Biological and Morphological Characterization of *Xanthomonas campestris* Bacteriophages

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ABSTRACT

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Seven virulent bacteriophages of *Xanthomonas campestris* isolated from infested soils and seeds in Hawaii were characterized and compared with two phages each from Japan and North Carolina. The phages have hexagonal heads and fall into three morphological classes based on tail structure. Host specificity and morphology of phages OH_2 and OK_2 from Japan are similar to phages HP_1, HP_3, HT_7, and HT_{3h} from Hawaii. This group of phages has contractile tail sheaths surrounding rigid cores with narrow "neck" regions. Uncontracted tails average 18 × 115 nm and heads measure 55–65 nm in diameter. Hawaiian phages A342 and HXX are morphologically similar to North Carolina phages $P_{1\text{-}3a}$ and P_6 in having noncontractile flexuous tails that average 14 × 120 nm and heads that measure ~55 nm in diameter. Wisconsin phage RR68 has a short wedge-shaped tail 15 nm long. The phages differ in susceptibility to heat and in relative efficiency of plating at different incubation temperatures and were further characterized by the rates of adsorption onto homologous host bacteria and also other parameters measured during the one-step growth experiment.

Bacteriophages of plant pathogens are useful tools for detecting and differentiating bacterial strains (16,24,32). In the genus *Xanthomonas,* phages have been characterized for only a few nomenspecies (13,17,19–22,34,35). Phages that can attack the taxospecies, *X. campestris,* have been reported (15,28,30,31); however, none of the virulent phages were specific, and no *X. campestris*-specific phages have been studied and characterized except one temperate phage (31).

The purpose of the present study was to isolate host-specific phages for detecting and phage-typing *X. campestris* strains. Preliminary studies on isolation of some host-specific phages and their properties have been previously reported (3).

MATERIALS AND METHODS

Media. The following media were used: tryptone-yeast-glucose (TYG) growth medium—tryptone (5 g), glucose (5 g), yeast extract (3 g), $MgSO_4 \cdot 7H_2O$ (10^{-3}M) in 1 L of water; and phosphate-buffered tryptone water (PBT)—tryptone (7 g), K_2HPO_4 (2.4 g) and KH_2PO_4 (0.7 g) in 1 L of water. Bacterial isolations from infected cabbage tissues were made on Kelman's tetrazolium chloride (TZC) medium (18) while isolations from seed were made by placing them on Schaad's SX medium (26). Starch-hydrolyzing colonies with characteristics of known *X. campestris* colonies on SX medium were purified by repeated streaking on TZC plates prior to pathogenicity testing.

Bacterial strains and phage isolation. Materials used for bacterial and phage isolations and sources of strains are listed in Table 1. Only pathogenic strains, tested on cabbage plants by the notch inoculation method (29), were used. Cultures were stored at 4 C in sterile water tubes. Phages $P_{1\text{-}3a}$ and P_6, provided by E. Echandi (North Carolina State University, Raleigh 27650), and phages OH_2 and OK_2 from M. Goto (Shizuoka University, Shizuoka, Japan) were included in this study for comparison. Phages were isolated in our laboratory from soils collected from cabbage farm fields with a history of black rot and from infected seeds by using an enrichment technique (7). Seeds (1 g) were triturated with mortar and pestle, and soils (5 g) were sifted through a 1.2-mm (14-mesh) screen, before being added to 500-ml Erlenmeyer flasks which contained 100 ml of a suspension of *X. campestris* strains (Table 1) growing exponentially (18 hr) in TYG broth supplemented with 1 g of $CaCO_3$. After an incubation period of 18–20 hr at room temperature (~22 C) on a rotary shaker, culture fluids were centrifuged at 12,100 *g* for 10 min. The supernatants were then shaken with chloroform (10%, v/v) and tested for phage against the same bacterial strain used in the original enrichment (homologous host).

All phages were purified by four successive single-plaque isolations. A single plaque from the last plating was cut out and suspended in 2 ml of PBT and used for preparation of high-titre stocks by using the agar plate method (2). Plates showing nearly confluent lysis were washed with occasional shaking for 2 hr in 5 ml of PBT. The liquid extract was centrifuged by alternating two centrifugation cycles at 12,100 *g* for 10 min with one cycle at 32,800 *g* for 1 hr. After the last centrifugation, the pellet was resuspended in PBT and incubated at 6 C for 24–48 hr. The supernatant from the last cycle, with titres of 10^9–10^{12} plaque-forming units (PFU) per milliliter was stored over chloroform (~1%) at 4 C.

TABLE 1. Sources of *Xanthomonas campestris* strains and phages used to study phage biology and morphology

Phage isolated	Enrichment host	Source material	Location
HP_1, HP_3	KC 3-1-12	Soil I, cabbage farm	Hawaii
HT_7	KC 2-1-1	Soil II, cabbage farm	Hawaii
HXX	XC114[a]	Soil III, cabbage farm	Hawaii
HT_{3h}	PHW46[b]	Soil III, cabbage farm	Hawaii
A342	A342	Infected broccoli seeds (Honolulu Seed Co.)	Hawaii
RR68	RR68	Infected radish seeds (Lot RR68-549A)	Wisconsin
$P_{1\text{-}3a}$[a]	XC118[a]	Brussels sprout seeds	North Carolina
P_6[a]	XC114[a]	Infected rutabaga leaves	North Carolina
OH_2[c]	OH_2[c]	Chinese cabbage	Japan
OK_2[c]	OK_2[c]	Head cabbage	Japan

[a] Provided by E. Echandi, North Carolina State University, Raleigh.
[b] Provided by P. H. Williams, University of Wisconsin, Madison.
[c] Provided by M. Goto, Shizuoka University, Japan.

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Plaque count assays were made by the single agar layer method (25). Molten TYG 1% agar (5-ml aliquots) was maintained in a water bath at 45 C. PBT was used as phage diluent and host cells were suspended in TYG broth at $\sim 10^9$ cells per milliliter from cultures grown overnight on TYG agar slants. Assays were made by mixing 0.1 ml of the appropriate phage dilution with 0.5 ml of the bacterial host suspension in a molten agar tube and pouring the tube contents into a petri plate, which was incubated upside-down at 28 C and read after 8–18 hr. Relative efficiency of plating (EOP) (11) using this method, in comparison to the conventional double layer method (2), ranged from 0.85 to 1.38. The EOP is defined as the probability of plaque formation by a phage particle and is expressed as the fraction of infective centers which produces plaques (11).

Adsorption rate, one-step and intracellular growth. All growth experiments were performed in aerated tubes held in a 28 C water bath with the homologous bacterial host unless otherwise indicated. Log-phase bacteria cultured in aerated tubes were adjusted turbidimetrically to $\sim 10^8$ cells per milliliter prior to each experiment. Adsorption rates were measured with the chloroform inactivation technique of Fredericq (12). Phage was added at input multiplicity of infection (m.o.i.) (8) of 0.05 to 0.01. One-step growth experiments were essentially as described by Ellis and Delbruck (11) with an m.o.i. of ~0.1. After adsorption for 20 min (32–95% adsorption, depending on the phage), the cultures were diluted with prewarmed TYG broth into growth tubes and also assayed for unadsorbed phages. The aerated growth tubes were assayed at intervals up to 4 hr for total infective centers and for intracellular phages by using a chloroform technique (27).

Efficiency of plating (EOP). Temperature effects on relative EOP were determined by using the titre at the optimal temperature as the standard. Plates incubated at 24, 28, 31, and 35 C were read after 24 hr; plates incubated at 16 and 12 C required 2 days, while those incubated at 6 C required 4 days of incubation. Relative EOP also was compared between homologous and heterologous bacterial hosts. Phage titres of 10^7, 10^4 and 10^2 PFU/ml were tested against each heterologous combination at 28 C.

Thermal inactivation. Heat stability of phages at 40, 45, 50, 55, 60, 65, and 75 C were tested in a temperature-regulated water bath. Phages, diluted to 2×10^3 PFU/ml with TYG broth, were heated for 10 min and then rapidly chilled in crushed ice before being assayed. Phage survival tests at selected temperatures also were performed at 5-min intervals up to 30 min.

Electron microscopy. Phage morphology and adsorption were examined by the negative staining method with a Hitachi HS-8-1 electron microscope operated at 50 kV. Phages, suspended in 0.1 M ammonium acetate neutralized with 1N KOH, were mixed in equal volumes with 2% neutral potassium phosphotungstate (PTA) and placed on carbon stabilized Formvar-covered specimen grids. For adsorption studies, bacteria at $\sim 10^8$ cells per milliliter were infected at 50–100 m.o.i. in TYG broth and droplets placed on specimen grids. After 30 min of adsorption time, grids were floated on droplets of distilled water, then stained for 1 min in PTA. Excess stain was removed with filter paper and then dried in the specimen chamber of the microscope before being examined.

Tests for lysogeny and bacteriocinogenicity. Three test methods were used as follows:

Method 1. Agar overlay method of Vidaver et al (33): Eighty-five *X. campestris* strains were tested as indicators against each other in all possible combinations, by mixing log-phase cells with 0.7%

TABLE 2. Characteristics of *Xanthomonas campestris* bacteriophages[a]

Phage	Adsorption rate[b] constant (K)	Eclipse[c] period (min)	Latent period[c] (min)	Rise period[c] (min)	Burst size (nm)	Thermal inactiviation[d] (c)	Morphology[e]
HP_1	$1.7–2.2 \times 10^{-10}$	65	65–70	70–80	75–98	70	O, CT
HP_3	$5.3–8.3 \times 10^{-11}$	65	65–70	45–50	25–42	70	H, CT
HT_{3h}	$5.2–8.8 \times 10^{-11}$	55	55–65	65–75	9–15	55	H, CT
HT_7	$1.9–5.8 \times 10^{-10}$	55	60–65	65–75	94–98	>70	H, CT
OH_2	$1.2–3.7 \times 10^{-10}$	55	55–65	40–55	23–37	70	H, CT
OK_2	$2.1–3.8 \times 10^{-10}$	55	55–65	55–65	25–45	70	H, CT
HXX	$4.4–6.2 \times 10^{-11}$	95	100–110	60–65	15–50	>70	H, NT
P_{1-3a}	$3.2–7.6 \times 10^{-10}$	45	50–55	35–40	50–73	55	H, NT
P_6	$1.7–4.9 \times 10^{-10}$	45	50–55	35–40	22–43	55	H, NT
A342	$6.2–7.2 \times 10^{-10}$	110	130–140	65–75	32–44	50	H, NT
RR68	$2.9–5.7 \times 10^{-10}$	95	105–115	85–100	220–242	70	H, ST

[a] Growth on homologous host at 28 C; two-to-five experiments.
[b] As described by Adams (1).
[c] Including 20-min adsorption period.
[d] Exposure for 10 min to inactivate 2×10^3 PFU.
[e] O, octahedral head; H, hexagonal head; CT, contractile tail; NT, noncontractile tail; ST, short wedge-shaped tail.

TABLE 3. Relative efficiency of plating (EOP) of bacteriophages on homologous and heterologous strains of *Xanthomonas campestris*[a]

Phage	Bacterial host strain								
	KC3-1-12	KC2-1-1	OH_2	OK^2	PHW46	RR68	XC118	XC114	A342
HP_1	1[b]	0.8	1	10^{-4d}	0[c]	10^{-4}	0	0	0
HP_3	1[b]	1	1.1	10^{-4}	0	10^{-4}	0	0	0
HT_7	1	1[b]	1	10^{-4}	0	10^{-4}	0	0	0
OH_2	0.8	0.8	1_b	10^{-4}	10^{-7d}	10^{-4}	0	0	0
OK_2	1	0.9	1	1[b]	10^{-7}	10^{-4}	0	0	0
HT_{3h}	0.9	1	0.8	0.4	1[b]	0.2	0	0	0
RR68	0[c]	0	0	0	0	1[b]	0	0	0
P_{1-3a}	0	0	0	0	0	0	1[b]	0.8	0
P_6	0	0	0	0	0	0	1.2	1[b]	0
HXX	0	0	0	0	0	0	0	1[b]	0
A342	0	0	0	0	0	0	0	0	1[b]

[a] Three experiments; EOP compared with homologous host as standard.
[b] EOP with homologous host.
[c] No plaques observed when plated with 10^7 plaque-forming units (PFU) per milliliter.
[d] Plaques observed only when plated with more than 10^4 or 10^7 PFU/ml.

molten water agar (45 C) and pouring 2.5-ml aliquots over chloroform-killed cultures grown on yeast-mineral salts (YS) plates (9). Overlaid plates were incubated at 28 C and read after 24 hr for inhibition zones.

Method 2. Mitomycin C induction: Seven milliliter volumes of log-phase cultures growing in YS broth at room temperature (~22 C) on a reciprocating shaker were treated with Mitomycin C (Sigma Chemical Co., St. Louis, MO 63178) to a final concentration of 0.2 μg/ml. A YS broth blank was used as control. After incubation for 2 hr, an equal volume of fresh YS broth was added to each culture and incubated for another 6 hr. The cultures were then centrifuged at 12,100 *g* for 10 min and the supernatant was used for spot-tests against the complete set of test bacteria, which were separately mixed with molten 0.6% water agar and layered over YS bottom agar. Plates were read for inhibition zones after 24 hr of incubation at 28 C.

Method 3. Ultraviolet light induction: Sixteen cultures, selected for resistance to a set of virulent *X. campestris* phages, were tested. Twenty-four-hour bacterial growth on YS agar slants was washed once with 0.85% saline and then suspended in 0.2 M phosphate buffer (pH 7.2) at a cell concentration of ~10^7 cells per milliliter. Two-milliliter aliquots were poured into open petri plates and exposed with gentle rocking of plates to a Westinghouse Sterilamp 782L-20 at a distance of 35 cm for 1 min. Fresh double strength YS broth (2 ml) was then added to each plate and incubated in the dark at 28 C for 8 hr before being centrifuged. Supernatants were then spot-tested against 85 *X. campestris* strains by the procedure described above.

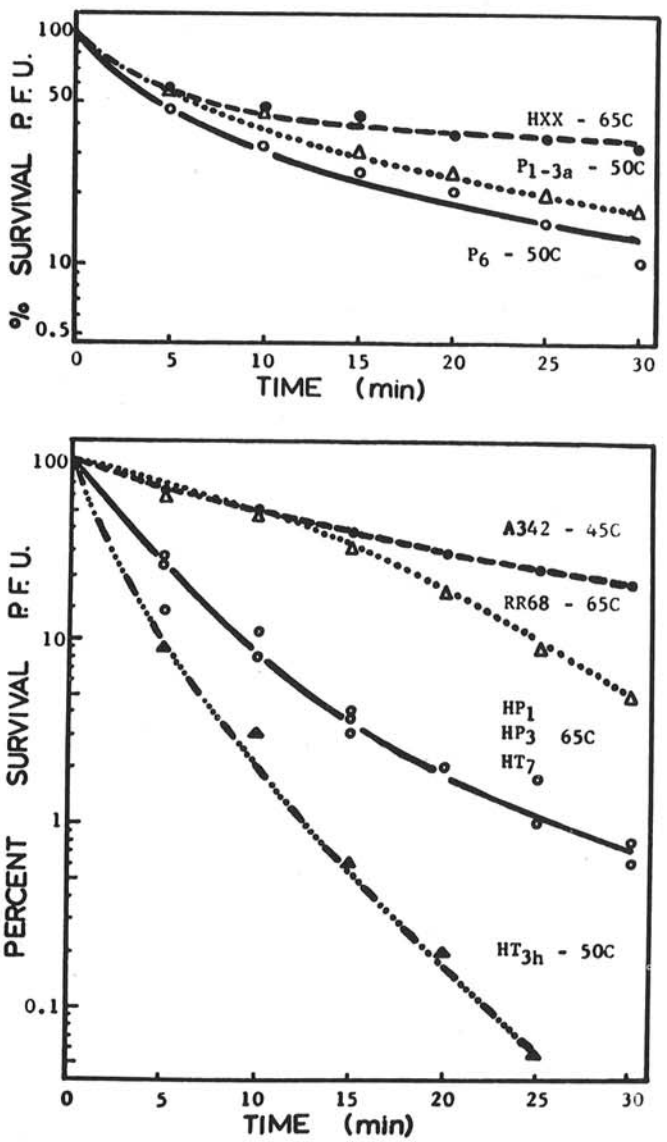

Fig. 1. Survival curves of *Xanthomonas campestris* phages at selected temperatures. Tubes containing 2×10^3 PFU/ml were cooled in crushed ice at 5 min intervals before assay.

RESULTS

Phage isolation and plaque morphology. All phages isolated were virulent and formed clear plaques except for A342, HXX, and HT_{3h} which were turbid. Plaques of HP_1, HP_3, $P_{1\text{-}3a}$, P_6, OH_2, and OK_2 had haloes and diameters of 1.5–2.5 mm except for P_6 which measured 2.0–3.5 mm. All were round with entire margins except for those of HP_3 which were slightly irregular. Plaques of HT_7 and RR68 formed no haloes, those of RR68 differing from HT_7 in having smaller diameters (0.5–1.5 mm) and slightly angular margins. Plaque diameters of A342 and HXX were 0.5–1.0 mm while those of HT_{3h} were variable in size (1.0–2.5 mm) with indistinct margins.

Phage HP_1 represents a group consistently isolated from Hawaiian soils. Phage isolations were attempted from 10 seed lots without any success (Takii & Co., Ltd., four; P. H. Williams, two; Honolulu Seed Co., two; T. Sakata & Co., one; Northrup King, one).

Adsorption rates, latent period, and burst size. Rates of phage adsorption varied for the different phages and are listed in Table 2 together with their other growth characteristics. Phages HXX, HT_{3h}, and HP_3 are notable for low adsorption rates and HXX, A342, and RR68 for relatively long latent periods.

Efficiency of plating. The EOP for homologous and heterologous phage-bacteria combinations are listed in Table 3. Phages A342, RR68, and HXX are very specific, whereas the remaining phages fall into two interacting groups. Temperature effects on relative EOP on their homologous hosts varied with the phages tested. The relative EOP of the *X. campestris* phages was high at temperatures of optimal bacterial growth except for A342 which reached its maximum at 18 C. All bacteria grew at 35 C, but no phages were infective at this temperature. Infectivity of A342, HT_{3h}, HXX, and OK_2 was markedly reduced at the lower temperatures, the first two being completely inhibited at 12 C.

Thermal inactivation. Phages A342, HT_{3h}, and P_6 were most sensitive to heat and were partially inactivated at 40 C. Partial inactivation of the other phages began at 45–50 C except for the more heat-tolerant HP_3, HT_7, and HXX which were partially inactivated at 55 C. Temperatures for complete inactivation of 2×10^3 PFU are summarized in Table 2. Phage survival curves at selected temperatures (Fig. 1) were all the multicomponent type except for phage RR68 which was of the multihit type (8).

Electron microscopy. Morphology of the different phages from partially purified lysates are shown in Fig. 2. All phages have polyhedral heads and may be separated into three groups based on their tail structures. Phages HP_1, HP_3, HT_7, HT_{3h}, OH_2, and OK_2 are morphologically similar with a rigid tail made up of a contractile tail sheath surrounding an inner core which terminates in a base plate. Uncontracted tails average 18 × 115 nm while contracted tail sheaths measure about 24 × 58 nm. The heads (60 nm in diameter) are supported by a narrow "neck" region (9 nm). Phage HP_1 has a distinct octahedral head. HXX, A342, $P_{1\text{-}3a}$, P_6, and RR68 have slightly smaller heads averaging 55 nm in diameter. The first four have long flexuous noncontractile tails averaging 14 × 120 nm whereas RR68 has a very stubby wedge-shaped tail about 15 nm long. Cellular adsorption was observed for A342, RR68, and all the phages with contractile tails. For phages HXX, $P_{1\text{-}3a}$, and P_6, adsorption onto cellular debris only was observed. In all cases, orientation of adsorption was tail-first.

Tests for lysogeny and bacteriocinogencity. All tests for lysogeny or bacteriocinogencity were negative. There were no signs of any definite indicator cell lysis. *X. campestris* isolates A240-4, Wai 2, and PHW-109 when used as indicators for the supernatants prepared from Mitomycin C induction, showed slightly less dense bacterial growth at more than 50% of the test spots, indicating slight growth inhibition. Spot-tests were repeated for these isolates as indicators with the same results. For each isolate, the soft agar

from five zones showing the most apparent inhibition was scraped off and suspended separately in 1 ml of YS broth. Tenfold dilution series were then prepared for each and 0.1-ml aliquots were plated out with their corresponding indicator. Results also were negative in this confirmatory test.

DISCUSSION

Seven virulent bacteriophages isolated in Hawaii were characterized and compared with two phages originating from Japan and two from North Carolina. Three distinct groups based on their tail structures were identified. These fall into morphological groups A, B, and C of Bradley's classification (5) and are inferred to have double-stranded DNA (1). Phages previously reported to infect *X. campestris* belong only to morphological group B (30,31). The two Japanese phages, OH_2 and OK_2, are morphologically very similar to four of the phages isolated from soils collected from commercial cabbage farms in Hawaii. These two phages can cross-infect with the Hawaiian *X. campestris* strains while their host bacteria also were susceptible to

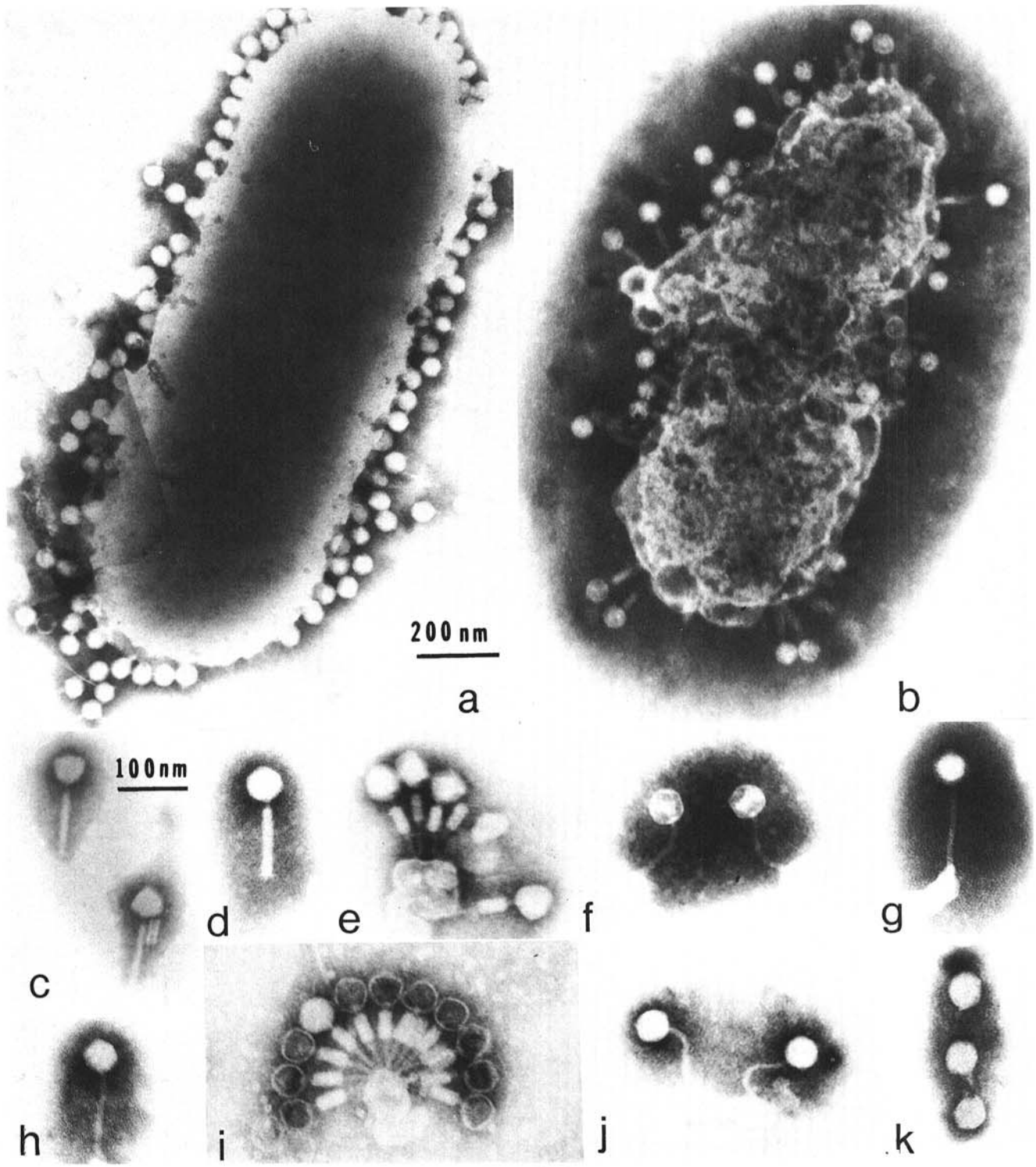

Fig. 2. Cellular adsorption and morphology of *Xanthomonas campestris* phages. Cellular adsorption of **a,** phage RR68 and **b,** phage HT_7 onto host cells. Note contracted tail sheaths on HT_7 (× 57,500). **c,** Phage HP_1, showing octahedral heads. **d,** Phage HT_{3h}, showing noncontracted tail sheath. Adsorption onto cellular debris by: **e,** phage OK_2; **f,** phage A342; **g,** phage HXX; **h,** phage $P_{1\text{-}3a}$; **i,** phage HP_3 (all except one show empty capsids); **j,** phage P_6; **k,** phage RR68, showing wedge-shaped tail. Magnifications of C–K, × 92,000.

four of the Hawaiian phages (Table 3). This group of phages and their bacterial hosts may be related since nearly all the cabbage seeds used by commercial farms are of Japanese origin, and the outbreak in 1970 of cabbage black rot that still continues to afflict many cabbage farms today was thought to have originated from infected seeds (T. Hori, *personal communication*).

Host-virus interactions for the other phages were very specific. The two North Carolina phages P_{1-3a} and P_6, formed a close interacting group with XC118 and XC114, their respective propagating hosts. Phage HXX shared the same host (XC114) as P_6 and was the only phage with a group B morphology (similar to the two North Carolina phages) that was isolated from Hawaiian soils. However, its lytic pattern and low adsorption rate excludes it from the interacting North Carolina group. This may reflect the selection pressure for a host-specific phage exerted by the enrichment technique used in isolation. Phages A342 and RR68 also lysed only their respective hosts. Detailed studies on the host range and lysotype of a collection of *X. campestris* of different geographical origins will be reported separately (23). Phage RR68 was unique in morphology, the nature of its thermal inactivation curve, and its large burst size (Table 2). So far as we know, this is the first report of a phage with this morphology known to infect the genus *Xanthomonas*. Other unique xanthomonad phages are the filamentous Xf and *X. oryzae* (20) and the tailless icosahedral phage CP_2 of *X. citri* (5,35).

Tolerance of phage to heat bore no correlation to higher relative EOP at higher incubation temperatures; the most heat-tolerant phage (HXX) and the least tolerant phage (A342) were similarly noninfective at 31 C while the heat-susceptible HT_{3h} was still infective at this temperature. Failure to infect at elevated and/or reduced temperatures may be due to failure of the phage to adsorb irreversibly onto the host cell because of modified host cell receptors or phage attachment structures, or to failure to grow and mature intracellularly. In *X. pruni,* reduced adsorption of phages to host cells grown at 35 C in comparison to those grown at 27 C was suggested to be due to host cell wall structural alterations that affected adsorption (6).

Only one lysogenic strain has been reported for *X. campestris* (31). In our laboratory, tests of 85 *X. campestris* strains for lysogenicity and/or bacteriocinogenicity were unsuccessful. Within the genus, lysogeny has been reported for *X. pruni* (10), and for *X. citri* (14,35,36).

The phages and their bacterial hosts have been deposited in the American Type Culture Collection, Rockville, MD 20802.

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