Characterization of two novel filamentous phages of Xanthomonas

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Two filamentous phages of Xanthomonas campestris pv. vesicatoria and Xanthomonas oryzae pv. oryzae were isolated and designated φXv and φXo, respectively. They were similar to other filamentous phages of Xanthomonas in (i) shape, (ii) restrictive host specificity, (iii) high stability, (iv) an ssDNA genome, (v) a dsDNA as the replicative form (RF), (vi) propagation without lysis of host cells and (vii) ability to integrate into the host chromosome. These phages showed sequence homology to filamentous phage φL of X. c. pv. campestris. φXv was inactivated by antisera against φXv, φXo and φL, whereas φXo and φL were inactivated only by their respective antisera and the anti-φXv serum. Both the single-stranded phage DNAs and the RF DNAs of φXv, φXo and φL were able to transfect X. c. pv. vesicatoria, X. o. pv. oryzae and X. c. pv. campestris. Physical maps of φXv and φXo were constructed for the RF DNAs. Genome sizes were estimated, based on mapping data, to be 6.8 kb for φXv and 7.6 kb for φXo, larger than that of the φL genome (6.0 kb). The difference in genome sizes appeared to result from insertions of large DNA fragments. These fragments and the regions mediating integration were localized in the physical maps.

Xanthomonas campestris includes more than 123 pathovars (pv.) and is the major species of the genus Xanthomonas, a genus including several Gram-negative bacteria pathogenic to plants (Vauterin et al., 1990). In addition to being the organism of choice for industrial production of xanthan gum (Slodki & Cadmus, 1978; Sandford & Baird, 1983), X. c. pv. campestris is also the causal agent of black rot in crucifers (William, 1980). Other Xanthomonas strains are also pathogenic. For example, X. c. pv. vesicatoria causes foliage and fruit spot disease in peppers and tomatoes (Minsavage et al., 1990), and X. oryzae pv. oryzae causes bacterial leaf blight in rice plants (Ou, 1972; Swings et al., 1990). These bacteria form yellow-pigmented, mucoid colonies, making morphological identification difficult. Members of the genus Xanthomonas share common biochemical characteristics (Bradbury, 1984), possibly indicating a similar genetic background among these plant pathogens.

Previously we isolated and characterized a filamentous bacteriophage, φL, from X. c. pv. campestris that has a genome of 6008 bases with 10 possible open reading frames (ORFs) (Tseng et al., 1990; Wen, 1992). The φL genome is approximately 1300 bases smaller than that of the filamentous phage Cfc of X. c. pv. citri (7308 bases; Kuo et al., 1991); we were interested in the genomic organization of filamentous Xanthomonas phages of different sizes. However a comparison was not possible since ORFs were not identified in the Cfc genome.

Here we report the characterization of two filamentous phages obtained from X. c. pv. vesicatoria (Xcv) and X. oryzae pv. oryzae (Xo), respectively.

Fifty-eight strains of X. c. pv. vesicatoria and 31 strains of X. o. pv. oryzae, from local fields, were used for isolation of filamentous phages. Bacteria were grown at 28 °C in Luria broth (Miller, 1972) with vigorous shaking or on plates of Luria broth containing 1.5% agar. Xcv36 and Xo21 were used for phage propagation and as indicator hosts for plaque assays carried out as described by Eisenstark (1967).

Spot tests were carried out as described previously (Tseng et al., 1990) using culture supernatants as phage sources and each of the strains as an indicator host. Two filamentous phages, named φXv and φXo, were obtained by single-plaque isolation from Xcv64 and Xo6, respectively. They were filamentous in shape, and measured approximately 1120 ± 200 x 8 nm for φXv and 1290 ± 200 x 8 nm for φXo (Fig. 1). Like other filamentous

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Fig. 1. Electron micrograph of phages \( \phi X_v \) (a) and \( \phi X_o \) (b) stained with 2% phosphotungstic acid. Bar represents 250 nm.

![Electron micrograph](image)

**Fig. 2.** Treatments of phage \( \phi X_v \) DNA with hydrolytic enzymes followed by agarose gel (0.8%) electrophoresis in Tris-borate buffer (Maniatis et al., 1982). The gel (5 x 6 cm), run at 100 V for 25 min, was stained in ethidium bromide solution (0.5 µg/ml) containing bovine pancreas RNase A (1.5 µg/ml; Sigma) for 15 min. Lane M, \( H_{ind}III \) DNA cut with HindIII as M, markers; lanes 1 and 2, RF DNA (approx. 200 ng) uncut and cut with \( Pvu_{II} \) (1 unit; New England Biolabs), respectively; lane 3, ssDNA (approx. 220 ng) treated with 1 unit of \( Pvu_{II} \) for 30 min; lanes 4, 5 and 6, ssDNA (approx. 220 ng) digested with 0.8 units of S1 nuclease (Promega) for 0.5, 1.0 and 3.0 min, respectively.

Phages, \( \phi X_v \) and \( \phi X_o \) formed turbid plaques of about 1 mm in diameter and multiplied without cell lysis or severe retardation of cell growth. Phage particles were released by carrier strains into the medium at a rate of approximately \( 10^3 \) to \( 10^5 \) p.f.u./ml. High phage titres were produced by mid-log phase cultures of Xcv36 and Xo21 after phage infection at an m.o.i. of 20, followed by additional growth for 8 h. The highest titres obtained were \( 4.3 \times 10^{11} \) p.f.u./ml for \( \phi X_v \) and \( 6.4 \times 10^{12} \) p.f.u./ml for \( \phi X_o \). Phage particles were so high that, without further concentration, phage DNAs could be visualized in agarose gels by vortexing 10 µl of cell-free culture supernatants with 2 µl of a gel-loading mix (1% SDS, 0.25% bromophenol blue and 30% glycerol) followed by gel (0.7%) electrophoresis (Maniatis et al., 1982) (data not shown). The phage DNAs, extracted by two cycles of phenol–chloroform treatment, were resistant to digestion with RNase A (Sigma) and restriction endonucleases \( B_{am}III \), \( E_{co}RI \) and \( Pvu_{II} \) (New England Biolabs) but sensitive to S1 nuclease (Promega) digestion, indicating that \( \phi X_v \) and \( \phi X_o \) have ssDNA genomes. One set of results for \( \phi X_v \) using ssDNA and replicative form (RF) DNA, extracted by the alkaline lysis method of Birnboim & Doly (1979) is shown in Fig. 2. Phages \( \phi X_v \) and \( \phi X_o \) were stored as crude suspensions at \( 4 \) °C, after removal of the cells by centrifugation (15000 g for 10 min) and Millipore filtration (0.45 µm). Stability tests using cell-free crude phage suspensions showed that storage at \( 4 \) °C for 6 months or incubation at \( 80 \) °C for 10 min did not affect their infectivity.

Host range tests showed that \( \phi X_v \) and \( \phi X_o \) infected only \( X. \) c. pv. vesicatoria and \( X. \) o. pv. oryzae, respectively, and not other \( X. \) campestris pathovars including \( X. \) campestris, \( c. \) citri, \( p. \) phaseoli and \( m. \) manihotis. \( Pseudomonas \) aeruginosa, \( Agrobacterium \) tumefaciens and \( E. \) coli were not infected by phages \( \phi X_v \) or \( \phi X_o \). To determine whether the phages could multiply in heterologous hosts, RF DNAs of \( \phi X_v \), \( \phi X_o \) and \( \phi L_f \) were each electroporated (Wang & Tseng, 1992) into strains Xcv36, XcP20H (indicator host for \( \phi L_f \)) and Xo21 and the infective centres were detected by plaque assay on the respective indicator hosts. In three experiments with duplicate samples, Xcv36, Xo21 and XcP20H were found to be transfected at frequencies of \( 1.7 \times 10^5 \) to \( 6.4 \times 10^5 \) p.f.u. per µg RF DNA of \( \phi X_v \), \( \phi X_o \) and \( \phi L_f \) (Table 1). Phage particles released by heterologous hosts were plaque-assayed after growing the transfected cells for 2 h, assuming that multiplication would not be initiated during the period of incubation. The numbers

Table 1. Electroporation of RF DNA of phages \( \phi X_v \), \( \phi X_o \) and \( \phi L_f \) into Xanthomonas and the phage titres released

<table>
<thead>
<tr>
<th>Phage</th>
<th>Xcv36</th>
<th>Xo21</th>
<th>XcP20H</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \phi X_v )</td>
<td>( 3.5 \times 10^9 )</td>
<td>( 92 )</td>
<td>( 1.7 \times 10^5 )</td>
</tr>
<tr>
<td>( \phi X_o )</td>
<td>( 2.3 \times 10^6 )</td>
<td>( 18 )</td>
<td>( 6.4 \times 10^7 )</td>
</tr>
<tr>
<td>( \phi L_f )</td>
<td>( 3.4 \times 10^5 )</td>
<td>( 11 )</td>
<td>( 3.3 \times 10^5 )</td>
</tr>
</tbody>
</table>

*Transfectants were counted as the infective centres assayed immediately after electroporation and expressed as transfectants per µg DNA (c/0); the phage numbers released were calculated by dividing the titres counted at 2 h by the number of transfectants (c/0).
Table 2. Inactivation of phages $\phi Xv$, $\phi Xo$ and $\phi Lf$ by the antisera*

<table>
<thead>
<tr>
<th>Serum</th>
<th>$\phi Xv$ (%)</th>
<th>$\phi Xo$ (%)</th>
<th>$\phi Lf$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-$\phi Xv$</td>
<td>100</td>
<td>26.7</td>
<td>94.7</td>
</tr>
<tr>
<td>Anti-$\phi Xo$</td>
<td>96.1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Anti-$\phi Lf$</td>
<td>50.2</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

* Data are expressed as percentage of phage inactivated over the controls, which were prepared by adding sera taken before the first injections.

obtained were 7 to 18 p.f.u. per transfected heterologous cell (Table 1). It was interesting to note that, in the transfection by homologous phages, $Xo$21 released more phage particles (128) per transfected cell than did $Xv$36 (92) and $XcP20H$ (95) (Table 1). This is in agreement with the fact that $Xo$ had higher yield ($6.4 \times 10^{12}$ p.f.u.) than that of $Xv$ ($4.3 \times 10^{11}$ p.f.u.), as described above. Since phages $Xv$, $Xo$ and $Lf$ failed to infect heterologous hosts, successes in transfection suggest that the receptor site appears to be an important determinant of host specificity for filamentous phage infection. The phage ssDNAs were also capable of transfection; however, the efficiencies were about 100-fold lower than those observed using RF DNAs (data not shown).

Rabbit antisera specific for $Xv$, $Xo$ and $Lf$, respectively, were prepared by three consecutive intradermal injections of a purified phage (approx. 0.2 mg/ml) emulsified in Freund's adjuvant (Sigma), at intervals of 1 week. The increase of antiserum titre over time was determined using an ELISA (Ma et al., 1984). The titres of the antisera, collected by sacrificing the rabbits at 6 weeks after the first injections, had A405 values of 0.36, 1.29 and 0.41 for $Xv$, $Xo$ and $Lf$, respectively, after 500-fold dilutions. Cross-reactivity was tested by mixing 150 µl of a phage suspension, containing 1000 ± 200 p.f.u. in sterile distilled water, with an equal volume of the antiserum, followed by assaying the survival titres after incubation at 4 °C for 1 h. Under these conditions, the three phages were 100% inactivated by their respective antisera. The results of DNA hybridization (see below), using $Lf$ as a probe, were used as references to help align the fragment order. The physical maps constructed are shown in Fig. 3. The genome sizes of $Xv$ and $Xo$ were calculated to be 6.8 and 7.6 kb, respectively, based on the mapping data. Thus, the $Xo$ and $Xv$ genomes are 1.6 and 0.8 kb larger than that of $Lf$ (6.0 kb), respectively. These differences correlate with the variations in the particle length of $Xo$ (1290 nm), $Xv$ (1120 nm) and $Lf$ (1000 nm; Tseng et al., 1990).

Southern blot hybridization (Maniatis et al., 1982) was carried out to compare sequence homology between these phages. $Lf$ RF DNA was labelled with [$\alpha$-32P]dCTP (Amersham) by using a random priming kit (Promega). Hybridization with the RF DNAs of $Xv$ and $Xo$, digested with restriction enzymes, demonstrated homology to $Lf$ in most regions of these phages (data not shown). Regions that did not hybridize with $Lf$ were found and localized in the restriction maps. As shown in Fig. 3, these regions are a fragment from map unit (m.u.) 2.3 to 2.85 in $Xv$, and in $Xo$ a $MluI$ fragment (m.u. 2.1 to 2.85) and a $KpnI$ fragment (m.u. 4.3 to 4.5). In view of these data, the differences between the sizes of the

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**Fig. 3.** The physical maps of $Xv$ (a) and $Xo$ (b). Points on the maps are measured in m.u. (shown in parentheses). The thickened portions represent the fragments that did not hybridize with $Lf$. The inner lines marked 'int' represent fragments that mediate integration. Abbreviations for restriction endonucleases: Hc, HindIII; Hd, HindII; K, KpnI; M, MluI; P, PvuII; R1, EcoRI; RV, EcoRV; Sm, Smal.
φXv, φXo and φLf genomes seem to result primarily from insertion of large DNA fragments. Other filamentous phages of *Xanthomonas* have previously been described, e.g. Xf and Xf2 of *X. o. pv. oryzae* (Kuo et al., 1969; Kamiunten & Wakimoto, 1979), Cf, Cflt and Cflc of *X. c. pv. citri* (Dai et al., 1980, 1988; Kuo et al., 1991) and φL of *X. c. pv. campestris* (Tseng et al., 1990). Several have been shown to integrate into the host chromosomes (Dai et al., 1987; Chang, 1989; Pai, 1989; Fu et al., 1992). In this study, fragments of φXv and φXo RF DNA were cloned into pOK12 (Vieira & Messing, 1991), a pACYC177 derivative which cannot be maintained in *Xanthomonas*, and electroporated into the respective indicator hosts, selecting for kanamycin resistance conferred by the vector. The 1.8 kb *Pvu*II fragment of φXv (m.u. 6’4 to 1’4) and the 1.9 kb *EcoRI*-SmaI fragment of φXo (m.u. 1’7 to 3’6) were shown to mediate integration into the host chromosomes (Fig. 3).

Phages φXo, Xf and Xf2 (Kuo et al., 1969; Kamiunten & Wakimoto, 1979) all specifically infect *X. o. pv. oryzae*. However, only limited comparisons can be made, because little is known about the similarities between these phages. The only data available for Xf at molecular level is the amino acid sequence of its major coat protein (Frangione et al., 1978), which will be a good reference for comparison. Until now, filamentous phages specific to *X. c. pv. vesicatoria* have not been documented; φXv appears to be the first reported. These phages are useful for studying genomic organization, phage-host relationships, phage assembly and DNA replication. Sequence determination of the φXv and φXo genomes is currently under way in our laboratory, in the hope of understanding the genomic organization of these important phages.

In conclusion, based on the characteristics described in this study, phages φXv and φXo are two filamentous phages that can be classified as members of the genus *Inovirus*, including coliphage fd as the type species, belonging to the family *Inoviridae* (Francki et al., 1991).

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