Partial Characterization of Bacteriophages for *Clavibacter michiganense* subsp. *nebraskense**

Yukio SHIRAKO**, Anne K. VIDAVER*** and Hans-W. ACKERMANN****

Abstract

Bacteriophages Clm11, ClmX and ClmXC for *Clavibacter* (*Cl.*) *michiganense* subsp. *nebraskense* were characterized with respect to biological and physical properties. All phages produced clear plaques on strain CN18-5, the original propagating host. On strain CN76-2, ClmX produced predominantly turbid plaques with a few clear plaques, from which ClmXC was derived. The host range of the three phages was restricted to strains of *Cl. michiganense* subsp. *nebraskense*. Adsorption rate constants and burst sizes of the three phages were in the range of 2.9 to 9.4×10^{-9} ml/min and 6 to 19 virions/cell, respectively. All phages were morphologically similar with a hexagonal head about 60 nm in width and a flexuous tail approximately 235 nm long. The type of nucleic acid was double-stranded DNA. Agarose gel electrophoresis of restriction endonuclease digests of the phage DNAs indicated that the three phages are similar, but distinguishable in DNA structure. ClmXC was probably a deletion mutant of ClmX.

(Received June 20, 1986)

Key words: *Clavibacter michiganense* subsp. *nebraskense*, bacteriophages, characterization.

Introduction

There are only a few reports of phages for coryneform bacteria^{3,11,15}. In plant pathogenic species, there are basic descriptions for phages specific for *Corynebacterium* (*C.*) *flaccumfaciens* subsp. *flaccumfaciens*¹², *Cl. michiganense* subsp. *insidiosum*⁵, *Cl. michiganense* subsp. *michiganense*^{8,21} and *Cl. rathyai*⁴. This paper describes some biological and physical properties of selected phages active against *Cl. michiganense* subsp. *nebraskense*, the causal agent of Goss's bacterial wilt and blight of corn¹⁹. The guidelines of Ackermann *et al.*¹ were followed in choosing properties for phage characterization and nomenclature. The taxonomy of plant pathogenic coryneform bacteria is that of Davis *et al.*⁶.

Materials and Methods

Media and growth conditions. A nutrient broth-yeast extract (NBY) medium¹⁷ was used in liquid or solid form as previously described²⁰. Bacteria were grown in liq-

* This work was supported in part by USDA-RRF funds (NC-135) to A. K. Vidaver.

** Faculty of Agriculture, Tohoku University, Sendai 980, Japan 東北大学農学部

*** Department of Plant Pathology, University of Nebraska, Lincoln 68583, USA.

**** Felix d'Herelle Reference Center for Bacterial Viruses, Department of Microbiology, Faculty of Medicine, Laval University, Quebec 10, P. Q., Canada G1K 7P4.

uid culture at 25 C, on a rotary shaker at 250 rpm and used as plating cells when 0.3–0.5 A_{640} was reached.

Phages and the host. Among the phages isolated previously^{18,19}, CN8, CN11 and CNX, which are renamed Clm8, Clm11 and ClmX, respectively, in accordance with published guidelines¹¹, were further characterized in this study. *Cl. michiganense* subsp. *nebraskense* strain CN18-5¹⁸ was used as the propagating host of the phages.

Host range determinations. In addition to 67 strains of *Cl. michiganense* subsp. *nebraskense* used in phage typing¹⁸, the following bacteria were tested for phage sensitivity. *Cl. michiganense* subsp. *michiganense* (2); *Cl. michiganense* subsp. *sepedonicum* (2); *Cl. michiganense* subsp. *insidiosum* (2); *Cl. michiganense* subsp. *tessellarius* (2); *Cl. iranicum* (1); *Cl. rathayi* (1); *Cl. tritici* (1); *C. flaccumfaciens* subsp. *flaccumfaciens* (1); *C. flaccumfaciens* subsp. *betae* (1); *C. flaccumfaciens* subsp. *oortii* (1); *C. ilicis* (1); *C. fascians* (1); *Micrococcus luteus* (1); *Pseudomonas syringae* pv. *syringae* (1); *P. cepacia* (1); *Xanthomonas campestris* pv. *phaseoli* (1); and *Erwinia carotovora* subsp. *atroseptica* (1). The number of strains tested is in parentheses. A routine test dilution (RTD) was determined for strain CN18-5, according to the method of Parker¹⁶. Ten μ l of purified lysate in RTD and 100-times concentrated RTD were deposited on freshly prepared bacterial lawns as previously described²⁰. After 2 days of incubation at 25 C, the presence and types of plaques were recorded.

Purification of phages. The procedures were essentially as previously described²⁰. High titer lysates were routinely prepared from confluent lysis plates by adding 5 ml of NBY broth to each of 10 to 20 plates. After about 20 min, the soft-agar layers were scraped off with a bent glass rod. The crude lysates were clarified twice by low speed centrifugations at $15,000 \times g$ for 15 min and then at $18,000 \times g$ for 10 min. The supernatant was centrifuged at $65,000 \times g$ for 60 min over 6 ml of a 20% sucrose per tube in a Beckman type 30 rotor. The pellet was suspended in 12.5 mM phosphate buffer, pH 7.1 (PB), and centrifuged at $175,000 \times g$ for 30 min through a linear 10–40% sucrose density gradient prepared in PB. The gradient was fractionated with an ISCO model 183 fractionator and an ISCO UA-5 absorbance monitor. The phage fraction was diluted in PB and pelleted at $65,000 \times g$ for 60 min. The final pellet was suspended in PB and stored at 4 C until needed.

Electron microscopy. Purified phages were washed twice in 0.1 M neutral ammonium acetate with centrifugation at $72,400 \times g$ for 90 min. The suspensions were deposited on copper grids with carbon-coated Formvar films and were stained with 2% potassium phosphotungstate, pH 7.2 (PT), or 4% uranyl acetate, pH 4.2 (UA), supplemented or not with 50 μ g/ml of bacitracin for wetting¹⁰. The virions were examined in a Philips EM 300 electron microscope. Magnification was monitored with catalase crystals¹³.

Isolation of nucleic acid. Nucleic acid was isolated from the purified phage with either of the following two methods. (1) The purified phage was dissociated in 2% sodium dodecyl sulphate (SDS), 1% 2-mercaptoethanol, 1 mM disodium ethylenediaminetetraacetate (EDTA), 30 mM sodium phosphate, pH 9.0, at 75 C for 15 min. The liberated nucleic acid was separated from the residual protein through a 10–40% linear sucrose density gradient prepared in 150 mM NaCl, 15 mM sodium citrate, pH 7.0, cen-

trifuged at $175,000\times g$ for 5 hr. The UV-absorbing, nucleic acid fraction was collected with an ISCO model 183 fractionator and an ISCO UA-5 absorbance monitor. The nucleic acid was precipitated in 70% ethanol at -20°C overnight, pelleted by a centrifugation and suspended in 10 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5. (2) After dissociating the phage as described above, the denatured protein was precipitated by adding potassium acetate to 0.5 M at 0°C for 1 hr. The precipitate containing the denatured protein was removed by a low speed centrifugation. The nucleic acid in the supernatant was precipitated with ethanol as before and suspended in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5, containing $10\text{ }\mu\text{g/ml}$ of RNase A⁹.

Determination of nucleic acid type. The isolated nucleic acid was incubated under four different conditions: (1) in 10 mM NaCl at 37°C for 1 hr; (2) in 0.1 M NaOH at 37°C for 2 hr, followed by neutralization with an equal volume of 0.2 N HCl, 30 mM sodium phosphate, pH 7.2; (3) in $20\text{ }\mu\text{g/ml}$ of DNase I, 1 mM MgCl_2 at 37°C for 1 hr; and (4) in $2\text{ }\mu\text{g/ml}$ of RNase A at 37°C for 1 hr. After incubation, the preparations were centrifuged through linear 10–40% sucrose density gradients prepared in 0.3 M NaCl, 20 mM Tris-HCl, pH 7.0, at $175,000\times g$ for 3 hr. The gradients were scanned at A_{254} as before.

Restriction endonuclease digestion and agarose gel electrophoresis. The isolated nucleic acid and λ DNA were digested with Hind III, Bam HI, Eco RI and Sal I (BRL, USA) at 37°C for 30 min. Reaction buffers for each enzyme were prepared according to the recommendation of BRL. The reaction was stopped at 70°C for 5 min. The intact and enzyme-treated nucleic acids were electrophoresed in 0.7% agarose (Bio-rad, USA) in 40 mM Tris-acetate, pH 8.0, 2 mM EDTA. Electrophoresis was run on a horizontal apparatus at 1 V/cm for 12 hr at room temperature. After electrophoresis, the gel was stained and visualized as previously described⁹.

Results

Host range

Clm8, Clm11 and ClmX infected only *Cl. michiganense* subsp. *nebraskense* strains of bacteria examined, producing clear plaques of 0.5 to 1.5 mm in diameter, with the exception that ClmX produced a few clear plaques but mostly turbid ones on strain CN76-2¹⁹. The turbid plaque formers from strain CN76-2 always gave rise to both plaque types with this host, whereas clear plaque formers consistently produced clear plaques on all the strains of *Cl. michiganense* subsp. *nebraskense*, including strain CN76-2. This clear plaque-forming derivative was purified by ten successive plaque pickings on strain CN76-2 and designated as ClmXC.

Adsorption rate constant, latent period and burst size

The adsorption rate constants, latent periods and burst sizes of the three phages are shown in Table 1. The phage lytic cycles are longer than the doubling time of the host (120–210 min).

Morphology

All four phages were morphologically identical and had isometric heads and long, flexuous, non-contractile tails (Fig. 1). Base plates or collars were not seen. PT-stained

Table 1. Biological properties of *Cl. michiganense* subsp. *nebraskense* bacteriophages^{a)}

Phage	Adsorption rate constant K ml/min	Latent period min	Rise period min	Burst size relative increase virions/cell
Clm11	4.2×10^{-9}	180	90	8
ClmX	2.9×10^{-9}	210	90	9
ClmXC	9.4×10^{-9}	180	120	16

a) Host strain CN18-5 was used.

Fig. 1. *Cl. michiganense* subsp. *nebraskense* phages after staining with phosphotungstate (PT) and uranyl acetate (UA). Bars indicate 100nm. (A) Phage Clm8 showing a hexagonal head, a structureless tail and club-shaped spikes in a petal-like arrangement; bacitracin-PT. (B) Phage ClmXC showing tail striations and relatively small head; UA. (C) Pentagonal capsid in phage Clm8; UA. (D) Positively stained, shrunken capsid in phage ClmXC; UA.

phages showed relatively large heads and apparently flattened, structureless tails. Addition of bacitracin revealed the presence of 5 to 6 club-shaped spikes (Fig. 1-A). UA-stained phage tails showed distinct cross striations (Fig. 1-B). Heads were icosahedra as evidenced by the simultaneous occurrence of capsids with hexagonal and pentagonal outlines (Fig. 1-C). UA-positively stained phage heads were always shrunken (Fig. 1-D) and were not measured. Their dimensions are listed in Table 2. Phages Clm11, ClmX and ClmXC were chosen for further study.

Nucleic acid type

Clm11 and ClmX were purified from CN18-5 lysates and ClmXC, from those of CN76-2.

Table 2. Dimensions of *Cl. michiganense* subsp. *nebraskense* bacteriophages stained with phosphotungstate or uranyl acetate^{a)}

Phage part	Phosphotungstate	Uranyl acetate
Head: apical diameter, nm	62±3	59±3
sides, nm	30	29
Tail: length, nm	234±6	234±4
diameter, nm	11	9
striations, number	not seen	55
striations, periodicity, nm	not seen	5
spikes, number	6?	not seen
spikes, length, nm	23	not seen

a) Twenty particles were measured per stain.

Fig 2. Sedimentation profiles of phage Clm11 nucleic acid after different treatments. (A) Incubated in 2 µg/ml of RNase A at 37°C for 1 hr; (B) Incubated in 20 µg/ml of DNase I, with 1 mM MgCl₂ at 37°C for 1 hr.; (C) Incubated in 0.1 N NaOH at 37°C for 2 hr, then neutralized with 0.2 N HCl in 30 mM phosphate buffer, pH 7.2; (D) Incubated in 10 mM NaCl at 37°C for 1 hr.

When purified phage was dissociated in buffer at 75°C for 15 min and centrifuged through 10–40% linear sucrose density gradients at 175,000×*g* for 5 hr, only a single UV-absorbing peak was observed in the center of the gradient. The isolated nucleic acid was resistant to RNase A (Fig. 2-A) and sensitive to DNase I (Fig. 2-B), indicating that it was DNA. The faster sedimentation of the nucleic acid after alkaline treatment, followed by neutralization, than that of the control (Fig. 2-C, D), indicated that the DNA was linear or open circular double-stranded (only the result of Clm11 nucleic acid is shown). If the DNA was covalently closed, double-stranded circular, single-stranded linear or circular, it would be expected to sediment at the same rate as the control.

Restriction endonuclease digestion of phage DNA

DNA from Clm11, ClmX and ClmXC were examined (Fig. 3). λ-DNA digests were used as size markers. DNA from Clm11, ClmX and ClmXC were readily digested by

Fig. 3. Restriction endonuclease digested DNA. The lanes contain the following DNA samples: (A through D) Undigested DNA of λ , Clm11, ClmX and ClmXC, (E through H) Hind III digests of λ , Clm11, ClmX and ClmXC, (I through L) Bam HI digests of λ , Clm11, ClmX and ClmXC, (M through P) Eco RI digests of λ , Clm11, ClmX and ClmXC and (Q through T) Sal I digests of λ , Clm11, ClmX and ClmXC.

Hind III, Bam HI and Sal I and apparently incompletely by Eco RI. The size and number of the fragments of the three phage DNA were very similar to each other but differences were also readily and reproducibly detected. ClmXC DNA digested with Bam HI and Sal I had fewer bands and some were of a lower molecular mass than ClmX.

Discussion

Phages specific for the phytopathogens *C. flaccumfaciens* subsp. *flaccumfaciens*¹²⁾, *Cl. michiganense* subsp. *michiganense*^{8, 21)}, *Cl. michiganense* subsp. *insidiosum*⁵⁾ and *Cl. rathayi*⁴⁾ have been reported. The phages reported here are specific for *Cl. michiganense* subsp. *nebraskense*. Such phage specificity may reflect differences in receptor site composition or structure, as well as differences in restriction. The phages have been deposited in the Felix d'Herelle Reference Center for Bacterial Viruses, Quebec, P. Q., Canada. Accession numbers for phages Clm8, Clm11 and ClmXC are HER 88, HER 89 and HER 92, respectively.

Our phages are of the same morphological type as other phages of coryneform bacteria; all known phages for this group have isometric heads and long, non-contractile tails^{3, 5, 8, 11, 12, 21)}. Among other *Cl. michiganense* subsp. *michiganense* phages, they closely resemble phage CMPI, but differ from phages MiP₁ and MiP₁₁²¹⁾ by their much longer tail. The true capsid diameter of our phages is probably about 60 nm, in between di-

mensions of PT- and UA-stained particles. PT-stained particles often appeared flattened, whereas UA frequently induces shrinkage of phage heads²⁾. The wetting agent bacitracin, is recommended for the visualization of tail fibers¹⁰⁾. The morphology and the type of nucleic acid indicate all the phages specific for corynebacteria belong to styroviridae¹⁴⁾.

The close relationship of the phages Clm11, ClmX and ClmXC was revealed by restriction endonuclease analysis. It should be recalled that phage ClmXC was isolated from a clear plaque produced by phage ClmX on strain CN76-2, on which the majority of plaques were turbid. Strain CN76-2 did not appear to be lysogenized by ClmX under our conditions, nor was there evidence that strain CN76-2 contained a temperate phage active against any other test strain (data not shown). If the strain, nevertheless, was induced upon infection with phage ClmX, the probability is that the phage would not be closely related to ClmXC. Therefore, it seems most likely that phage ClmXC is a deletion mutant originating from phage ClmX.

We thank M. K. Brakke and L. C. Lane for advice and helpful discussion, and P. Lambrecht for technical assistance.

Literature cited

1. Ackermann, H. -W., Audurier, A., Berthiaume, L., Jones, L. A., Mayo, J. A. and Vidaver, A. K. (1978). Adv. Virus Res. 23 : 1-24.
2. Ackermann, H. -W., Jolicœur, P. and Berthiaume, L. (1974). Can. J. Microbiol. 20 : 1093-1099.
3. Berthiaume, L. and Ackermann, H. -W. (1977). Pathol. Biol. 25 : 195-201.
4. Bradbury, J. F. (1973). CMI Descriptions of Pathogenic Fungi and Bacteria, No. 376. Kew.
5. Cook, F. D. and Katznelson, H. (1960). Can. J. Microbiol. 6 : 121-125.
6. Davis, M. J., Gillaspie, Jr., A. J., Vidaver, A. K. and Harris, R. W. (1984). Int. J. Syst. Bacteriol. 34 : 107-117.
7. Davis, R. W., Botstein, D. and Roth, J. R. (1980). Advanced Bacterial Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
8. Echandi, E. and Sun, M. (1973). Phytopathology 63 : 1398-1401.
9. Gonzalez, C. F. and Vidaver, A. K. (1979). J. Gen. Microbiol. 110 : 161-170.
10. Gregory, D. W. and Pirie, B. J. S. (1973). J. Microsc. 99 : 261-265.
11. Holmes, R. K. and Barksdale, L. (1970). J. Virol. 5 : 783-794.
12. Klement, Z. and Lovas, B. (1959). Phytopathology 49 : 109-112.
13. Luftig, R. (1967). J. Ultrastruct. Res. 20 : 91-102.
14. Matthews, R. E. F. (1982). Intervirology 17 : 1-199.
15. Nagington, J. and Carne, H. R. (1971). J. Gen. Virol. 13 : 167-172.
16. Parker, M. T. (1972). Methods of Microbiology, Vol. 7B, Academic Press, London. pp. 1-28.
17. Vidaver, A. K. (1967). Appl. Microbiol. 15 : 1523-1524.
18. Vidaver, A. K., Gross, D. C., Wysong, D. S. and Doupnik, B. L. (1981). Plant Disease 65 : 480-483.
19. Vidaver, A. K. and Mandel, M. (1974). Int. J. Syst. Bacteriol. 24 : 482-485.
20. Vidaver, A. K. and Schuster, M. L. (1969). J. Virol. 4 : 300-308.
21. Wakimoto, S., Uematsu, T. and Mizukami, T. (1969). Ann. Phytopath. Soc. Japan. 35 : 168-173.

和 文 摘 要

白子幸男・Anne K. VIDAVER・Hans-W. ACKERMANN : *Clavibacter michiganense* subsp. *nebraskense* 感染性バクテリオファージの性状

Clavibacter michiganense subsp. *nebraskense* 感染性バクテリオファージ Clm11, ClmX および ClmXC の生物学および物理学的性状を調べた。全てのファージは増殖用 CN18-5 菌株に対し、透明な溶菌斑を形成した。ClmX は CN76-5 菌株に対し、多数の不透明溶菌斑と共に少数の透明溶菌斑を形成し、その透明溶菌斑から単溶菌斑分離法により ClmXC が分離された。宿主範囲は *Cl. michiganense* subsp. *nebraskense* に限定され、吸着速度係数は $2.9 \sim 9.4 \times 10^{-9}$ ml/min, バーストサイズは 6~19個だった。粒子形態は幅 60 nm の六角形状の頭部と長さ約 235 nm の屈曲性を持つ尾部からなるオタマジャクシ状であった。核酸は 2 本鎖 DNA で、制限酵素分解産物のフラグメント数と大きさにおいて各ファージ間に若干の違いが認められた。ClmXC は ClmX の欠失変異株と考えられた。