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Characterization of phage ϕL7 and transfection of *Xanthomonas campestris* pv. *campestris* by the phage DNA

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Abstract. A virulent phage, ϕL7, which specifically infects *Xanthomonas campestris* pv. *campestris* was isolated. This phage is tadpoled-shaped, having a hexagonal head that measured 60 nm in diameter and a noncontractile flexuous tail that averaged 10 x 180 nm. It is stable at temperatures between 4°C and 70°C, but inactivated at pH lower than 4.0. It possesses a double-stranded DNA of approximately 37 kb as determined by the sum of restriction fragment length. Since the transformation system of *X. campestris* has not yet been established, we tried to work out the appropriate conditions starting with transfection. In this study, we found that treatments with Tris-hydrochloride containing $CaCl_2$ and polyethylene glycol 6000 rendered the cells competent, and the transfection frequency obtained was approximately 1.2 x 10^3 plaque-forming units per μg phage ϕL7 DNA.

Key words: ϕL7; Transfection ; Virulent phage; *Xanthomonas campestris*.

Introduction

The usefulness of bacteriophage is several fold. In molecular biology, bacteriophages per se are interesting life systems (Luria *et al.*, 1978); also, they offer a way to develope cloning vectors (Zinder and Boeke, 1982). In plant pathology, phage typing is useful for studying the occurence and distribution of lysotypes of causal bacteria (Liew and Alvarez, 1981b).

Relatively little information is available concerning the phages that attack *Xanthomonas campestris* pv. *campestris* (for short *X. campestris*), the pathogen causing black rot in crucifers (Williams, 1980) and the industrial microorganism for xanthan gum production (Jeanes, 1974). Sutton *et al.* (1958) described a phage that attacks numerous *Xanthomonas* including *X. campestris*. Sutton and Quadling (1963) reported a temperate phage that specifically attacks *X. campestris* and lysogenizes the host cells. Tseng and Lo (1986) isolated a filamentous, single-stranded DNA phage that specifically infects *X. campestris* without lysis and is able to integrate its genome into the host chromosome (Tseng *et al.*, 1989). Virulent specific phages for *X. campestris* have been reported by Watanabe *et al.* (1980) and Liew and Alvarez (1981a). However, none of the *X. campestris* phages has yet been developed as cloning vector.

In this report, we describe the isolation and characterization of the virulent phage ϕL7 for *X. campestris* and the procedures for transfection with the phage DNA.

Materials and Methods

Bacteria, Media and Cultivation

Xanthomonas campestris pv. *campestris* 11 has been described elsewhere (Yang and Tseng, 1988). *X. campestris* pv. *oryzae* and *X. campestris* pv. *phaseoli* were obtained from S. T. Hsu. *X. campestris* pv. *citri*

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was obtained from W. C. Wu. Bacteria were all cultured in LB medium (Miller, 1972) with vigorous shaking (280 rpm) at 28°C. XOL medium (Chu and Tseng, 1984), a basal salt medium, was used as the suspending buffer for bacteriophage and bacterial cells.

Isolation of Phage ϕL7

Phage ϕL7 was isolated from the soil collected in a garden where cabbage showed black rot symptoms. 10 g of the soil was added into 40 ml of exponentially growing culture of *X. campestris* 11 in LB medium. After overnight incubation at 28°C, the culture fluid was centrifuged at 10,000 x g for 10 min. The supernatant was shaken with chloroform (10% v/v) and tested for phage by applying a drop of the aqueous fluid on the LA plate containing *X. campestris* 11 as the indicator host. The clearing spot was picked and suspended in XOL medium and then purified by three sucessive single-plaque isolations.

Plaque Assay

The double-layered plate method described by Adams (1959) was adopted for plaque assay. One tenth ml of a phage sample, after appropriate dilution with XOL medium, together with 3.5 ml of molten LA soft agar (0.75%) containing 0.5 ml of an overnight culture of *X. campestris* 11 was poured onto an LA agar plate. After overnight incubation at 28°C the plaques that appeared were counted.

Test of Host Specificity

To test the susceptibility of different hosts to the phage ϕL7, a double-layered plate was made as described above, except that the individual host was included in each plate and the phage was spotted onto the surface of the plate. Clearing zone indicated a positive result.

Preparation of Phage ϕL7 and Phage DNA

The protocol used to prepare the phage ϕL7 DNA was previously described by Fu and Tseng (1990). The host for propagation was *X. campestris* 11.

Enzymes and Restriction Digestions

Restriction endonucleases, DNase, S1 nuclease, RNase and proteinase K were purchased from BRL (Besthesda Research Laboratories) and used according to the instructions provided by the supplier.

Agarose Gel Electrophoresis

DNA fragments were electrophoretically separated on 0.7% agarose gel with TAE buffer (40 mM Tris-acetate, pH 8.0, containing 2 mM EDTA-Na_2) as described elsewhere (Maniatis *et al.*, 1982).

Electron Microscopy

For electron microscopic observations, the ϕL7 was purified by two sucessive block gradient according to Miller (1972). The purified phage sample was stained with 1% uranyl acetate and examined with a Hitachi Hu-11A electron microscope.

Transfection

Thirty ml of *X. campestris* 11 cultured in LB at early log phase (0.6 unit of OD at 550 nm) was harvested and washed once with 250 mM Tris-HCl, pH 7.5 at 4°C. The cell suspension was centrifuged immediately at 8,000 x g for 5 min at 4°C and resuspended in 50 mM Tris-HCl, pH 9.0 containing 100 mM $CaCl_2$, then incubated at 25°C for 30 min. The cells were pelleted and resuspended in 1 ml of XOL medium, and then kept on ice and used immediately. To a 1.5 ml eppendorf tube, were added in order 400 μl of the cells and then 2 μg of the ϕL7 DNA in 200 μl TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA-Na_2) and 400 μl of 40% polyethylene glycol (PEG) 6,000 in 100 mM Tris-HCl, pH 7.2. The mixture was incubated on ice for 30 min and moved to 35°C for 2 min. Subsequently, 4 ml of LB was added and followed by incubation at 28°C for 20 min. The numbers of transfected cells were counted as infective centers by double-layer assay.

Stability Tests of ϕL7

To test the thermal stability of ϕL7, the crude lysate (2×10^{11} PFU/ml) was diluted 100-fold with XOL medium and incubated in a waterbath at appropriate temperature for 10 min and then stored in ice bath for survival assay. To test the effects of pH on the infectivity of ϕL7, the crude lysate was diluted 100-fold into an appropriate buffer and incubated for 30 min before the survival titer was measured. For pH 7 to 10, 50 mM Tris-HCl buffer was used, whereas 50 mM Na-acetate was used for pH 4 to 6. To test the effects of organic solvents on the ϕL7 infectivity, equal volumes of a solvent and the diluted (100-fold) crude extract were well mixed, and left standing at room

temperature for 30 min. The survival titer was subsequently examined.

Results

Isolation of Phage ϕL7

From the soil of a local garden, where cabbage showed symptom of black rot, some plaque-forming agents against *X. campestris* 11 were detected. After single-plaque isolation, one of them was isolated and designated ϕL7. This phage could lyse a growing culture of *X. campestris* 11 and form clear plaques of 2-3 mm in diameter, and pass through a millipore nitrocellulose membrane filter, demonstrating the properties of a virulent phage.

Morphology and Host Specificity

The phage ϕL7 was tadpole-shaped, having a regular hexagonal head that measured approximately 60 nm in diameter, and a non-contractile flexuous tail that averaged 10 x 180 nm (Fig. 1). It infected 33 Taiwan isolates of *X. campestris* pv. *campestris*, but showed no infection toward the *X. campestris* pv. *citri*, *X. campestris* pv. *oryzae* or *X. campestris* pv. *phaseoli* tested.

Stability of ϕL7

The phage was stable in crude lysate and could be stored for 6 months at 4°C. At temperatures lower than 58°C, the phage remained 100% infective for at least 10 min; at higher temperatures the infectivity declined abruptly. At temperatures over 70°C for 10 min, the infectivity was disappeared (Fig. 2). The phage remained active in Tris-HCl, pH 10.0. However, it became inactivated at pH 4.0 or lower in either Na-acetate or Tris-HCl buffer. Chloroform and ether, added to the crude lysate at 1:1 ratio, caused the phage to lose about 53% and 46% of its infectivity, respectively, whereas similarly added methanol and acetone had no effect on the infectivity of ϕL7.

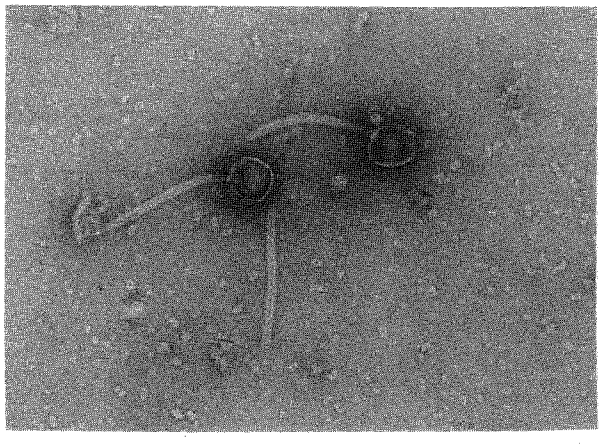

Fig. 1. Electron micrograph of phage ϕL7 stained with 1% uranyl acetate. (×40,000)

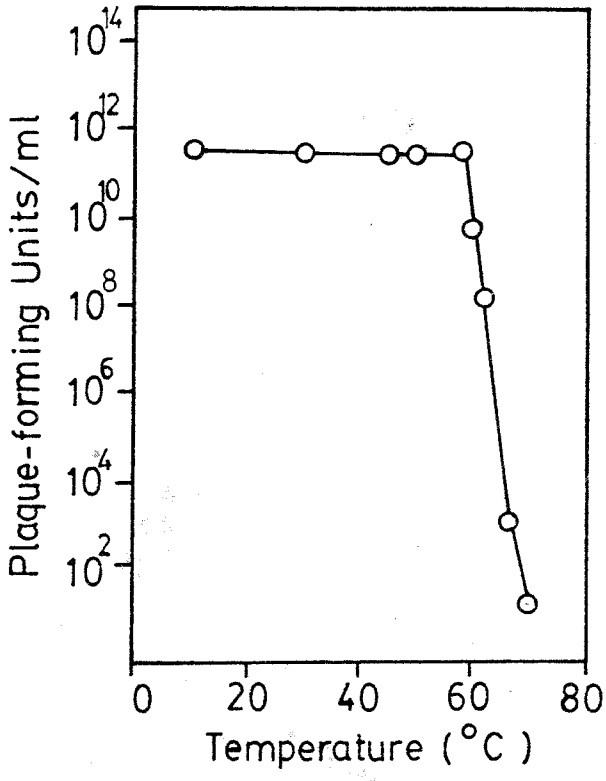

Fig. 2. The temperature effect on the stability of phage ϕL7. The phage suspension prepared as described in the Materials and Methods was incubated at various temperatures for 10 minutes before survival assay.

The Genome of ϕL7

The genomic DNA of ϕL7 was resistant to Sl nuclease, RNase and protease, but sensitive to DNase and restriction endonucleases, indicating that the genome was a double-stranded DNA molecule. When the DNA was subjected to restriction enzyme digestions, distinct patterns were revealed on a 0.7% agarose gel electrophoresis. Fig. 3 shows one of the representative electrophoretogram of such digestion products. The restriction fragment lengths obtained from the digestions with *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Nco*I and *Pvu*II,

which gave suitable pattern, were summed up and the genomic size was calculated to be 37 kb (Table 1).

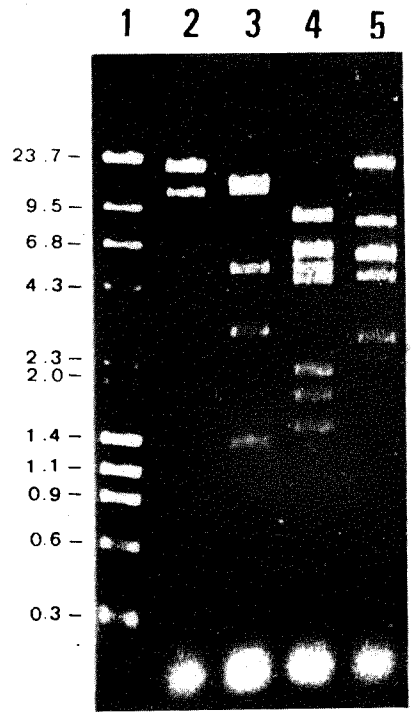

Fig. 3. Agarose gel electrophoresis of restriction endonuclease fragments of ϕL7 DNA digested with various restriction enzymes. Lane 1 is *Hind*III-digested λ DNA plus *Hae*III-digested ϕX174 DNA as the molecular weight standard. Lanes 2-5 are ϕL7 DNA digested with *Eco*RI, *Eco*RI+*Bam*HI, *Eco*RI+*Eco*RV and *Eco*RI+*Pst*I, respectively. The fragments were separated on a 0.7% agarose gel.

Transfection of ***X. campestris*** *by* ϕ*L7*

Two sources of hint suggested that treatments with a combination of Tris-HCl, $CaCl_2$ and PEG might work to induce competence in *X. campestris*. First, our previous observations indicated that *X. campestris* pv. *oryzae* released the major portion of their periplasmic alkaline phosphatase into the medium when the cells were treated with Tris-HCl at pH values higher than 8.0 (Chu and Tseng, 1984). Second, $CaCl_2$ and Tris-HCl, separately or in combination with PEG, have been used to achieve competence for some bacteria during transformation procedures (Fornari and Kaplan, 1982; Takahashi *et al.*, 1983; Sladek and Maniloff, 1983). In this study, we found that treatments with Tris-HCl at alkaline pH, containing $CaCl_2$ and PEG 6,000, did render the cells of *X. campestris* competent to uptake the phage DNA. Under the working conditions described in the Materials and Methods, a transfection frequency of 1.2 x 10^3 PFU/μg DNA was obtaind. Several points were noticed from the experiments. First, the PEG concentration was critical. At concentrations lower than 13% it was not effective, but concentrations higher than 20 % caused severe killing effect. Second, while the transfection frequency increased following the increase of DNA amount, the system was saturated when the DNA was increased up to 2 μg (Fig. 4). Third, a 2 min heat shock at 35°C was essential to accomplish the DNA uptake.

Discussion

In this study, we have isolated and characterized

Table 1. *Determination of the molecular weight of ϕL7 DNA (units in Kb) from DNA fragments generated by cleavage with restriction enzymes*[a,b]

Enzyme	molecular weight of the fragments generated by restriction enzymes	Total molecular weight of the fragments
*Eco*RI	22, 13	35.0
*Eco*RI+*Bam*HI	15, 13, 5.2, 3.0, 1.2	37.4
*Eco*RI+*Eco*RV	9.0, 6.6, 6.0, 5.5, 4.5, 2.0, 1.8, 1.4	36.8
*Nco*I	9.4, 5.3, 4.3, 4.1, 3.7, 3.5, 3.0, 2.5, 1.2, 1.1, 0.9	39.0
*Hind*III	6.2, 5.6, 5.4, 4.8, 4.2, 3.5, 2.8, 2.7, 1.4, 1.25, 1.05	38.9
*Pvu*II	9.4, 5.5, 3.7, 3.4, 3.2, 2.25, 2.15, 1.9, 1.0, 0.95, 0.85, 0.8, 0.7	35.8

[a] The data were calculated from Fig. 3 and from the agarose gel electrophoresis not shown in this paper.
[b] The molecular weight of ϕL7 DNA fragments were determined from their moving distance relative to those of the standard DNA fragments (λ DNA digested with *Hind*III).

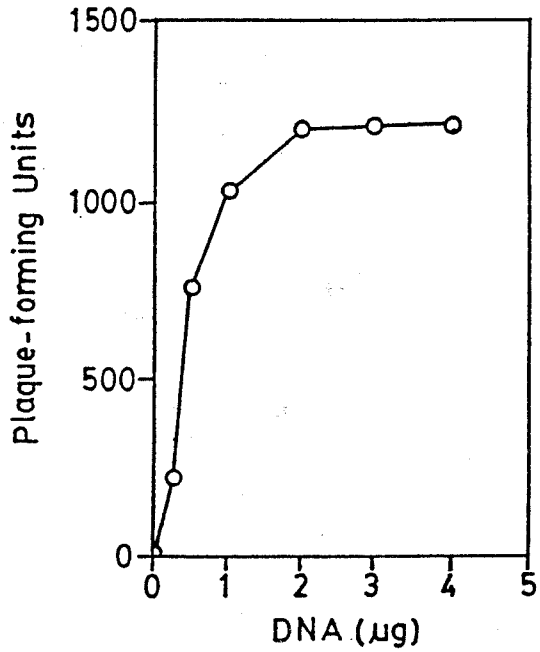

Fig. 4. Effect of ϕL7 DNA concentration on the frequency of transfection. The standard conditions described in the Materials and Methods were followed, except that the amount of DNA was varied in different tubes.

the virulent phage ϕL7. This phage attacks specifically *X. campestris* (32 out of 35 strains tested) without affecting *X. campestris* pv. *citri*, *X. campestris* pv. *oryzae*, *X. campestris* pv. *phaseoli* or *X. campestris* pv. *vesicatoria*. It possesses a hexagonal head and a non-contractile flexuous tail as its surface structure, and a double-stranded DNA of about 37 kb as its genome.

Specific virulent phages for *X. campestris* have been reported by Liew and Alvarez (1981a) and Watanabe *et al.* (1980). Since no information was presented concerning the size or the structure of the genomes and antisera against these phages are not available, comparisons between ϕL7 and these phages are therefore difficult. The morphology revealed by electron microscopy is the only means feasible for comparisons. In terms of this parameter, ϕL7 is similar to phage A342 of Liew and Alvarez (1981a) but not the remaining 6 phages reported simultaneously or the 3 phages described by Watanabe *et al.* (1980). In addition, Liew and Alvarez (1981b) have carried out phage typing to differentiate *X. campestris* strains of different geographical distributions. The observations that ϕL7 did not attack the pathovars other than *campestris*, and 3 of the 35 *X. campestris* strains were not susceptible to ϕL7 indicate that ϕL7 is also useful for phage typing.

Previously, attempts have been made to transform *X. campestris* with plasmid DNA. Murooka *et al.* (1987) reported that *X. campestris* was transformed, at a frequency of 8×10^2 transformants/μg DNA, by plasmid pBR328 (Soberon *et al.*, 1980). However, our efforts to use pBR322 and pBR325 (Bolivar *et al.*, 1977; Bolivar, 1978), the isologous plasmids of pBR328, was not successful (unpublished results). It was also shown that conjugally transferred pSUP202 (Simon *et al.*, 1983). pBR325 carrying *mob* gene, was not maintained in *X. campestris* (unpublished results). Atkins *et al.* (1987) transformed *X. campestris* with pKT230 and other broad-host range plasmids using procedures similar to those routinely used for *E. coli*, and frequency of 8×10^2 transformants/μg DNA was obtained. It was noticed that DNA prepared from *X. campestris*, which was descended from the DNA previously mobilized by conjugation, gave higher frequencies of transformation (Atkins *et al.*, 1987). In this study, *X. campestris* was transfected by ϕL7 DNA at a frequency of 1.2×10^3 PFU/μg DNA. Considering the fact that ϕL7 DNA (37 kb) is 3.1 times the size of pKT230 (11.9 kb), our protocol is probably better than that used by Atkins *et al.* (1987). Presumably the ϕL7 DNA is indigenous to *X. campestris* and therefore is immuned to the restriction system, or the treatments with a combination of Tris -HCl, $CaCl_2$ and PEG are more effective than the $CaCl_2$ treatment routinely used for *E. coli* (Saunders *et al.*, 1984). Although a frequency of 1.2×10^3 transformants/μg DNA is not high enough for the need of genomic bank construction, it would be useful for delivery of cloned genes into *X. campestris*. Efforts are being made in our laboratory to further optimize the conditions towards a higher frequency of transformation.

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十字花科蔬菜黑腐病菌之噬菌體ϕL7及其對宿主之轉染

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ϕL7爲自本地分離出來，能專一地感染十字花科蔬菜黑腐病菌 *Xanthomonas campestris* pv. *campestris* 之噬菌體。其頭爲直徑約 60 nm 之六角體，有約 10×180 nm 長，不收縮而微彎的尾巴。此噬菌體在 4 °C 到 70 °C 之間均呈穩定，在低於 4.0 的 pH 之下則不穩定。經由鑑識內核酸酶切割等分析，計算出其基因體爲約 37 kb 之雙股 DNA。由於 *Xanthomonas compestris* 之轉形方法迄未建立，本試驗乃以ϕL7 DNA 爲材料進行轉染(transfection)試驗，供建立轉形系統。結果發現，以 Tris-HCl、$CaCl_2$ 及 PEG 6000 處理宿主細胞，可使ϕL7 DNA 的轉染達 1.2×10^3 PFU/μg 之頻率。