

Xanthomonas campestris pv. campestrisファージの諸性質について

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Some Properties of *Xanthomonas campestris* pv. *campestris* Phage

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渡辺 実*・内藤きよ美**・金子邦夫***・南波佐間 仁****・細川大二郎* :
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Abstract

Xanthomonas campestris pv. *campestris* phages (3 isolates of phage S, K and '76) were isolated from black rot diseased leaves of cabbage, cauliflower and turnip, and some of their fundamental properties were investigated. The particle of phage S was tadpole-like with a polyhedral head of about 50×60 nm and a tail of about 110×10 to 12 nm. Phages S and K attacked specifically *X. campestris* pv. *campestris*, but not *X. campestris* pv. *citri*, pv. *cucurbitae*, pv. *oryzae*, pv. *phaseoli* and pv. *pruni*. Phages S and K showed different host range to bacterial isolates of *X. campestris* pv. *campestris*. The thermal inactivation point of phage S was 53 C in distilled water and 65 C in potato ring-rot liquid medium by 10 min incubation. Phage S was more stable in its lytic activity in potato ring-rot liquid medium or in phosphate buffer than in distilled water. Phage S was also stable at pH 7.0, but not at pH 4.9 or 9.1. Throughout all conditions of different three dispersants and five pH levels phage S was more stable at 5 C than at 28 C. One-step growth experiments showed that multiplication of phage '76 was optimal at 28 C. The latent period, rise period and average burst size in the case of single infection were 75 min, 40 min and 13 plaque forming units, respectively. At 30 C or at the temperatures below 26 C, however, the latent period was prolonged and the average burst size became smaller.

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Introduction

Black rot disease has caused serious damage to many crucifer plants especially to cabbage and cauliflower at Tama district, Tokyo.

Although many phages which attack corresponding plant pathogenic bacteria have been isolated, only a few reports were found on the phage which attacks *Xanthomonas campestris* pv. *campestris*, the causal bacterium of black rot disease of crucifers^{4,5,18)}. The first report on the morphology, host range and one-step growth of *X. campestris* pv. *campestris* phage was made by Sutton *et al.*¹⁸⁾, who showed that the phage attacked a wide range of

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Xanthomonas species including *X. campestris* pv. *campestris*.

Several phage isolates were obtained from black rot diseased leaves, and some fundamental properties of these phages will be described here.

Materials and Methods

Isolation of host bacteria. *X. campestris* pv. *campestris* was isolated from black rot diseased leaves of cauliflower, turnip and cabbage in the experimental farm of Tokyo University of Agriculture and Technology in 1966 and also from diseased leaves of cabbage in 1976. The pathogenicity of these bacterial isolates was confirmed by the needle-prick inoculation to the healthy cabbage leaves.

Isolation and enrichment of phages. Phages were isolated from diseased leaves of cauliflower and turnip in 1966 and from those of cabbage in 1976 by the use of corresponding indicator strain of host bacterium, and they were designated as the phage S, K and '76, respectively.

Isolation was made as follows: lesion parts of diseased leaves were homogenized with sterilized distilled water, centrifuged at $7,000 \times g$ for 15 min and the supernatant was adequately diluted. The mixtures consisting of diluted supernatant, bacterial suspension of indicator strain and melted bouillon agar medium containing 0.6% agar were poured onto the basal layer of bouillon agar (2% agar) solidified previously in Petri dish, and were incubated at 28 C for 15 to 20 hr.

Small clear plaques of about 1 mm in diameter appeared as shown in Fig. 1. The phages in a single plaque were transferred into a broth culture of indicator strain in test tubes. After incubation by shake culture for 24 hr at 28 C, the bacterial cells were removed by centrifugation at $7,000 \times g$ for 15 min, and small quantities of the supernatant were re-transferred into the fresh bacterial broth culture. After these three serial enrichment of the phage, high titer phage lysates of approximately 10^9 plaque forming units (pfu) per ml were obtained by the soft agar layer method¹⁾.

Preparation of anti-phage serum. Phage suspension ($2.6 \times 10^5 \sim 3 \times 10^8$ pfu per ml), previously eliminated bacterial debris by filtrating with Millipore filter (pore size $0.22 \mu\text{m}$), was injected 11 times into the ear vein of rabbit at intervals of 2 to 3 days. Anti-serum was taken by routine method. The K-value (velocity constant of phage inactivation) of this anti-serum was about 50.

One-step growth experiment. In the experiments of single infection, 1 ml of bacterial suspension (5×10^8 cells per ml) and 1 ml of phage suspension (1×10^8 pfu per ml) were mixed in test tubes kept in a water-bath at 20, 22, 26, 28 and 30 C. After 10 min, free phages were inactivated by adding 4 ml anti-phage serum for 5 min, then 0.5 ml of the mixtures (bacterium-phage complex and anti-serum) was diluted to 10,000-fold with Cavfch medium²⁾ (so-called 'growth tube'). Bacterium-phage complex (0.1 ml) was taken out from the growth tube at settled intervals, and used for plaque counting.

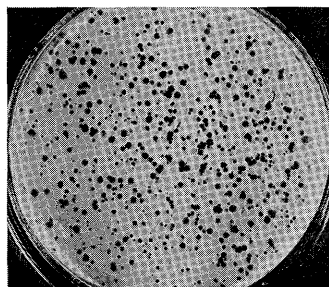


Fig. 1. Plaques of *X. campestris* pv. *campestris* phage.

In the experiments of multiple infection, 1 ml of

bacterial suspension (about 10^7 cells per ml) and 1 ml of phage suspension (about 10^8 pfu per ml) were mixed, and kept at 22, 26, 28 and 30 C. Other experimental procedures were the same as those in the experiment of single infection.

Results

Morphology of phage S particle

Phage S suspension was treated with twice repeating of ultra (at $50,000\times g$ for 40 min) and low speed differential centrifugation (at $7,000\times g$ for 15 min). The phage suspension thus treated was negatively stained with 1% alcoholic solution of phosphotungstic acid for electron microscopic observation.

This phage particle was typical tadpole-like, with a polyhedral head about 50×60 nm and a cylindrical tail about 110 nm in length and about 10 to 12 nm in diam. (Fig. 2 and 3).

Host range

Host range was studied by streaking or dropping the phage suspension onto the culture surface of an indicator strain on potato ring-rot agar medium²⁾. After the incubation at 28 C for 20 hr, the appearance of lysis was examined (Fig. 4).

Table 1 showed that phages S and K did not attack *Xanthomonas campestris* pv. *citri* (7 isolates), pv. *cucurbitae*, pv. *oryzae*, pv. *phaseoli* (1 isolate of each) and pv. *pruni* (3 isolates). As to the isolates of *X. campestris* pv. *campestris*, phage S lysed 5 bacterial isolates out of 9 tested, whereas phage K attacked only 2 out of 4 which were not lysed by phage S. Therefore, it was clear that phages S and K were complementary in their host range.

Thermal inactivation points of phage S

Phage S was suspended in distilled water (pH 5.4), phosphate buffer (pH 7.0) and potato ring-rot liquid medium (pH 7.0) at the density of several hundreds pfu per 0.1 ml. One ml of the suspension was poured into a test tube and was immersed in a water-bath at given temperature. After 10 min incubation, the test tube was cooled rapidly with running tap water. Then, pfu in 0.1 ml of phage suspension was determined by the

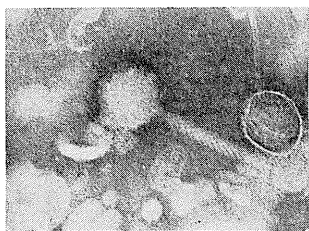


Fig. 2. Electron micrograph of phage S negatively stained by 1% phosphotungstic acid (PTA). $\times 150,000$

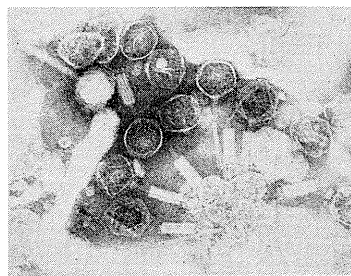


Fig. 3. Ghost particles adsorbed to bacterial debris with its tail. Electron micrograph of phage S negatively stained by 1% PTA. $\times 100,000$

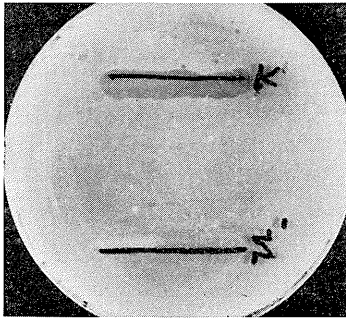


Fig. 4. Host range test of phage isolates. Phage suspension was streaked onto agar surface on which test bacterium was inoculated. One phage isolate (upper line) lysed test bacterium, but the other phage isolate (under line) did not.

plaque counting method after incubation at 28 C for 20 hr.

Table 2 showed that the thermal inactivation points of phage S in distilled water (pH 5.4), phosphate buffer (pH 7.0) and potato ring-rot liquid medium (pH 7.0) were 53 C, 62 C and 65 C, respectively by 10 min treatment.

Time course of phage S inactivation

Phage S was suspended in three dispersive solutions mentioned above at the density of 1,000 pfu per 0.1 ml. Fifteen ml of the suspension were poured into test tubes and were incubated at 5 C and 28 C. Then, plaque counting was made at the settled intervals.

Table 3 showed that the phages were rapidly inactivated in distilled water at either 28 C or 5 C, most of the phage particles was inactivated within 1 to 2 days. Less inactivation was observed in phosphate buffer and potato ring-rot liquid medium, in which more than half

of original pfu was retained even after 10 days at 28 C. Furthermore, a tendency was observed that the inactivation was greater at 28 C than at 5 C in all three dispersive solutions tested, especially in distilled water.

The effects of pH and temperature on the inactivation were investigated with the phages suspended in the Britton-Robinson's buffer solution at pH 4.9, 6.0, 7.0, 8.1 and 9.1 by similar methods described above.

At 28 C, the inactivation was remarkable within 1 day at pH 4.9, but it became less with approaching to the neutral, and was the least at pH 7.0 (Table 4). However, at pH higher than 7.0, inactivation increased again, and was remarkable after 7 days at pH 9.1. A similar tendency of inactivation was also observed at 5 C, but the phages were found to be more stable at 5 C than at 28 C in any pH tested.

Table 1. Host ranges of S and K phage of *X. campestris* pv. *campestris*

Bacteria	Phage isolates		Bacteria	Phage isolates	
	S	K		S	K
<i>X. campestris</i>			<i>X. campestris</i>		
pv. <i>campestris</i> - 1	- ^{a)}	+	pv. <i>citri</i> (7 isolates)	-	-
" - 2	-	+	pv. <i>cucurbitae</i> (1 isolate)	-	-
" - 3	+	-	pv. <i>oryzae</i> (1 isolate)	-	-
" - 4	+	-	pv. <i>phaseoli</i> (1 isolate)	-	-
" - 5	+	-	pv. <i>pruni</i> (3 isolates)	-	-
" - 6	+	-			
" - 7	+	-			
" - 8	-	-			
" - 9	-	-			

a) + : Lysis occurred, - : not occurred.

Table 2. Thermal inactivation point of S phage

Dispersant	Treated temperature (C) ^{a)}					
	Room temp.	51	52	53	54	55
Dist. water (pH 5.4)	514 ^{b)}	5	2	0	0	0
Phosphate buffer (pH 7.0)	Room temp.	59	60	61	62	63
	458	23	2	1	0	0
Potato ring-rot liquid medium (pH 7.0)	Room temp.	62	63	64	65	66
	407	4	2	1	0	0

a) Treated by 10 min incubation.

b) Values are the average plaque numbers among four plates of two replications.

Table 3. Time course of inactivation of S phage in different dispersants

Dispersant	Temperature (C)	Time course (days)							
		0	1	2	3	4	5	7	10
Dist. water (pH 5.4)	28	100 ^{a)}	4	0.3	0.5	0.1	0.2	0.07	0.03
	5	100	42	17	12	9	13	6	2
Phosphate buffer (pH 7.0)	28	100	92	80	71	69	65	48	55
	5	100	94	90	80	86	82	81	78
Potato ring-rot liquid medium (pH 7.0)	28	100	91	72	65	71	71	68	67
	5	100	94	81	82	85	80	78	65

a) Relative plaque numbers of each time course compared with that of zero time. Based on the average numbers among 9 plates of three replications.

Table 4. Inactivation of S phage under different conditions of pH and temperatures

pH	Temperature (C)	Time course (days)							
		0	1	2	3	5	7	10	14
4.9	28	100 ^{a)}	44	32	17	42	22	16	14
	5	100	65	52	61	58	54	48	29
6.0	28	100	75	61	50	65	57	57	50
	5	100	76	74	75	61	77	70	61
7.0	28	100	79	73	61	80	68	61	60
	5	100	91	86	88	91	88	81	73
8.1	28	100	54	51	59	66	54	64	50
	5	100	84	92	81	80	85	78	71
9.1	28	100	69	60	46	41	11	4	4
	5	100	96	98	91	69	60	56	55

a) Relative plaque numbers of each time course compared with that of zero time. Based on the average numbers among 9 plates of three replications.

Effects of temperature on multiplication of phage '76

In order to clarify the effects of temperature for the multiplication and the growth curve of phage, one-step growth experiment¹⁾ was conducted using phage '76 and its indicator bacterial strain under several temperature conditions. One-step growth curve at different temperatures in the case of single infection was shown in Fig. 5, and the latent periods, rise periods and average burst sizes at various temperatures in both single and multiple infection were shown in Table 5.

The results showed that optimal multiplication temperature of this phage was 28 C, which coincided with that of host bacterium. The latent period, rise period and average burst size at 28 C were 75 min, 40 min and 13 pfu, respectively in the case of single infection, and were 70 min, 55 min and 12 pfu, respectively in the case of multiple infection. At the temperatures below 26 C or at 30 C, the latent periods were prolonged and the average burst sizes became smaller.

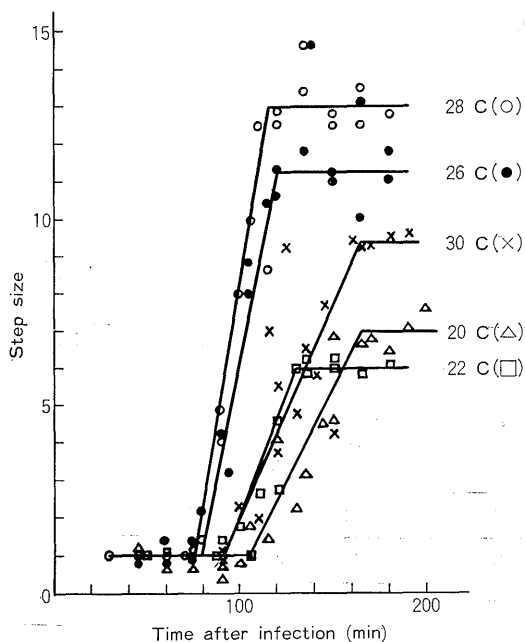


Fig. 5. One-step growth curve of '76 phage at several temperature conditions (single infection). Step size: ratio of pfu at each time to initial pfu at latent period.

Table 5. Results of one-step growth experiments in both single infection and multiple infection of '76 phage^{a)}

Temperature (C)	Types of infection	Latent period (min)	Rise period (min)	Average burst size (pfu ^{b)})
20	S ^{c)}	100	60	7
22	S	90	40	6
	M	80	55	5
26	S	80	40	11
	M	70	60	9
28	S	75	40	13
	M	70	55	12
30	S	90	75	9
	M	90	65	8

a) Data are the average of duplicated experiments.

b) Plaque forming units.

c) S: single infection, M: multiple infection.

Discussion

The polyvirulent *Xanthomonas* phage Pg 125 was isolated by Sutton *et al.*¹⁸⁾ from a suspension of macerated swede seed, compost and sewage which were added in nutrient broth heavily inoculated with *X. campestris* pv. *campestris*. Pg 125 was found to attack *in vitro* numerous phytopathogenic xanthomonads; such as *X. campestris* pv. *campestris*,

pv. *malvacearum*, pv. *phaseoli*, pv. *pruni* and pv. *vesicatoria*, etc. The present phages S and K, however, attacked only a homologous pathovar, *X. campestris* pv. *campestris*. It has been reported in Japan that phages of *X. campestris* pv. *citri*⁸⁾, pv. *oryzae*^{3, 26)}, pv. *pruni*¹⁹⁾, *Pseudomonas syringae* pv. *mori*¹⁵⁾, pv. *tabaci*¹⁴⁾, *P. solanacearum*¹¹⁾ and *Erwinia carotovora*¹⁷⁾ attacked only its homologous species or pathovars; on the other hand, certain phage strains of *X. campestris* pv. *campestris*⁴⁾, pv. *citri*⁴⁾, pv. *translucens* f. sp. *oryzicola*³⁾, *P. syringae* pv. *eriobotryae*¹⁰⁾ and pv. *mori*¹⁶⁾ attacked several heterologous species or pathovars in addition to its homologous ones.

Goto *et al.*⁴⁾ described that *Xanthomonas* phages which could frequently be found in soil, in general, showed very broad host ranges including many heterologous nomenclatures; in contrast, those which were isolated from diseased plant tissues usually showed limited host ranges. A *Xanthomonas campestris* pv. *campestris* phage isolated from diseased cabbage by Goto⁴⁾ attacked 3 isolates out of 93 isolates of *X. campestris* pv. *citri*, and also 1 isolate of pv. *blepharidis* and of pv. *phaseoli* in addition to pv. *campestris*. The discrepancy between our results and those by Goto *et al.*⁴⁾ may be due to the number of bacterial strains used.

The presence of phage strains which attack the different bacterial strains within a same species has commonly been observed, for instance, *X. campestris* pv. *citri* phage CP₁, CP₂²⁵⁾, pv. *oryzae* phage OP₁²¹⁾, OP_{1h}⁷⁾, OP_{1h2}²⁴⁾, OP₂²⁷⁾, BP₁~BP₇³⁾, pv. *pruni* phage PP₁^{9, 19)}, PP_{1h1}¹⁹⁾, PP_{1h2}¹⁹⁾, pv. *translucens* f. sp. *oryzicola* phage SP₁~₆³⁾, *P. syringae* pv. *eriobotryae* phage EP₁~₃¹⁰⁾, pv. *mori* phage MP₁, MP_{1h}¹⁵⁾, pv. *tabaci* phage No. 1~4¹⁴⁾, *P. solanacearum* phage^{11, 12)} and *E. carotovora* phage Ap, Bp, Cp²⁰⁾.

The thermal inactivation point of phage S was lowest (53 C) in distilled water (pH 5.4) and was considerably higher (62 C) in phosphate buffer (pH 7.0) and in potato ring-rot liquid medium (pH 7.0) (65 C). Similar results have been reported in the experiments using *X. campestris* pv. *oryzae* phage OP₁²¹⁾ and OP_{1h}⁷⁾, in which the thermal inactivation point was higher in liquid medium than in distilled water. In the case of pv. *oryzae* phage OP₂²⁷⁾, however, no difference was observed between liquid medium and distilled water.

Phage S was considerably stable in its lytic activity in both phosphate buffer and potato ring-rot liquid medium, but not in distilled water. This result may be related to the difference of thermal inactivation point among dispersants as above mentioned. Phage S was found to be most stable in a neutral condition (pH 7.0) and less stable in both acidic and alkaline conditions (pH 4.9 and pH 9.1, respectively). Moreover, the phage was more stable at 5 C than at 28 C regardless of the sort of dispersants and pH values. Similarly, it has been reported that *X. campestris* pv. *oryzae* phage OP₁²¹⁾ was unstable under higher temperature and in distilled water. Wakimoto²³⁾ reported that if OP₁ phage was preserved by freeze drying with protein solution (10⁻²~10⁻³ g protein per ml) or with potato semi-synthetic liquid medium, inactivation of phage could be considerably prevented. The reason why phage S was more stable in potato ring-rot liquid medium than in distilled water might partly be due to the protective action of proteinous components in the former medium.

When compare our results of one-step growth experiment (single infection at 28 C) with those obtained by Sutton *et al.*¹⁸⁾, latent period was markedly different; that is, Pg 125 was so long as 210 min, while the present phage '76 was 75 min. An average burst

size of phage '76 was 13 pfu, while Pg 125 was varied from 4 to 12 through three replicated experiments. When compare the characteristics of phage '76 with those of *X. campestris* pv. *oryzae* phage; latent and rise periods of phage '76 were about two times longer than those of OP₁ phage²²⁾, and were roughly coincided with those of OP₂ phage²⁷⁾. It had been reported that *X. campestris* pv. *citri* phage CP₂⁶⁾ showed about 150 min of latent period, and *P. solanacearum* S₁ phage¹³⁾ and *P. syringae* pv. *tabaci* phage¹⁴⁾ showed 190 and 70 pfu of average burst size, respectively.

The present *X. campestris* pv. *campestris* phages differed apparently in their host range from polyvirulent Pg 125 reported by Sutton *et al.*¹⁸⁾. It will be necessary to clarify further the host range in detail, not only to bacterial strains in *X. campestris* pv. *campestris* but also to heterologous bacterial species or pathovars by using many phage isolates. Furthermore, the behavior of *X. campestris* pv. *campestris* in the field should be elucidated by the use of adequate phage isolates.

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和 文 摘 要

Xanthomonas campestris pv. *campestris*

ファージの諸性質について

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アブラナ科植物黒腐病菌のファージ3系統 (S, K および '76 ファージ) をカンラン, ハナヤサイ, カブの罹病葉から分離し, その基礎的諸性質を調査した。S ファージの形態はオタマジャクン型で, 頭部は約50×60 nm の多面体, 尾部は長さ約 110 nm, 幅約 10~12 nm であった。S および K ファージの宿主範囲は, 供試 *Xanthomonas* 属細菌のうちアブラナ科植物黒腐病菌のみを侵し, その他のカンキツかいよう病菌, カボチャ褐斑細菌病菌, イネ白葉枯病菌, インゲン葉焼病菌, 核果類せん孔細菌病菌は侵さなかった。また, S および K ファージは黒腐病菌の分離株に対して異なる宿主範囲を示す2種の系統であった。S ファージの不活化温度は蒸留水中では53℃, 10分間と最も低く, ジャガイモ輪腐病菌液体培地中では65℃, 10分間で, かなり高かった。S ファージの経時の不活化状況は, 蒸留水 (pH 5.4) 中で最も不安定であり, ジャガイモ輪腐病菌液体培地およびりん酸緩衝液 (ともに pH 7.0) 中ではかなり安定であった。異なる pH の緩衝液中では pH 7.0 で最も安定であり, pH 4.9 および pH 9.1 では不安定であった。上記3種の分散媒および5種の pH の緩衝液中で, S ファージは28℃より5℃で安定であった。数段階の温度条件下での一段増殖実験の結果から, '76ファージの増殖には28℃が最適であり, 単数感染の場合に潜伏期75分間, 上昇期40分間, 新生ファージ平均放出数13であった。温度が30℃, あるいは26℃以下のときは28℃の場合より潜伏期が長びき, 新生ファージ平均放出数は小となった。