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 φLf of *X. c. pv.* *campestris*. φXv was inactivated by antisera against φXv, φXo and φLf, whereas φXo and φLf 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 φLf 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 φLf 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, φLf, 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 φLf 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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phages, φXv and φXo 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 $10^6$ 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 φXv and $6.4 \times 10^{12}$ p.f.u./ml for φXo. 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 BamHI, EcoRI and PvuII (New England Biolabs) but sensitive to S1 nuclease (Promega) digestion, indicating that φXv and φXo have ssDNA genomes. One set of results for φXv using ssDNA and replicative form (RF) DNA, extracted by the alkaline lysis method of Birnboim & Doly (1979) is shown in Fig. 2. Phages φXv and φXo were stored as crude suspensions at 4 °C, after removal of the cells by centrifugation (15000g 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 φXv and φXo infected only X. c. pv. vesicatoria and X. o. pv. oryzae, respectively, and not other X. campestris pathovars including campestris, citri, phaseoli and manihotis. Pseudomonas aeruginosa, Agrobacterium tumefaciens and Escherichia coli were not infected by phages φXv or φXo. To determine whether the phages could multiply in heterologous hosts, RF DNAs of φXv, φXo and φLf were each electroporated (Wang & Tseng, 1992) into strains Xcv36, XcP20H (indicator host for φLf) 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 $6.4 \times 10^7$ p.f.u. per μg RF DNA of φXv, φXo and φLf (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 φXv, φXo and φLf into Xanthomonas and the phage titres released

<table>
<thead>
<tr>
<th>Host</th>
<th>Xcv36</th>
<th>Xo21</th>
<th>XcP20H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phage</td>
<td>$c_o$</td>
<td>$c_i/c_o$</td>
<td>$c_o$</td>
</tr>
<tr>
<td>φXv</td>
<td>$3.5 \times 10^9$</td>
<td>92</td>
<td>$1.7 \times 10^6$</td>
</tr>
<tr>
<td>φXo</td>
<td>$2.3 \times 10^6$</td>
<td>18</td>
<td>$6.4 \times 10^7$</td>
</tr>
<tr>
<td>φLf</td>
<td>$3.4 \times 10^5$</td>
<td>11</td>
<td>$3.3 \times 10^6$</td>
</tr>
</tbody>
</table>

* Transfectants were counted as the infective centres assayed immediately after electroporation and expressed as transfectants per μg DNA ($c_o$); the phage numbers released were calculated by dividing the titres counted at 2 h by the number of transfectants ($c_i/c_o$).
**Table 2. Inactivation of phages φXv, φXo and φLf by the antisera***

<table>
<thead>
<tr>
<th>Serum</th>
<th>φXv (%)</th>
<th>φXo (%)</th>
<th>φLf (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-φXv</td>
<td>100</td>
<td>26.7</td>
<td>94.7</td>
</tr>
<tr>
<td>Anti-φXo</td>
<td>96.1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Anti-φLf</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, Xo21 released more phage particles (128) per transfected cell than did Xcv36 (92) and XcP20H (95) (Table 1). This is in agreement with the fact that φXo had higher yield (6.4 x 10^{12} p.f.u.) than that of φXv (4.3 x 10^{11} p.f.u.), as described above. Since phages φXv, φXo and φLf 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 φXv, φXo and φLf, 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 φXv, φXo and φLf, 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 antiserum. The results of cross-reactivity test showed that anti-φXv serum was able to inactivate φXo and φLf; φXv was inactivated by both anti-φXo and anti-φLf sera, and no cross-reactivity was observed between anti-φXo and φLf or between anti-φLf and φXo (Table 2). These results suggest that φXv may be phylogenetically more related to both φXo and φLf than φXo is to φLf.

To construct physical maps, RF DNAs of φXv and φXo were cut partially or completely using one or more enzymes. When it was applicable, digested DNA fragments were recovered after agarose gel electrophoresis and then subjected to a second cut. The results of DNA hybridization (see below), using φLf as a probe, were used as references to help align the fragment order. The physical maps constucted are shown in Fig. 3. The genome sizes of φXv and φXo were calculated to be 6/8 and 7.6 kb, respectively, based on the mapping data. Thus, the φXo and φXv genomes are 1.6 and 0.8 kb larger than that of φLf (6.0 kb), respectively. These differences correlate with the variations in the particle length of φXo (1290 nm), φXv (1120 nm) and φLf (1000 nm; Tseng et al., 1990).

Southern blot hybridization (Maniatis et al., 1982) was carried out to compare sequence homology between these phages. φLf RF DNA was labelled with α-32PdCTP (Amersham) by using a random priming kit (Promega). Hybridization with the RF DNAs of φXv and φXo, digested with restriction enzymes, demonstrated homology to φLf in most regions of these phages (data not shown). Regions that did not hybridize with φLf 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 φXv, and in φXo a MluI fragment (m.u. 0.4 to 1.9), a HincII fragment (m.u. 2.1 to 2.85) and a KpnI–MluI 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 φXv (a) and φXo (b).** Points on the maps are measured in m.u. (shown in parentheses). The thickened portions represent the fragments that did not hybridize with φLf. The inner lines marked 'int' represent fragments that mediate integration. Abbreviations for restriction endonucleases: Hc, HincII; Hd, HaelII; K, KpnI; M, MluI; P, PvuII; Rl, EcoRI; RV, EcoRV; Sm, Smal.
\( \phi Xv, \phi Xo \) and \( \phi Lf \) 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), \( Cf \), \( Cf1t \) and \( Cf1c \) of *X. c. pv. citri* (Dai et al., 1980, 1988; Kuo et al., 1991) and \( \phi Lf \) 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 \( \phi Xv \) and \( \phi Xo \) RF DNA were cloned into pOK12 (Vieira & Messing, 1991), a pACYC177 derivative which cannot be maintained in *X. c. pv. oryzae*. Cloning of *\phi Xv* and *\phi Xo* RF DNA was cloned into pOK12 (Vieira & Messing, 1991), a pACYC177 derivative which cannot be maintained in *X. o. pv. oryzae*.

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