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Cultural Characteristics of *Xanthomonas axonopodis* pv. *citri* Bacteriophages CP_1 from Korea

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Bacteriophage of *Xantomonas axonopodis* pv. *citri*, a causal agent of citrus canker disease, was studied for its cultural characteristics. The relative efficiency of plating (EOP) of 11 phages used to 13 strains of *X. axonopodis* pv. *citri* tested ranged from 0.8 to 1, indicating that the phages are homogeneous. Homogeneity of the phages suggests that citrusphage belongs to a single group CPK as reported in a previous study. Typical one-step growth of a phage P5 selected from the citrusphages was observed. The EOP of the P5 was dependent upon the media, pH, and temperature. It was observed that multiplication of the phage cultured in Wakimotos potato semisynthetic media at 25°C was more effective than that in other temperatures, regardless of the bacterial strains and media used. It was observed that pH 6.5 is optimal for multiplication of the phage. In comparison of the EOP among citrusphages CP_1, CP_2, and P5, multiplicative characteristic of phage P5 in the bacteria on time-course was similar with that of phage CP_1. Thus, it was concluded that citrusphage group CPK from Korea is CP_1 based on host specificity of the phage as described in a previous study, homogeneity, and its multiplication pattern.

Keywords : Bacteriophage, CP_1, cultural characteristics, *Xanthomonas axonopodis* pv. *citri*.

Bacteriophages of plant pathogenic bacteria may play an important role as components of microbial communities with respect to plant diseases (Civerolo, 1973; Civerolo and Keil, 1969; Erskine, 1973; Okabe and Goto, 1963; Zeitoun and Wilson, 1969). The ecological significance of the phage can be evaluated by understanding the effects of various factors on phage-host interactions in natural environments.

Infection of bacteriophage is limited by attachment to surface of bacterial cell (Garen and Kozloff, 1959). Although the influence of various *in vitro* factors on *Xanthomonas axonopodis* pv. *pruni* and its phage (Civerolo, 1973; Civerolo, 1974; Mandell and Eisenstark, 1953), and on *Bacillus subtilus* and its phage (Lamontagne and McDonald, 1972) have been described previously, little is known about the effects of factors on citrusphage-its host interaction. In a previous study, citrusphage distributed in Korea was presumed as CP_1 based on its specificity on lysotype A (Myung et al., 2001). Thus, the present study was undertaken to get fundamental information about cultural characteristics that may be affected by the phage-*X. axonopodis* pv. *citri* interaction, and to compare the multiplicity of citrusphage P5 from Korea with that of CP_1 and CP_2 described in previous study (Obata et al., 1969) to prove the Korean strain of phage to be CP_1.

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Materials and Methods

Bacteria, phage propagation, and phage count assay. Bacteria and phages used in this study are listed in Table 1. Bacteria and phages were cultured as in a previous study (Myung et al., 2001). Briefly, strains of *X. axonopodis* pv. *citri* were routinely cultured at 27°C on peptone sucrose agar (PSA), and suspended in peptone sucrose broth (PSB) for phage propagation. Phages were propagated on *X. axonopodis* pv. *citri* (BC 1) cultured in PSB for 24 hours at 25°C. A double layer method was routinely used for plaque count assay. Two (2) or 3 ml of phage-bacteria mixture in 0.7% soft agar was overlaid on the basal medium.

One-step and intercellular growth experiment. Phage P5 was added at a ratio of phage to bacteria (BC 28) of 0.01 to 0.9 ml of bacteria at about 1×10^9 cfu/ml in a water bath at 25°C. For the one-step growth experiment, the phage was allowed to adsorb the bacteria for 20 minutes (about 80% adsorption), diluted into Wakimoto's potato semisynthetic broth [WPSB, 2.0 g $Na_2HPO_4 \cdot 12H_2O$, 0.5 g Ca $(NO_3)_2 \cdot 4H_2O$, 5.0 g peptone, 20 g sucrose, and 15 g agar added to 1 liter of boiled 300 g potato, pH 7.0], and assayed for unadsorbed phage. The cultures were incubated at 25°C on a rotary shaker (250 rpm) and were assayed at every 20-minute interval up to 80 minutes. Intracellular growth of phage to the bacteria was investigated in two different methods, shaking and stationary cultures of the bacteria. The cultures were incubated at 25°C on a rotary shaker (250 rpm) and assayed at 0, 1, 2, 2.5, 3, 3.5, 4, 4.5, 5, and 6 hours. Phage P5 (1×10^7 pfu/ml) was added to a loop of freshly cultured bacteria.

Table 1. *Xanthomonas axonopodis* pv. *citri* strains, phage, hosts, origin, and isolation year

Bacterium and phage	Host	Year of isolation	Location[a]
Bacterial strains			
BC 1	*Citrus reticulata* cv. *unshu*	1992	Korea
BC 3	*C. lemon*	1993	Korea
BC 7	*C. natsudaidai*	1993	Korea
BC 20	*C. reticulata* cv. *unshu*	1993	Korea
BC 27	*C. grandis*	1993	Korea
BC 28	*C. grandis*	1993	Korea
BC 41	*C. reticulata* cv. *unshu*	1993	Korea
BC 47	*C. reticulata* cv. *unshu*	1993	Korea
BC 50	*C. natsudaidai*	1993	Korea
BC 57	*C. natsudaidai*	1993	Korea
BC 59	*C. reticulata* cv. *unshu* × *C. sinensis*	1993	Korea
BC 61	*C. natsudaidai*	1993	Korea
BC 62	*C. hassaku*	1993	Korea
Phage strains			
P5	*C. reticulata* cv. *unshu*	1993	Korea
P14	*C. sinensis*	1993	Korea
P18	*C. reticulata* cv. *unshu*	1993	Korea
P25	*C. natsudaidai*	1993	Korea
P26	*C. sinensis*	1993	Korea
P50	*Poncirus* Raf. × *C. sinensis*	1993	Korea
P57	*C. reticulata* cv. *unshu*	1993	Korea

[a] Cited in Myung et al. (2001).

Effect of different media and temperatures on phage multiplicity. The following media were used to determine the effect of media and temperatures on phage multiplicity: PSA (Bacto-peptone 10 g, sucrose 10 g, sodium glutamate 1 g, distilled water 1 L), and nutrient agar (NA, pH 7.0, Difco, Maryland, USA). The medium containing bacteria-phage mixture was incubated at 20, 25, 30, and 35°C as described above. Plates incubated at 20, 25, and 30°C were read 24 hours after incubation, while plates incubated at 35°C were read 48 hours after incubation. One (1) ml of phage suspension [1×10^7 plaque-forming unit (pfu)/ml] was added to 9 ml of soft agar of each medium containing strains of *X. axonopodis* pv. *citri*. Three (3) ml of the mixture was overlaid on the bottom layer.

Relative efficiency of plating (EOP) of bacteriophage on bacterial strains. Bacteria and phages used for determining the relative EOP are listed in Table 2. Two (2) ml of the phage mixture, which contained 1×10^7 pfu/ml of phages and 1×10^9 cfu/ml of bacteria were overlaid on the bottom agar. Plates were recorded pfu 24 hours after incubation at 25°C.

Effects of pH on relative EOP. WPSA was used for determining the effect of pH on multiplicity of phage. Plates were adjusted to pH 4.5 to 11 at interval of 0.5 with 1 N HCl or NaOH. Two (2) ml of the phage mixture, which contained 1×10^7 pfu/ml of phages and 1×10^9 cfu/ml of bacteria, were overlaid on the bottom agar. Plates were recorded pfu after incubation for 18 hours at 25°C.

Results

One-step and intercellular growth of phage. Typical one-step growth of a phage P5 was observed (Fig. 1). Latent period for the phage P5 was ca. 40 minutes, the rise period ca. 40 minutes, and the average burst size ca. 60%. Infectious phage was released rapidly for the next 20 minutes, reaching a maximum rise period of about 20 minutes. Eighty percent (80%) adsorption of phage to host was observed during the first 60 minutes after addition of phage to bacterial suspension (data not shown). Intracelluar growth of phage decreased during the first hour, recognized after two hours shaking culture, and then a sharp logarithmic rise of phage was observed (Table 2).

Relative efficiency of plating (EOP) of bacteriophage. Relative EOP of seven phages on the strains of *X. axonpopdis* pv. *citri* is shown in Table 3. The EOP, ranging from 0.8 to 1, of these seven virulent *X. axonpopdis* pv. *citri* phages was homologous to 12 strains of *X. axonpopdis* pv. *citri*. Homogeneity of the phages suggests that citrusphage belongs to a single group CPK as reported in a previous study (Myung et al., 2001).

Effect of temperatures and media on EOP of bacteriophage. The EOPs of bacteriophage to bacterial strains of *X.*

Table 2. Relative efficiency of plating (EOP)[a] of bacteriophages on strains of *Xanthomonas axonopodis* pv. *citri*

Phages	Strains of *Xanthomonas axonopodis* pv. *citri*												
	BC 1	BC 3	BC 7	BC 20	BC 26	BC 28	BC 41	BC 47	BC 50	BC 57	BC 59	BC 61	BC 62
P5	1	1	1	1	1	1	1	0.9	1	1	1	0.9	0.9
P14	0.8	1	1	1	0.9	1	1	1	1	1	1	1	1
P18	1	1	1	1	1	1	0.9	0.9	0.9	1	1	1	1
P25	0.9	0.9	1	1	1	1	1	1	0.8	0.9	1	1	1
P26	1	1	1	1	0.9	1	1	1	1	1	0.9	1	1
P50	1	0.9	1	1	1	1	1	1	1	1	1	0.8	1
P57	1	1	0.9	0.8	1	1	1	1	1	1	0.9	1	1

[a] EOP compared with BC 28 as standard. Data represent an average of three experiments with three replicates each.

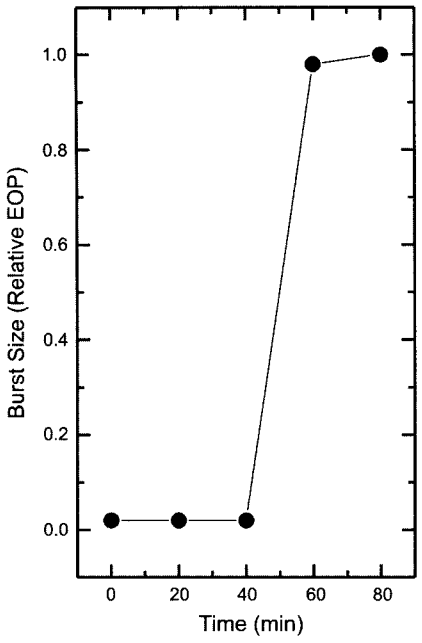

Fig. 1. One-step growth of citrusphage P5 grown in the strain BC 28 of *Xanthomonas axonopodis* pv. *citri* at 25°C in WPSA. Phage P5 was added at a ratio of phage to the strain of 0.01 to 0.9 ml of bacteria at about 1×10^9 cfu/ml in a water bath at 25°C. Data represent an average of three experiments with two replicates each.

Table 4. Effect of temperatures and media on relative efficiency of plating (EOP)[a]

Strain	Medium[b]	Temperature (°C)			
		20	25	30	35
BC 1	PSA	0.51	0.57	0.47	0
	WPSA	0.86	1.00	0.86	0
	NA	0.52	0.54	0.50	0
BC 7	PSA	0.49	0.50	0.50	0
	WPSA	0.92	0.97	0.90	0
	NA	0.53	0.54	0.51	0
BC 20	PSA	0.44	0.44	0.41	0
	WPSA	0.94	1.00	0.95	0
	NA	0.53	0.54	0.51	0
BC 28	PSA	0.49	0.52	0.51	0
	WPSA	0.93	1.00	0.94	0
	NA	0.55	0.59	0.53	0
BC 41	PSA	0.53	0.55	0.51	0
	WPSA	0.90	0.98	0.94	0
	NA	0.57	0.58	0.52	0

[a] Plaque forming units (pfu) of temperatures and media/pfu at WPSA and 25°C.
[b] PSA = peptone sucrose agar; WPSA = Wakimoto's semisythetic potato sucrose agar; NA = nutrient agar. Data represent an average of two experiments with three replicates each.

axonopodis pv. *citri* at different temperatures and media were determined (Table 4). In experiments on the effect of temperatures on the EOP, phages multiplied at a range of 20° to 30°C. It was observed that multiplication of phage cultured at 25°C was the best regardless of bacterial strains and media used in this study. No plaque was observed at 35°C in all media used, although bacterial lawns were developed at that temperature within 24 hours.

The effect of media on the relative EOP on different *X. axonopodis* pv. *citri* strains is shown in Table 4. Infection of phages in WPSA to all bacterial strains in the different temperatures was twice more effective than that in the other media.

The effect of pH on relative EOP. Relative EOP varied depending on the pH of the media where the phage-bacteria mixtures were incubated (Fig. 2). The optimal pH for multiplicity of phage ranged from 6.5 to 7.0. Increase in pH from 4.5 to 6.5 was accompanied with increase in relative EOP, whereas, increased pH from 6.5 to 9.5 reduced the EOP. Burst size at pH 10 was slightly increased, and then decreased. In addition, bacterial lawn was developed at a range of pH from 5 to 11, but did not develop at pH 4.5.

Discussion

This study found out that the multiplicity of citrusphage in its host was limited to temperature, pH and media. Phage attachment is generally determined by infection of phage to its host bacterium. The attachment to host cell is a primary factor for determining multiplicity of phages in cells, and is affected by several factors (Garen and Kozloff, 1959).

The first step for infection was a reversible binding of the phage to the bacterium through the interaction primarily of

Table 3. Comparison of relative efficiency of plating (EOP) among citrusphage P5 from Korea, CP1 and CP2

Phage	Incubation time (hour)										Remarks
	0	1	2	2.5	3	3.5	4	4.5	5	6	
P5	115[a]	46	139	960	5350	6690	42500	34850	292000	585000	In this study
CP1	NT[b]	86	466	NT	3130	NT	26100	NT	500000	NT	Obata et al. (1969)
CP2	NT	16	8	NT	123	NT	264	NT	792	NT	Obata et al. (1969)

[a] Data represent an average of three experiments with three replicates each.
[b] NT=not tested. Cells were cultured in shaking incubator at 25°C.

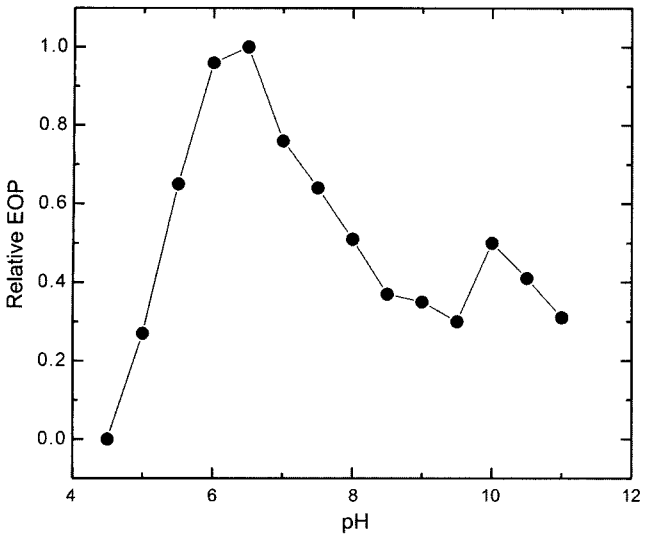

Fig. 2. Effect of pH on relative efficiency of plating (EOP). Phage P5 was added at a ratio of phage to a strain BC 28 of *Xanthomonas axonopodis* pv. *citri* of 0.01 to 0.9 ml of bacteria at about 1×10^9 cfu/ml. Plates were recorded as plaque forming unit after incubation for 18 hours at 25°C. Data represent an average of three experiments with three replicates each.

ionic groups on the two surfaces (Garen, 1954). The surface charges are generally determined by the pH of the environment of the two cells. Therefore, observation peaked at pH 6.5, and zero at an extreme pH of 4.5 in the relative EOP suggests that ionic carboxyl and amino groups are needed for attachment of citrusphage to its host cells. In addition, slight increase in burst size at pH 10 indicates that another ionic strength on its host cell surface may promote the attachment of the phage to the bacterium.

Attachment usually begins upon addition of an inorganic salt because phage and cell surfaces carry a net negative charge. Thus, inorganic salts play an important role of attachment of phage to its host cell (Garen and Kozloff, 1959). The result that burst size of phage in the WPSA medium containing Ca^{++} and Na^{+} was larger than that of phage cultured in other media. It indicates that inorganic salts may affect the attachment of citrusphage to its host, *X. axonopodis* pv. *citri*.

Effect of temperatures on phage- host interaction have reported by Liew and Alveriz (1980) and Civerolo (1974). Their results suggested that failure to infect bacterial cell at high temperature may be due to the phage was not able to adsorb irreversibly onto the host cell because of modified host cell receptors or phage attachment structures, and did not grow and mature intracellulary. The non-yielding of phage from *X. axonopodis* pv. *citri* cells cultured at 35°C could be explained although data about the specific location, and the chemical and structural nature of phage receptor sites on *X. axonopodis* pv. *citri* cells are not available.

In view of the ecological balance between phage and its usual host, *X. axonopodis* pv. *citri* phages may not attack their host in hot weather, which would be immune to high temperature. Therefore, spread of *X. axonopodis* pv. *citri* and development of citrus bacterial canker in nature may occur most rapidly and extensively at high temperature when other environmental conditions are favorable.

Myung et al. (2001) showed that in Korea, only a single group CPK was distributed, and it was presumed that the phages might be CP_1 based on their host specificity. Multiplicative pattern of phage CP_1 in its host is different with that of CP_2 (Obata et al., 1969). Multiplication of phage CP_1 tends to decrease during the first hour, and the increase of CP_1 was recognized after two hours shaking and then showed a sharp rise. The result as shown in Table 2 implies that multiplication of phage P5 is similar to that of CP_1. Thus, it can be concluded that the citrusphage from Korea is the same as CP_1 based on host specificity of phage P5 in the previous study, the homogeneity of phages on their hosts, and their multiplicative pattern as observed in this study.

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