Characterization of filamentous bacteriophage \( \phi \)Lf from \textit{Xanthomonas campestris} pv. \textit{campestris}

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A filamentous phage, \( \phi \)Lf, which specifically infects \textit{Xanthomonas campestris} pv. \textit{campestris} was isolated. The phage particle measured 1000 (± 200) \( \times \) 8 nm. It formed turbid plaques of about 1 mm in diameter. During multiplication, the progeny virions extruded into the medium without retarding host cell growth. Stocks were stable for 6 months at 4 °C and survived treatment at 80 °C for 10 min. Treatment with chloroform, ethanol or acetone completely destroyed infectivity; ethyl ether and methanol inactivated 98 to 99% of the phage. SDS-polyacrylamide gel electrophoresis showed a major coat protein band of approximate \( M_r \) 4000 whereas an immunoprecipitation test detected the existence of two coat protein species. The phage genome was shown to be a single-stranded DNA molecule. A physical map was constructed and the DNA size was calculated to be 5.9 kb. Cells treated with Tris-HCl containing CaCl\(_2\) and polyethylene glycol 6000 were transfected by replicative form DNA at a frequency of \( 3.4 \times 10^3 \) p.f.u./\( \mu \)g.

\textit{Xanthomonas campestris} pv. \textit{campestris} is important agriculturally as the causal agent of black rot in crucifers (Williams, 1980) and industrially as the xanthan gum producer (Slodki & Cadmus, 1978). Owing to the absence of suitable genetic analysis systems, the molecular biology of this species remains largely unknown. The only method presently available in this regard is conjugal transfer which has been used to introduce cloned genes from \textit{Escherichia coli} into \textit{X. campestris}. However, the plasmids being used, e.g. pRK 290 (Ditta et al., 1980) or its derivatives, are over 20 kb in size. When subclones are needed, cloning efficiency is inevitably low owing to an increasing degree of difficulty in ligation and transformation. A more suitable vector and transformation system for \textit{X. campestris} are needed.

Filamentous phages such as M13 and fd have been used successfully as vectors for gene cloning and DNA sequencing in \textit{E. coli} (Messing, 1983). Several filamentous phages of \textit{Xanthomonas} have been reported, but none was shown to infect the pathovar \textit{campestris} (Dai et al., 1980; Kuo et al., 1969). Therefore, it was necessary to start work on the isolation and characterization of a filamentous phage for \textit{X. campestris}. This report describes our first efforts along these lines. Preliminary results of this work have been presented (Tseng & Lo, 1986).

Thirty-two local strains of \textit{X. campestris} (designations not listed) were freshly isolated and tested for the occurrence of phage by spot tests, in which samples (10 \( \mu \)l) from the culture supernatant were dropped onto a preformed double layer (Eisensfark, 1967) of TYG containing indicator host cells. TYG medium contained 10 g/l tryptone, 6 g/l yeast extract, 1 mM-MgSO\(_4\) and 5 g/l glucose. Culture supernatant from strain XL5 formed a lytic zone which was then found to contain phages. After three successive single plaque isolations, a well separated plaque was obtained. Electron microscopy (staining with 1% uranyl acetate) showed that the phage particles were filamentous in shape and 1000 (± 200) \( \times \) 8 nm in size (Fig. 1). This new phage was designated \( \phi \)Lf.

In plaque assays (Eisensfark, 1967), using a TYG double layer, \( \phi \)Lf formed turbid plaques of about 1 mm in diameter on a lawn of 3DA, a non-mucoid mutant strain (Yang et al., 1987), after 12 h at 28 °C. In spot tests, \( \phi \)Lf was found to infect \textit{X. campestris} specifically, but not \textit{E. coli} JM101 and HB101, \textit{Agrobacterium tumefaciens}, \textit{Pseudomonas syringae} or 10 strains of \textit{Xanthomonas} representing four different pathovars, i.e. \textit{X. citri}, \textit{X. oryzae}, \textit{X. phaseoli} and \textit{X. vesicatoria}. In a culture of XL5, the \( \phi \)Lf-carrying strain, phage particles were released continuously accompanying cell growth. About 10\(^4\) p.f.u./ml was produced during the mid-logarithmic phase, then the titre declined to 10\(^2\) p.f.u./ml after the
culture entered the stationary phase (Fig. 2). However, when a strain not carrying the phage, such as 3DA, was grown in TYG broth and infected with φLψ at an m.o.i. of 20 and then incubated with vigorous shaking (280 r.p.m.) at 28 °C, the titre finally reached $2 \times 10^{11}$ p.f.u./ml in 8 h and cell growth was unaffected. Therefore, in this study, 3DA was used as the host for propagation of φLψ under these conditions. These cultures were good sources of cells for replicative form (RF) DNA extraction by following the protocols of Birnboim & Doly (1979) for plasmid preparation, and the culture supernatants were suitable for preparation of φLψ genomic DNA by the method of Messing (1983) for preparation of M13 ssDNA. To purify phage φLψ, the supernatants were filtered through a Millipore membrane filter (0-45 μm), precipitated in the presence of 0-5 M-NaCl and polyethylene glycol (PEG) 6000 and then purified by CsCl discontinuous density gradient centrifugation (Miller, 1972). The φLψ phage particles were found to band in the area corresponding to a density of 1.335 g/ml.

The crude suspension of φLψ, after removal of the cells, was stable for 6 months at 4 °C. At 80 °C, the crude suspension kept 100% infectivity for more than 10 min; however, infectivity was absent after 10 min at 95 °C. Treatment with 10% chloroform, ethanol or acetone at 28 °C for 30 min completely destroyed its infectivity whereas methanol and ethyl ether inactivated 98 to 99% of the phage. Tris–HCl (10 mM, pH 8.0) and CaCl$_2$ were found to inactivate 13 and 77%, respectively, of the phage infectivity.

Coat structures of filamentous phages are composed of more than 2500 copies of a major protein of 4500 to 5200 $M_r$ and a few copies of each of one or more minor proteins (Day et al., 1988). SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) of the φLψ coat proteins showed a single band of approximate $M_r$ 4000 (Fig. 3), which is similar to the $M_r$ of monomers for other filamentous phages. In contrast to this, the purified fd major coat protein was shown to be highly aggregated in aqueous solution and dissociated into a stable dimeric form in SDS–polyacrylamide gels or Sephadex columns containing SDS (Cavalieri et al., 1976). Ouchterlony double diffusion tests (Ouchterlony & Nilsson, 1978) of...
phage particles against antiserum formed two prominent precipitin lines (Fig. 4), showing that φLF contains at least a small amount of a second protein as a minor coat component which, though not detected in SDS–PAGE, is strongly antigenic.

The genomic DNA of φLF was sensitive to S1 nuclease, a single strand-specific nuclease, but resistant to digestion by RNase and all the restriction enzymes tested. On the other hand, the RF DNA was cut by the restriction enzymes and gave rise to distinct patterns on agarose gel (0.7%) electrophoresis (Maniatis et al., 1982), but was resistant to RNase and S1 nuclease. These results indicate that the φLF genome is an ssDNA, like those of all other filamentous phages previously reported. Restriction endonucleases EcoRV, PstI and XhoI each cut the RF DNA into a linear form of approximately 5-9 kb. EcoRI, HincII, HindIII and MluI each cut the RF DNA into two fragments. By using these restriction enzymes, a physical map (Fig. 5) of the RF DNA was constructed. The size of the φLF genome, 5-9 kb, is smaller than the 7-4 kb of that of Xf (Day et al., 1988) and the 7-6 kb of that of Cf (Yang & Kuo, 1984). The restriction map of Cf (Yang & Kuo, 1984) and restriction patterns of Xf (Pai, 1989) are also different from those of φLF (Fig. 5).

In summary, on the basis of the results presented above, φLF is a new phage of X. campestris, with ssDNA as have other filamentous phages, such as Ff, Pf, Xf and Cf (Day et al., 1988).

The purpose of this study was to find a filamentous phage possessing a dsDNA RF small enough to be used as a cloning vector and suitable for development of a transformation system. Therefore, transfection by φLF DNA was tested. To prepare competent cells, an overnight culture of 3DA was diluted 80-fold into 40 ml of TYG and grown until OD_{550} was 0.5; the cells were then harvested by centrifugation and suspended in 10 ml of cold TC buffer (0.25 M-Tris–HCl pH 7.2, containing 0.1 M-CaCl_2), then incubated on ice for 10 min. The suspension was centrifuged again and the pellet was resuspended in 1 ml of cold TC buffer and kept on ice until use. The transfection mixture (400 µl) contained 100 µl of competent cells, 0.5 µg RF DNA or 1.0 µg ssDNA, 150 µl of 40% PEG 6000 in 0.1 M-Tris–HCl pH 7.2 and sterile distilled water. The mixture was incubated on ice for 30 min followed by heat shock at 30°C for 2 min. After heat shock, 3.6 ml of TYG broth was added immediately and the mixture was incubated at 28°C with shaking for 30 min. Transfected cells were counted as infective centres by double-layer plaque assay. RF DNA transfection resulted in 3-4 × 10^3 p.f.u./µg DNA, whereas transfection by viral ssDNA gave only 1 × 10^2 p.f.u./µg DNA. The concentration of PEG 6000 was critical; it was less effective at concentrations lower than 13%, but at concentrations higher than 16% it started to exhibit an inhibitory effect. Although the frequency or transfection by φLF is higher than the transformation frequency (8 × 10^2 transformants/µg pKT230 DNA) obtained by Atkins et al. (1987) and is superior to conjugal transfer by not requiring counter selection, it is only useful for introducing cloned genes into X. campestris. For direct gene cloning, optimization of the procedures to obtain a higher frequency is needed. Localization of the dispensable area in the intergenic region (Schaller, 1987) of the φLF genome, where a DNA fragment can be inserted, is being carried out. Once the work is finished, a vector and transformation system will be available for X. campestris. In the meantime, we have shown that φLF exhibits an integration mechanism that facilitates the insertion of a cloned DNA into the chromosome of X. campestris (Tseng et al., 1989). Therefore, φLF should be useful as an integration vector.
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References


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