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Studies on the multiplication of OP_1 phage (*Xanthomonas oryzae* bacteriophage). 2

Interference phenomena in the multiplication between OP_1 and OP_{1t}, the growth temperature mutant obtained from OP_1*

SATOSHI WAKIMOTO and HAZIME YOSHII

INTRODUCTION

The multiplication of bacteriophage starts by the injection of the contents—desoxyribonucleic acid—of the phage adsorbed on the surface of the host cell. The penetrated DNA from the parent phage plays in the cell the principal role of the reproduction of new phages as the genetic marker.

When a bacterial cell is infected by two kinds of the virulent phages, the interference phenomena between the phages will take place. Phages are classified as the related with each other or as the unrelated on the standpoint of their serological reaction and morphological relationship. And the interference phenomena between two phages are different in accordance to their mutual relationships.

In the case of the unrelated phages, as it is reported with *Escherichia coli* phage α (T1), δ (T7) and γ (T2), the mutual exclusion phenomena and depressor effect have taken place.[5,6] Namely, in the case of simultaneous infection of two unrelated phages, the contents of one of the two phages penetrate and multiply, while the other phage cannot self-reproduce due to the exclusion of the previously penetrated phage. The excluded phage which adsorbed on the cell surface, in its turn, interferes or depresses the multiplication of the intruded phage.

In the case of the related phages, both two phages penetrate and multiply without any interference between them, if they simultaneously attack the same host cell. In the progenies, besides the reproduction

* Contribution from the Laboratory of Plant Pathology.

of normal phages of the characters of the respective parents, are produced the recombinants having the characters of both two parents as the results of recombination of the parent characters. When infection is unsimultaneous, lapse of time necessary for the excluding action of the prior phage against the succeeding phage is determined by the nature of the former. Interference phenomena of related phages were detailed in the experiments with T-even phages by Dulbecco.[8, 9]

The authors isolated a mutant OP_{1t} phage—which differs in its optimum growth temperature from the wild type OP_1 (*Xanthomonas oryzae* phage). Many kinds of phage mutant such as host range mutant,[7, 13] plaque morphology mutant,[7, 12] adsorption cofactor mutant,[3] proflavine resistant mutant,[11] and heat resistant mutant[2] were reported. The OP_{1t} phage is a new type mutant and is named the growth temperature mutant.

The writers report the experiments on the interference of OP_{1t} to OP_1 phage multiplication studied by the single-burst experiment[4, 10] and the one-step growth experiment.[10]

Materials

OP_1 phage: A host specific bateriophage of *Xanthomonas oryzae*, the causal organism of the bacterial leaf blight of rice, and some physiological and biological characters of the phage were reported.[15]

OP_{1t} phage: The growth temperature mutant obtained from OP_1, and has the same serological and several other characters except in its optimum growth temperature with OP_1.

X. oryzae no. 60: Newly isolated strain obtained from *X. oryzae* no. 49.

CaVfCh medium: Vitamin free caseinhydrolysate medium with $CaCl_2$. The protocol of this medium was reported in the previous paper.[15] This medium is profitable for both OP_1 and OP_{1t} phage multiplications but is not so preferable for the multiplication of *X. oryzae* as the semiartificial medium.

Anti-OP_1 phage serum: The method to make this was also previously reported.[15] It reacts with both OP_1 and OP_{1t} phages in equal activity, and inactivates them with the same inactivation constant.

Method

The single-burst experiment is the method with which the reproduced phages from each infected single bacterial cell are assayed quantitatively. If two kinds of phages attack a bacterial cell simultaneously or unsimultaneously, the interference, if present, between both phages in the same host cell will be cleared by this method. The

principal of this method is to divide the phage infected bacterial suspension to the test tube—growing tube 2 in Protocol 1—, before bacterial lysis takes place, so that each tube will contain, on the average, less than one cell of the infected bacteria. After the burst of all infected bacteria in each tube under definite environmental condition, the produced phage progenies are quantitatively assayed by usual plaque count method.

The procedures of the single-burst experiment used in this experiment are indicated in Protocol 1 and 2.

Protocol 1

Protocol of single-burst experiment (1)

			Temp.(°C)	Time
1. Adsorption tube:				
OP_1 phage suspension (2.0×10^6/ml.)	0.5 ml.			
OP_{1t} phage suspension (3.3×10^7/ml.)	0.5 ml.		30	5 min.
Bacterial suspension (5.0×10^5/ml.)	0.5 ml.	0.1 ml. (1)		
2. Serum tube: Anti-phage serum	0.5 ml.	(2)		5 min.
3. Dilution tube 1: CaVfCh medium	0.9 ml.	(3) 0.1 ml.		
4. Dilution tube 2: CaVfCh medium	9.9 ml.	(4) 0.1 ml.		30 min.
5. Growing tube 1: CaVfCh medium	50.0 ml.	(5)	25	
6. Growing tube 2: 50 tubes	1.0 ml. aliquot			30~60 min.
7. Plating for plaque making				15 hrs.
8. Change of the incubation temperature				3 hrs.
9. Plaque counting				5 hrs.

Protocol 2

Protocol of single-burst experiment (2)

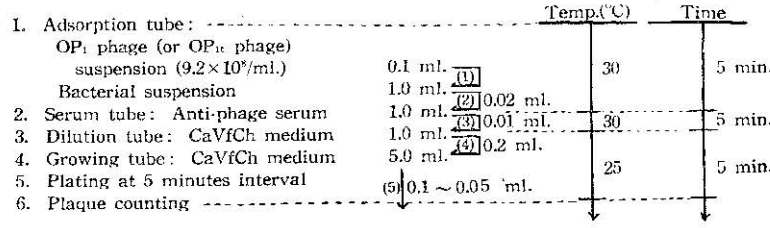
			Temp.(°C)	Time
1. Adsorption tube:				
OP_1 phage suspension (2.0×10^6/ml.)	0.1 ml.			
OP_{1t} phage suspension (3.3×10^7/ml.)	0.2 ml.		30	5 min.
Bacterial suspension	0.5 ml.	0.01 ml. (1)		
2. Serum tube: Anti-phage serum	0.5 ml.	(2) 0.1 ml.	30	5 min.
3. Dilution tube 1: CaVfCh medium	0.5 ml.	(3) 0.1 ml.		
4. Dilution tube 2: CaVfCh medium	9.9 ml.	(4) 0.1 ml.		35 min.
5. Growing tube 1: CaVfCh medium	50.0 ml.	(5)	25	
6. Growing tube 2: 50 tubes	1.0 ml. aliquot			35~60 min.
7. Plating for plaque making				15 hrs.
8. Change of the incubation temperature			30	3 hrs.
9. Plaque counting			25	5 hrs.

The method used for the one-step growth experiment of OP_1 and OP_{1t} phages is as Protocol 3.

The discriminating method of OP_1 from OP_{1t} phage by plaque morphology is as follows.

OP_1 phage forms clear plaques at 25°C. or 30°C. on *X. oryzae* no. 49 (Plate , figs. 1 et 3), while it forms indistinct plaques at 35°C. (Plate

Protocol 3
Protocol of one-step growth experiment



1, fig. 5) Namely, *X. oryzae* no. 49 cells infected with OP_1 phage are lysed prefectly under these temperatures unless at 35°C. While OP_1 phage forms clear plaques at 25°C., and cannot at 30°C. The ability of these phages to lyse the infected bacteria might be depressed by some unknown reasons under such high temperatures.

After plating the mixture of OP_1 and OP_{1t} phages by usual method, and placing the culture plate successively at 25°C. (15 hrs.), 30°C. (3 hrs.) and 25°C. (several hrs.), clear rings will appear in every plaque of OP_{1t} phages on account of lysis inhibition of the infected bacteria under 30°C., while there are normal plaques in the case of OP_1 phages. By the presence or absence of the rings in plaques, OP_1 and OP_{1t} phages are distinguishable from each other easily (Plate , figs. 2 et 4).

If the plate of the bacteria infected alone with OP_1 phages is incubated successively at 25°C., 35°C. and 25°C. each under the appropriate period the plaques having clear rings will appear as when OP_{1t} infected bacteria is incubated at 25°C., 30°C. and 25°C. successively. These are based on the fact that both phages differ from each other only phenomena in their optimum growth temperatures.

RESULTS

The result obtained by conforming to Protocol 1 is showed on the table of Result 1.

Result 1
Result of single-burst experiment

Tube no.	No. of plaques		Tube no.	No. of plaques	
	OP_1	OP_{1t}		OP_1	OP_{1t}
1	0	0	6	0	22
2	15	9	7	2	32
3	20	14	8	4	24
4	6	9	9	2	34
5	13	19	10	3	27

11	7	26	31	0	44
12	0	39	32	0	27
13	0	1	33	0	43
14	0	0	34	0	0
15	26	26	35	0	43
16	0	2	36	10	15
17	13	3	37	17	2
18	0	0	38	0	16
19	17	16	39	43	23
20	0	2	40	1	20
21	0	20	41	7	23
22	0	5	42	1	28
23	7	3	43	9	37
24	0	0	44	11	13
25	14	17	45	0	32
26	6	7	46	0	53
27	0	40	47	6	23
28	0	31	48	47	26
29	0	35	49	0	39
30	19	13	50	0	4

Total tubes	Tubes containing bacteria infected with OP_1	Nos. of phages in tubes		
		OP_1	OP_{1t}	Total
50	26	326	489	815

Check (no. of free phages) per 0.1 ml. of dilution tube 2.

OP_1	OP_{1t}
2	6

The result obtained by conforming to Protocol 2 is showed on the table of Result 2.

Result 2

Result of single-burst experiment

Tube no.	No. of plaques		Tube no.	No. of plaques	
	OP_1	OP_{1t}		OP_1	OP_{1t}
1	0	57	6	0	81
2	0	85	7	0	81
3	3	59	8	0	88
4	0	118	9	0	112
5	0	70	10	7	120

11	1	95	31	4	69
12	22	54	32	0	100
13	0	90	33	0	100
14	0	56	34	7	120
15	0	90	35	0	140
16	21	75	36	37	112
17	0	96	37	28	90
18	0	92	38	0	95
19	0	120	39	1	140
20	0	58	40	0	150
21	0	110	41	0	95
22	15	140	42	14	65
23	15	96	43	0	120
24	7	82	44	16	70
25	25	45	45	0	120
26	0	79	46	0	70
27	0	112	47	0	70
28	13	160	48	15	200
29	29	102	49	1	170
30	0	67	50	0	190

Total tubes	Tubes containing bacteria infected with OP_1	Nos. of phages in 20 tubes		
		OP_1	OP_{1t}	Total
50	20	281	2064	2345

Check (no. of free phages) per 0.1 ml. of dilution tube 2.

OP_1	OP_{1t}
1	26

The multiplication formulae of OP_1 and OP_{1t} phages on *X. oryzae* no. 60 resulting from one-step growth experiment conforming Protocol 3 are given in Result 3.

Result 3

Result of one-step growth experiment under 25°C.

	Latent period (min.)	Rise period (min.)	Average burst size
OP_1	45	20	25 (22–28)
OP_{1t}	40	20	25 (22–28)

Discussion

As stated in the introduction, the interference phenomena between two kinds of phages on the site of reproduction are different according to whether the phages are related or not. When serologically or morphologically related phages attack simultaneously the same host cell, both phages will penetrate and multiply accompanied with some recombinations.

OP_{1t} is the phage derived from the wild type OP_1 and is related to the latter with the exception of its optimum growth temperature. It will be expected therefore that simultaneous penetration and multiplication of both two phages in the same host cell will occur when they are inoculated simultaneously. Protocol 1 is the single-burst experiment carried out with the expectation to confirm the existence of above phenomena and to confirm the possibility of alternation of phage reproducing ability of the double infected bacteria with that of the simple infected bacteria.

The distribution pattern of infected bacteria to 50 growing tubes in Result 1 is discussed with Poisson's distribution.

$$P(r) = \frac{n^r \cdot e^{-n}}{r!}$$

$P(r)$: Proportion of tubes containing bacterial cells infected with phages in number (r).

n: Average number of infected bacteria per tube.

The following results were obtained with OP_1 phage.

	No. of tubes (50·P)	No. of bacteria infected with OP_1 (r · 50 · P)
P (0) 0.48	24	0
P (1) 0.353	18	18
P (2) 0.131	7	13
P (3) 0.032	2	5
P (4) 0.006	0	0
		Total 36

where $n = 0.74$ because $P(0) = \frac{24}{50} = e^{-n} = e^{-0.74}$

Namely, out of 26 tubes which produced OP_1 phage, 18 tubes had contained one infected bacterial cell each, 7 tubes 2, 2 tubes 3, and no tubes were found to contain 4 or more bacterial cells infected with the phage. So, the total number of bacteria infected with OP_1 phage in 26 tubes was calculated at 36. The average burst size of OP_1 phage

in this experiment were therefore obtained by dividing 326—total yields of OP_1 phages by 36 · —the total infected bacterial number of 26 tubes.

$$N_{op_1} = \frac{326}{36} = 9$$

As to OP_{1t} phage, by dividing total yields of 489 in 26 tubes in Result 1 by total OP_1 infected bacteria of 36, the average burst size was obtained.

$$N_{op_{1t}} = \frac{489}{36} = 13.6$$

Considering it as a whole, 26 tubes containing 36 infected bacteria which produced both OP_1 and OP_{1t} phages; the total phages produced amounted to 815. So the average burst size of the mixed phages (N) was calculated as follows:

$$N = \frac{815}{36} = 22.6$$

These figures are nearly equal to those which were obtained from Protocol 3, Result 3. In other words, there are scarcely any differences on the phage producing ability of the bacteria when the case of mixed infection in compared with that of simple infection.

Protocol 2 is the experiment in which the proportion of the number of OP_{1t} phages to that of OP_1 is changed. From the table of Result 2, the average burst size of the bacteria regarding OP_1 phage is about 9 which is the same as that in the case of Result 1 obtained from the experiment Protocol 1. In other words, in the case of mixed infection, so far as this experiment in concerned, the rate of phage producing ability of the bacteria is not affected even if the ratio of the particle number of the inoculated phages is changed.

Finally, comparing the refults in the tables of Result 1 and 2 with those of Result 3, it is reasonable to conclude that the partition in constant proportion to the reproducing ability of the host bacteria of OP_1 and OP_{1t} phages, in the case of mixed infection, is due to the difference in the latent periods of both phages.

Summary

OP_{1t} phage, one step mutant of OP_1 (*Xanthomonas oryzae* phage), is found to be a new type mutant. It is called a growth temperature mutant because its optimum growth temperature (25°C.) differs from that of the wild type (30°C.).

When these two phages attack simultaneously the same host baterial cell, they both penetrate and multiply.

	Latent period (min.)	Rise period (min.)	Average burst size
OP_1	45	20	25 (22–28)
OP_{1t}	40	20	25 (22–28)

The phage producing potency of the cell is divided not in accordance with the different multiplicity of the inoculated phages but with their faculties or reproducing speed and the cell released both types of phage progenies in a definite proportion.

Scarcely any difference on the average burst size between the case of mixed infection and that of simple infection is found.

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Explanation of Plate 1

Fig. 1. Plaques formed by OP_1 phages, incubated for 15 hrs. at 30°C.

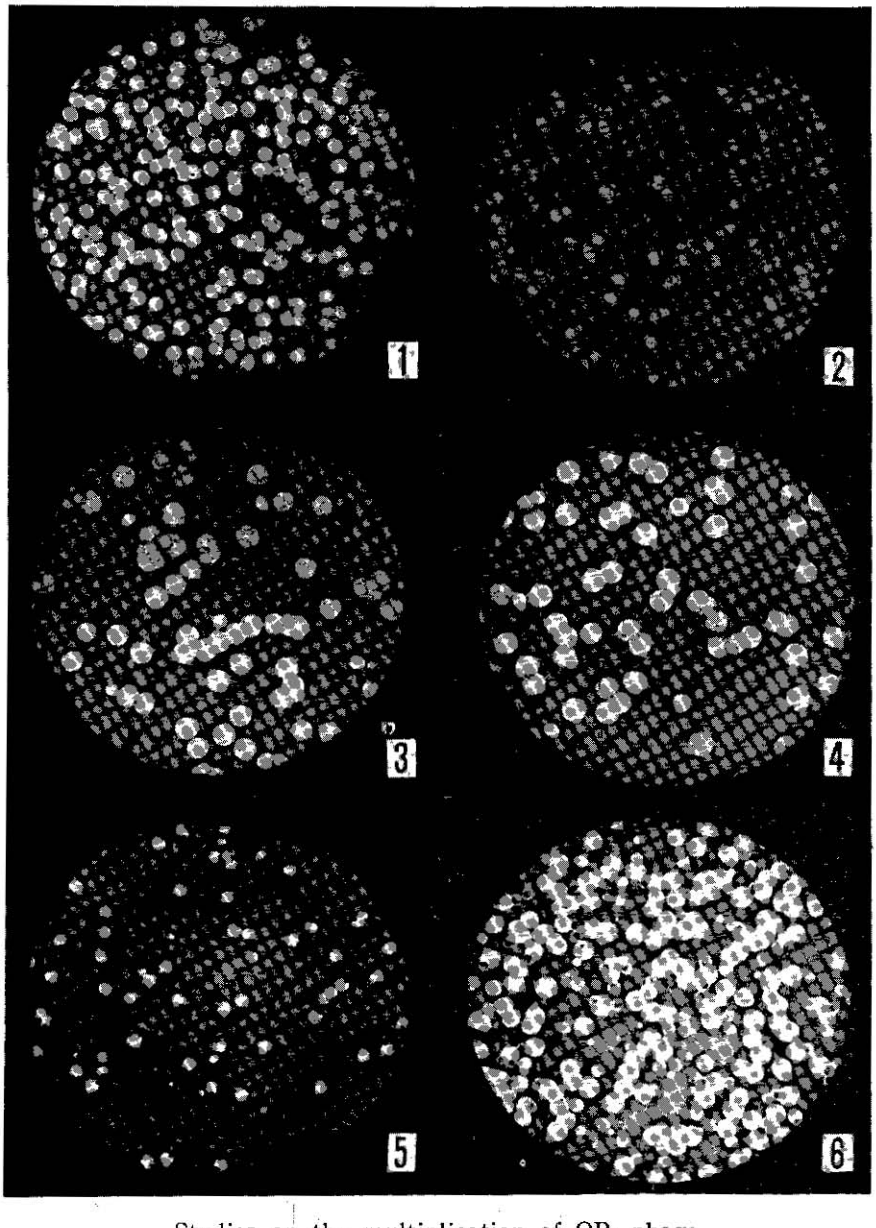
Fig. 2. Plaques formed by OP_{1t} phages, incubated for 15 hrs. at 25°C., 3 hrs. at 30°C. and 3 hrs. at 25°C. successively.

Fig. 3. Plaques formed by OP_1 phages, incubated for 15 hrs. at 25°C., 3 hrs. at 30°C. and 3 hrs. at 25°C. successively.

Fig. 4. Plaques formed by OP_1 mixed with OP_{1t} phages, incubated for 15 hrs. at 25°C., 3 hrs. at 30°C. and 3 hrs. at 25°C. successively. These plaques with rings on the upper left side are formed by OP_{1t}. While the others are formed by OP_1.

Fig. 5. Plaques formed by OP_{1t} phages, incubated for 15 hrs. at 30°C.

Fig. 6. Plaques formed by OP_{1t} phages, incubated for 10 hrs. at 30°C. and 5 hrs. at 25°C. successively.



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