Isolation and Characterization of a Bacteriophage for the Identification of Corynebacterium michiganense

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ABSTRACT

A bacteriophage designated CMP1, was isolated from overwintering tomato stems infected with *Corynebacterium michiganense*. CMP1 plaques are about 0.5- to 1.0-mm diam, clear, round, and entire, after 48 hr incubation. They are formed at 16, 20, and 24 C, but not at 28 C or above. CMP1 lysed virulent isolates of *C. michiganense* from western North Carolina and other parts of the country, but did not lyse species in *Agrobacterium, Bacillus, Erwinia, Pseudomonas, Xanthomonas*, and *Corynebacterium* other than *C. michiganense*. CMP1 was gradually inactivated at 45 C and inactivated in 10 min between 50 and 55 C. Antiserum neutralization of CMP1 required 20 min. About 82% of the phage particles were adsorbed to bacterial cells in 60 min. The latent period of CMP1 was 180 min, its rise period about 180 min and the average burst size 17 particles per cell. CMP1 has an hexagonal head about 68.3-nm wide and a thin tail about 244.9-nm long and 9-nm wide. This phage was found to be very useful for rapid diagnosis of *C. michiganense*.

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Additional key words: bacterial canker of tomato, phage identification.

Bacteriophages are useful tools for detecting bacterial plant pathogens (3). The specificity of bacteriophage lysis, and the simplicity of the procedure for detecting lysis offers a practical means for identification of bacterial pathogens and for diagnosis of bacterial diseases. A *Corynebacterium michiganense* phage was first isolated in 1947 by Thomas (4). The phage's properties or characteristics were not described. In 1969, Wakimoto et al. (5) isolated two phages specific to *C. michiganense* from tomato stems collected in Nagano, Japan. Detailed descriptions of the phages were given in their paper. The objective of our work was to isolate a highly specific bacteriophage that could be utilized to detect *C. michiganense*.

MATERIALS AND METHODS.—*C. michiganense* cultures were purified by three successive single colony transfers. Only pure cultures that produced typical bacterial canker symptoms were utilized. Their designation and source are listed in Table 1. Other bacterial species were obtained from the collection maintained in the Department of Plant Pathology at N. C. State University. The species and the number of isolates of each were: *C. fascians*, 2; *C. insidiosum*, 1; *Xanthomonas translucens*, 1; *X. hyacinthi*, 1; *X. pruni*, 1; *X. campestris*, 1; *X. phaseoli*, 1; *X. malvacearum*, 1; *X. vesicatoria*, 1; *Agrobacterium tumefaciens*, 4; *A. rhizogenes*, 2; *Erwinia tracheiphila*, 1; *E. chrysanthemi*, 1; *E. carotovora*, 1; *E. atroseptica*, 1; *Bacillus polymyxa*, 1; *Pseudomonas solanacearum*, 2; *P. tabaci*, 1; and *P. lachrymans*, 1. All cultures were grown in nutrient agar and stored at 4 C until ready to use.

The following media were used: (i) Potato semisynthetic agar (PSA) consisting of broth from 300 g of boiled potatoes, $Na_2HPO_4 \cdot 12H_2O$ (2g), $Ca(NO_3)_2 \cdot 4H_2O$ (0.5g), glucose (20g), peptone (5g), agar (15g), and water up to 1 liter; (ii) Potato dextrose agar (PDA); (iii) Yeast-dextrose-calcium carbonate agar (YDC); (iv) Nutrient agar (NA); (v) Nutrient broth (NB); and (vi) 7% water agar (WA). PSA, NA, and NB were adjusted to pH 7.4 with 0.1 M NaOH before autoclaving.

Phage isolation and characterization.—Dry tomato stems were collected from several fields in Madison County, N. C. in the winter of 1971-72. Tomatoes in these fields were heavily attacked by *C. michiganense* in the summer of 1971. Ten to 20 g of stems from each field were chopped in a blender with 200 ml of NB and dispensed in a 1-liter flask containing 5 g of powdered calcium carbonate. Cultures of C829L grown on YDC slants for 18-24 hr at 28 C were suspended in 5 ml of NB and added to the mixture. The mixture and bacteria were incubated for 24 hr at 24 C, filtered through glass wool and about 20 ml were centrifuged at 10,000 *g* for 10 min to remove debris and bacterial cells. The supernatant was transferred to a separatory funnel and shaken vigorously with 2 ml of chloroform to kill any remaining bacteria. After separation of the chloroform and aqueous phase, 0.01 ml drops of the aqueous phase were withdrawn with a platinum loop and placed on the solidified surface of C829L suspended in WA and layered over PSA in a petri plate. Plates were set aside for the drops to dry and then incubated for 48 hr at 24 C. Lytic spots were removed, diluted in NB, and plated by the double-layer technique (1) with C829L as the host bacterium. One phage plaque was picked and replated with the host bacterium. The process was repeated

three times. One plaque from the last plating was cut out, suspended in 3 ml of NB, homogenized and plated in a dilution series with C829L by the double-layer technique with five replicate plates per dilution. Plates with semi-confluent plaques were used to prepare stock phage suspensions. Five ml of NB or 0.001 M phosphate buffer at pH 7.3 were added to each plate and incubated 3-5 hr at 24 C with occasional shaking. Phage was harvested from the resulting liquid extract by centrifuging at 10,000 *g* for 10 min. The supernatant was diluted with NB or phosphate buffer, and vigorously agitated in a separatory funnel with chloroform (10%, v/v). After separation of chloroform, the aqueous phase was stored at 4 C in sterile screw-cap glass bottles.

The phage's host range was determined by spot and quantitative plaque tests. For the spot test about 10^8 host cells were mixed with 2.5 ml of liquid (40 C) WA and immediately poured on the surface of petri dishes with hardened PSA. Once the layer of WA solidified, 0.01 ml of the phage at a concentration of 1-4 $\times$ 10^7 plaque forming units (PFU)/ml was spotted on the surface with a platinum loop and plates were incubated 24-48 hr at 24 C. For the quantitative test and routine phage titrations, the double-layer technique was used. In the quantitative test, 0.2 ml of a phage suspension with 1 to 2 $\times$ 10^3 PFU/ml was mixed in 2.5 ml of liquid (40 C) WA with about 10^8 host cells and immediately poured over hardened PSA in petri dishes. After incubating for 48-72 hr, the number of plaques in each plate were counted with a colony counter or microscope at 10 $\times$ magnification. Plaques on the plates were graded as follows: ++, number of plaques equal to or 50% less than homologous host; +, 49% to 20% less than homologous host; ±, less than 20% of homologous host; −, no plaques.

Thermal inactivation was determined in thin-walled, 5-ml test tubes containing 1 ml of phage suspended in NB. Tubes were immersed for 10 min in a water bath regulated at 35, 40, 45, 50, 55, and 60 C, respectively. After treatment, the tubes were immediately placed in crushed ice, and the suspension assayed by the double-layer technique, with C829L as the propagating host.

Anti-phage serum was prepared by injecting rabbits intravenously with a semi-purified, buffered, phage suspension containing about 10^{10} to 10^{12} PFU/ml. Rabbits were injected five times at weekly intervals with 1 ml of phage suspension and bled 7 days after the final injection. Antisera titer and neutralization were determined as described by Adams (1). Antiserum was diluted in NB and tests were conducted at 24 C.

Adsorption and one-step growth characteristics of the phage were studied. Adsorption was determined by placing 1 ml of phage with 1.3 $\times$ 10^3 to 2.2 $\times$ 10^3 PFU/ml in 9 ml of C829L at 2 $\times$ 10^7 to 5 $\times$ 10^7 cells/ml at 24 C. Aliquots were diluted at intervals in NB-chloroform (10% v/v) and assayed for unadsorbed phage by the double-layer technique with the propagating host bacterium. Latent period and burst size were determined as described by Adams (1). Phage suspensions were added at 2.4 $\times$ 10^{10} PFU/ml to 2.1 $\times$ 10^8 cells/ml of C829L. The phage was allowed to adsorb for 60 min, then antiserum was added and left to react 20 min. The reactant was diluted and assayed by the double-layer technique with the propagating host. The phage-bacterium mixture was incubated at 20 C and assayed at intervals both for infected cells and unadsorbed phage.

Phage morphology was determined with a Siemens Elmiskop electron microscope. Phage preparations were made in 0.001 M phosphate buffer using two cycles of low- and high-speed centrifugation (10,000 and 19,000 *g*). Washed phage particles were stained with 2% uranyl acetate, and measured by the technique described by Luftig (2).

Bacteriophage-lysis to diagnose C. michiganense.—C. michiganense was isolated from infected tomato stems, petioles, and fruits. Infected plant parts were excised, surface disinfested 5 min in 10% Chlorox, ground, diluted with sterile water, and

TABLE 1. Reaction of *Corynebacterium michiganense* isolates to CMP1 phage

Source and designation	Spot test[a]	Quantitative plaque test[b]
Western N. C. (E. Echandi)		
3	C	++
4	C	±
23	C	++
26	C	±
33	C	+
35	C	++
36	C	±
37B	C	++
38C	C	±
39A	C	++
41	C	++
42	C	++
43	C	±
Southern Cal. (R. G. Grogan)		
728	O	−
Rochester, N. Y. (R. S. Dickey)		
CM1	O	−
Cheyenne, Wyo. (B. D. Thyr)		
CM3	O	−
Western N. C. (D. Huisingh)		
CM7A	C	++
Merced, Cal. (R. G. Grogan)		
CM761	C	++
Western N. C. (D. Huisingh)		
C 100	C	++
C 829	C	++
C 829L[c]	C	++
American Type Culture Collection		
C10202	O	−
Berkeley, Cal. (M. Schroth)		
12049	C	±

[a] C = clear lytic spot; O = opaque lytic spot.

[b] ++ = number of plaques equal to or 50% less than homologous host; + = 49% to 20% less than homologous host; ± = less than 20% of homologous host; − = no plaques.

[c] = homologous host.

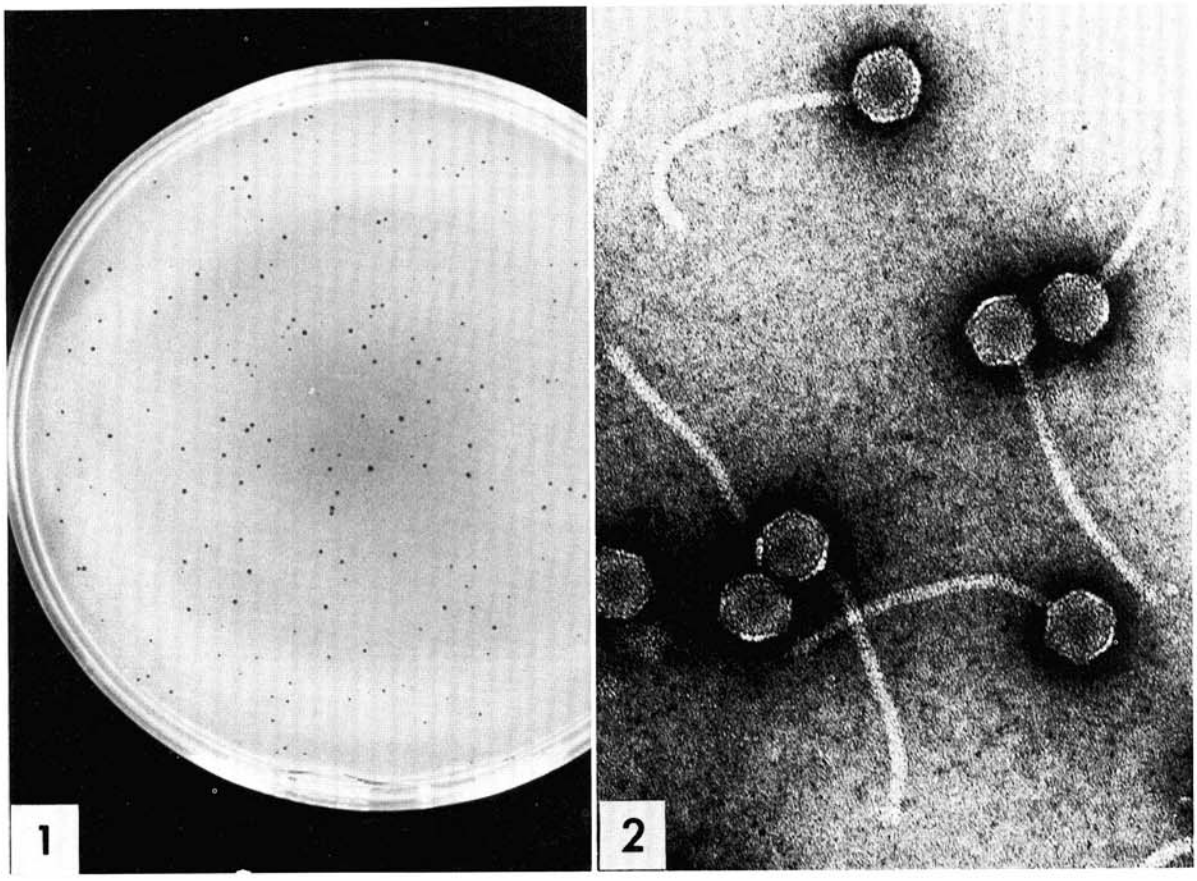

Fig. 1-2. 1) Plaques produced by phage CMP1 on *Corynebacterium michiganense* C829L after 48 hr at 24 C and **2)** CMP1 phage particles stained with uranyl acetate (× 40,000).

streaked on PDA plates. Cultures were incubated 48 hr at room temperature (27 C) and examined regularly for bacterial growth. Single corynebacterium-like colonies were selected. A small portion of each colony was transferred to a PDA slant and the remainder was diluted in 3 ml of NB. Dry surface of previously poured PSA or PDA plates were streaked with a sterile cotton swab moistened in the bacterial suspension. Excess fluid was eliminated from swabs by touching the inside wall of the container before streaking the agar. Once dry, the swab streaks provided a uniform band of inoculum where drops of phage (10^9 PFU/ml) were spotted with a sterile pipette drawn to a fine tip. Phage drops dried in a few minutes and plates were incubated at 22 C for 24 hr. A reaction was considered positive when lysis occurred.

RESULTS.—*Phage isolation and characterization.*—Infected tomato stems from western North Carolina yielded a phage designated CMP1. CMP1, when multiplied in its propagating host, produced clear plaques with sharp borders measuring 0.5- to 1.0-mm diam. Plaques began to appear after 24 hr incubation and were clearly visible and completely developed after 48 hr at 24 C (Fig. 1). Plaques were formed at 16, 20, and 24 C but not at 28 C or above.

The *C. michiganense* isolates listed in Table 1 were lysed by CMP1 with the spot test. Clear lytic spots were formed in all isolates except 728, CM1, CM3, and C10202 which developed opaque lytic spots (Table 1). In the quantitative plaque test, all isolates formed plaques except 728, CM1, CM3, and C10202, or the same isolates that formed opaque lytic spots in the spot test (Table 1). None of the other bacterial species were lysed by CMP1.

Thermal inactivation, serology, adsorption, and one-step growth characteristics of CMP1 were determined. CMP1 was partially inactivated in 10 min at 45 C and completely inactivated between 50 and 55 C. Titer of CMP1 antiserum was 1:200 when tested within a few days after being extracted. Antiserum neutralized CMP1 in 20 min. Normal rabbit serum did not neutralize the phage. About 82% of CMP1 particles were adsorbed to bacterial cells in 60 min at 24 C. CMP1 latent period was about 180 min, the rise period about 180 min, and its average burst size 17 particles per cell.

The gross morphology of CMP1 is shown in Fig. 2. Heads are hexagonal and average 68.3-nm diam and

the long and simple tails average 244.9 nm in length and 9.0 nm in width.

Bacteriophage-lysis to diagnose C. michiganense.—Seventy-five corynebacterium-like isolates were obtained from 96 infected tomato plants collected at random from 15 tomato farms in western North Carolina. Sixty-four of these isolates were lysed by CMP1. When 3-week-old tomato plants were inoculated with each of the 64 isolates, typical bacterial canker symptoms developed, confirming the identity of the pathogen. No symptoms developed in tomato seedlings inoculated with cultures not lysed by CMP1.

DISCUSSION.—Bacteriophage CMP1 was isolated and characterized. This phage is specific to *C. michiganense*, and has a wide host range within this bacterial species, as indicated by the spot tests and the quantitative plaque tests.

Although there is no report of lysogeny in *C. michiganense*, the formation of opaque lytic spots and the absence of plaques in isolates 728, CM1, CM3, and C10202 when subjected to the spot and the quantitative plaque tests could be an indication of lysogeny.

CMP1 is similar to MiPi and MiPih phages isolated in Japan by Wakimoto et al. (5) with respect to optimum temperature for plaque formation, thermal inactivation, and general morphology, but it differs by having a much longer and thinner tail than MiPi and MiPih.

C. michiganense was easily detected with CMP1. The procedure of isolating the pathogen and positively identifying it with CMP1 was found very useful in routine diagnosis and investigations of bacterial canker of tomatoes. Since most isolates tested were from North Carolina, the lysis test with CMP1 might have limited use with isolates from other regions.

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