a titer of 1×10^8 PFU per ml. All sera were used at a final dilution of 1:100, 1:500, 1:1000. One ml of phage dilutions was added to 1 ml of serum dilutions at 30°C. At 5 minutes intervals, 0.1 ml of the mixtures was taken out and added to 9.9 ml of G medium to stop antibody reaction. From these tubes, further serial dilutions were made, and phage survivals were assayed by the double layer method. The serological inactivation rate constant, K , was calculated at 90~99% neutralization by the following equation.

$$K = 2.3D/t \times \log P_0/P$$

in which P_0 =phage assay at zero time, P =phage assay at time t minutes, D =final dilution of antisera in the phage-serum mixture.

RESULTS AND DISCUSSION

Serological Grouping

Serology has become an important criterion for classifying phages, and, on the basis of neutralization reactions, Delbrück has suggested that serological characteristics of the T-phages are sufficient to divide these phages into genetically distinct groups.⁸⁾

To detect cross-neutralization reactions, antiserum was prepared against each phage, and then each antiserum was tested against its homologous phage and against all the other phages. As shown in Table I, the result obtained clearly indicated that the twenty-one phages could be divided into five groups. Group I contained ten phages, i.e., P61, P114, P401, P465, P508, P650, P204, P468I, Ap615 and L2. Group II contained five phages, i.e., P468II, Sl, P62, Ap72 and Ap85II. Group III contained four phages, i.e., P468III, Ap85III, Ap518 and

TABLE II. PLAQUE MORPHOLOGY OF TWENTY-ONE PHAGES

Serological group	Source of phage			Plaque size and morphology
	Fermentor	Air	Others	
I	P61			Large turbid (0.7~1.5 mm), fuzzy outline
	P114	Ap615	L2	Large clear (0.7~1.8 mm), sharp outline
	P401			
	P465			
	P468I			
	P508			
	P650			
	P204			
II	P468II	Ap62 Ap72 Ap85II	Sl	Medium clear (0.4~1.2 mm), fuzzy outline
III	P468III	Ap518 Ap85III Ap93		Small turbid (0.2~0.5 mm), fuzzy outline
IV	P4	—	—	Small clear (0.3~0.6 mm), sharp outline
V	—	—	L1	Medium clear (0.5~1.0 mm), sharp outline

⁸⁾ M. Delbrück, *Biol. Revs. Cambridge Phil. Soc.*, **21**, 30 (1946).

Ap93. Groups IV and V contained one phage each, P4 and L1, respectively. From the No. 468 abnormal broth three phages (P468 I, II and III), which showed the characteristic plaque size and morphology, were isolated. These three phages belonged to the different serological groups. Most of the phages isolated from the lysed culture broths belonged to group I. Seven Ap phages existed in the air of fermentation factory belonged to group II or III except one. These twenty-one phages were classified into six plaque morphological groups, as shown in Table II, and this grouping was in complete accord with the serological one, except that plaque type of original phage P61 differed from that of nine other phages under group I. Accordingly, it seems reasonable to assume that the second phage P114 is a plaque type mutant derived from an original parental phage P61.

Kinetics of Phage Neutralization

The neutralization curves of the group II phage, employing three different dilutions of its homologous antiserum, are shown in Fig. 2-a). As is evident in the figure, the logarithm

FIG. 2. Kinetics of Phage P468II Neutralization by Antiphage Sera.

D: Final dilution of sera

—a): with homologous antiserum, anti-468II

—b): with heterologous antisera

1) anti-468III, 85III, 61, 114, 401, 465, 650, 508 and 204

2) anti-S1

3) anti-85II

of the fraction, P/P_0 , of surviving phage decreases linearly with the time, t , the slope of the survival curve being inversely proportional to the dilution, D , of the antiserum in the reaction mixture. Fig. 2-b) presents the result of a neutralization test by heterologous antisera of the same dilution. The kinetics of neutralization of the other P- and Ap-series phages by their antisera also agreed satisfactorily with the first-order equation. The K values of respective homologous antisera against twenty-one phages ranged from 10 to 165 as shown in Table III.

TABLE III. NEUTRALIZATION RATE CONSTANTS OF P- AND Ap-PHAGES WITH HOMOLOGOUS ANTISERA

Group	Phage	K value
I	P61	10
	P114	8
	P401	14
	P465	10
	P508	15
	P650	9
	P204	9
II	P468II	72
	Ap85II	99
	S1	25
III	P468III	47
	Ap85III	45
IV	P4	165
V	L1	27

K values of Antisera to demonstrate Serological Relatedness

In all cases, the rate of neutralization has been greater with homologous phage and antiserum than with heterologous system.⁹⁾ The K value of an antiserum when tested with various phages can be taken as an indication of the degree of relatedness of the phages. Luria,⁹⁾ Hershey,¹⁰⁾ Gilmour and Ingalsbe¹¹⁾ found the host range mutant indistinguishable from the parent phage in K values. Hongo and Murata⁸⁾ used this method

9) S. E. Luria, *Genetics*, **30**, 84 (1945).

10) A. D. Hershey, *ibid.*, **31**, 620 (1946).

11) C. M. Gilmour and C. Ingalsbe, *J. Bacteriol.*, **78**, 193 (1959).

TABLE IV. THE *K* VALUES OF TWENTY-ONE PHAGES WITH HOMOLOGOUS AND HETEROLOGOUS ANTISERA

Antisera	Phages													
	P61	P114	P401	P465	P508	P650	P204	P468II	Ap85II	S1	P468III	Ap85III	P4	L1
Anti— 61	10	12	14	19	11	16	13	0	0	0	0	0	0	0
—114	11	8	12	9	11	11	9	0	0	0	0	0	0	0
—401	14	12	14	17	15	16	17	0	0	0	0	0	0	0
—465	9	10	12	10	11	12	10	0	0	0	0	0	0	0
—508	12	13	14	14	15	16	14	0	0	0	0	0	0	0
—650	8	8	7	7	7	9	7	0	0	0	0	0	0	0
—204	11	10	10	10	11	9	9	0	0	0	0	0	0	0
—468II	0	0	0	0	0	0	0	72	62	61	0	0	0	0
—85II	0	0	0	0	0	0	0	73	99	60	0	0	0	0
—S1	0	0	0	0	0	0	0	29	24	25	0	0	0	0
—468III	0	0	0	0	0	0	0	0	0	0	47	44	0	0
—85III	0	0	0	0	0	0	0	0	0	0	43	45	0	0
— 4	0	0	0	0	0	0	0	0	0	0	0	0	165	0
—L1	0	0	0	0	0	0	0	0	0	0	0	0	0	27

The *K* values represent averages of three trials for each combination, and were calculated at 90% or more neutralization.

TABLE V. HOST RANGE OF GROUP I PHAGES ON PHAGE-RESISTANT MUTANTS OF *Br. lactofermentum*

Resistant strains	P-series phages							
	66	114	401	465	468I	508	650	204
YO2 (original)	+	+	+	+	+	+	+	+
PF4 (YO2/61)	—	+	+	+	+	+	+	+
W413 (YO2/114/401)	—	—	—	+	+	+	+	+
W315 (YO2/114/401/465)	—	—	—	—	+	+	+	+
W401 (YO2/61/114/401/465)	—	—	—	—	—	+	+	+
K5 (YO2/61/401/465/508)	—	—	—	—	—	—	+	+
J2 (YO2/61/401/465/508/650)	—	—	—	—	—	—	—	+
W42 (YO2/61/401/465/508/650/204)	—	—	—	—	—	—	—	—

+: Lysis —: Lack of lysis

to test the relatedness of host range mutants of *Clostridium* HM-phages, and also obtained similar results. This method of antigenic analysis will thus offer a possibility to detect whether there exists a close correlation among these P- and Ap-series phages belonging to the same serological group.

The *K* values for the reciprocal neutralization, calculated with a serum diluted 1:100, are shown in Table IV. From the *K* values, it is clear that phages P4 and L1 are neu-

ralized only by its respective homologous antiserum and not at all by any of the heterologous antisera. As a result, no significant difference in *K* values of each antiserum when tested with the phages of the same group, was found in groups I, II and III. These data indicate that the phages within the same group are very closely related to each other. Therefore, it seems reasonable to assume that phages P61, P468II, P468III and P are the parental phages on the ground of first

appearance and the other phages belonging to groups I, II and III are mutants derived from them. Though the host ranges of the phages of group I on the phage-resistant mutants will be reported in next paper, a part of its results are shown in distinguishing the phage-mutations. Table V shows that the P-series phages of group I are host range mutants derived from an original parental phage P61. In view of these facts and of the order of appearance of the phages in the plant (Fig. 1), the first phage-infection in the plant was caused with an original phage P61, and the second with P114 which was plaque type mutant of P61. The phages which appeared from third to eighth were super-host-range mutants of P114. The fifth, however, was caused with the mixed infection of three phage species which differed in plaque type, host range and serological properties. The ninth was broken out with P4 which was the fourth phage species.

In regard to the phage-infection in acetone-butanol fermentation industry, Hongo and Murata⁵⁾ reported on the occurrence of the serial super-host-range mutations. In bacterial amylase¹²⁾ and cheese industry,¹³⁾ there are

some informations that show the occurrence of host range mutant phages.

A culture rotation system was suggested by Whitehead and Hunter¹⁴⁾ for preventing gross contamination of cheese milk with bacteriophages. Similar suggestion was also described by Hongo et al. in acetone-butanol fermentation.¹⁵⁾ Though the sources of the parental phages in the industrial process are unknown, various forms of culture rotation systems are now in effect to prevent the appearance of host range mutants. The cultures used in rotation must be unrelated with regard to bacteriophage-sensitivity.

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14) H. R. Whitehead and G. J. E. Hunter, *Proc. 12th Intern. Dairy Congr. Stockholm*, **2**, 629 (1949).

15) M. Hongo, A. Murata, R. Harada, K. Akahoshi, K. Nagata and S. Takahashi, *J. Agr. Chem. Soc. Japan*, **39**, 252 (1965).

12) K. Kitahara and T. Kaneko, *J. Agr. Chem. Soc. Japan*, **32**, 411 (1959).

13) F. J. Babel, *Adv. Appl. Microbiol.*, **4**, 51 (1962).