The A Protein of the Filamentous Bacteriophage Cf of Xanthomonas campestris pv. citri

MEI-KWEI YANG1* AND YEN-CHUN YANG2

Department of Biology, Fu Jen University,1 and Institute of Life Science, National Defense Medical Center,2 Taipei, Taiwan, Republic of China

Received 2 December 1996/Accepted 28 February 1997

Filamentous bacteriophages have very strict host specificities. Experiments were performed to investigate whether the A protein of the filamentous phage Cf, which infects Xanthomonas campestris pv. citri but not X. campestris pv. oryzae, is involved in determining Cf’s host specificity. The gene encoding the A protein of Cf was cloned and expressed in X. campestris pv. citri. The genomic DNA of another filamentous bacteriophage, Xf, which infects X. campestris pv. oryzae but not X. campestris pv. citri, was then introduced by electroporation into X. campestris pv. citri that had expressed the A protein of Cf. The progeny phages thus produced were able to infect both X. campestris pv. oryzae and X. campestris pv. citri, indicating that the A protein of Cf was incorporated into the viral particles of Xf and conferred upon Xf the ability to infect the host of Cf. Inactivation of the A protein gene abolished the infectivity of Cf. The results of this study indicate that the A protein of Cf is responsible for controlling the host specificity of Cf.

Cf and Xf are filamentous bacteriophages isolated from Xanthomonas campestris pv. citri and X. campestris pv. oryzae, respectively (5, 12, 13). X. campestris is a gram-negative rod. X. campestris pv. citri is a pathogenic bacterium causing citrus canker disease in oranges, whereas X. campestris pv. oryzae causes leaf blight in rice. These filamentous bacteriophages infect but do not kill their host cells. They continue to produce and release progeny phages from infected cells without seriously affecting the viability of host cells (6, 12).

When Cf and Xf phage lysates were electrophoresed on a polyacrylamide gel, two proteins of approximately 50 and 6 kDa were detected (26). The 50-kDa protein, which is the minor capsid protein, was designated the A protein, and the 6-kDa protein, which is the major capsid protein, was designated the B protein (26). These two proteins appear to be the only capsid proteins of both Cf and Xf. The A protein of Xf is slightly smaller than that of Cf. The nucleotide sequence of the entire Cf genome has been determined (14). The Cf genome is 7.3 kb and contains approximately 10 genes. The A protein gene of Cf encodes a protein of 419 amino acids with a calculated molecular mass of 44,676 Da, which is slightly smaller than the molecular mass estimated by polyacrylamide gel electrophoresis. It is unknown whether this increase in molecular mass is due to posttranslational modification. The B protein gene is 186 bp in length and encodes a 62-amino-acid protein with a calculated molecular mass of 6,070 Da. The amino acid sequences of both the A and B proteins of Cf have no apparent homology with those of analogous proteins of other filamentous bacteriophages. The identities of the other Cf genes are being determined. The nucleotide sequence of the Xf genome has not been reported, and no Xf genes have been identified.

Although the nucleotide sequence of the A protein gene of Cf has no homology with those of A protein genes of other bacteriophages, the genomic location and size of the A protein gene of Cf are analogous to those of the gIIIp gene of Ff phages (9). It plays an important role in the initiation of phage infection (8, 16) and is also called the adsorption protein (11, 15). gIIIp forms a knobbylike structure called the adsorption complex, which binds to the tips of F pili and brings the phage close to the cell surface (10), leading to release of phage DNA into the host cell and initiation of phage replication (2, 18, 22).

Although Cf and Xf bacteriophages are similar in morphology and life cycle (5, 13), they have different host specificities. Cf, isolated from X. campestris pv. citri, does not infect X. campestris pv. oryzae. Similarly, Xf, isolated from X. campestris pv. oryzae, does not infect X. campestris pv. citri. The production of bacteriophages may be determined at various steps, including attachment, adsorption, penetration, replication, and assembly (3, 18, 19, 22). A phage may be able to infect but unable to replicate or assemble inside the host; this situation would also contribute to host specificity. However, Xf or Cf phage progeny can be produced in nonpermissive hosts if the genomic DNA of Cf or Xf is introduced into host cells by electroporation (25). These observations suggest that the host specificities of these two phages are tied to the initial steps of infection, i.e., adsorption and penetration. Since capsid proteins of filamentous bacteriophages play a major role in adsorption and penetration, experiments were performed in this study to determine whether the A protein of Cf is involved in the determination of host specificity. Cf was studied because its entire genome has been sequenced and the location of its A protein gene has been determined.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. The bacterial strains, plasmids, and phages used in this study are listed in Table 1. Escherichia coli and X. campestris strains were grown in Luria-Bertani (LB) medium at 37 and 28°C, respectively. Plasmids were propagated in E. coli HB101. Culture and purification of Cf or Xf phage were performed as described previously (13).

Molecular genetic techniques. Plasmid DNAs and replicative form (RF) DNAs of filamentous phages were isolated from cultures by the method of Birnboim and Doly (1) and further purified by ethidium bromide-cesium chloride buoyant density centrifugation. Restriction endonuclease digestions and DNA ligations were performed according to the manufacturers’ instructions. Restriction fragments were isolated from agarose gel slices by...
TABLE 1. Bacterial strains, plasmids, and bacteriophages

<table>
<thead>
<tr>
<th>Strain, plasmid, or bacteriophage</th>
<th>Relevant genotype or characteristic(s)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli HB101</td>
<td>F' · hsdS2O (r Delete 13), lacI, lpdB6 ara-14 proA2 lacY1, metK2 araL20 (Strr) xyl-5, mtl-1 supE45</td>
<td>22</td>
</tr>
<tr>
<td>X. campestris pv. citri</td>
<td>Wild type, host of Cf phage</td>
<td>5</td>
</tr>
<tr>
<td>X. campestris pv. oryzae</td>
<td>Wild type, host of Xf phage</td>
<td>13</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pHC8</td>
<td>Derivative of pKK415, oriV, traF, TeⅠ</td>
<td>4</td>
</tr>
<tr>
<td>pCFFE2501</td>
<td>pBR325 derivative containing RF DNA of phage Cf</td>
<td>24</td>
</tr>
<tr>
<td>pUC-KIXX</td>
<td>E. coli gene cartridge vector</td>
<td></td>
</tr>
<tr>
<td>pCFA1</td>
<td>1.5-kb NalIV fragment from pCFFE2501 cloned into pHC8</td>
<td>Pharmacia This study</td>
</tr>
<tr>
<td>pCFA2</td>
<td>Same as pCFA1 but in different orientation</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Phages</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cf</td>
<td>Bacteriophage isolated from X. campestris pv. citri</td>
<td>5</td>
</tr>
<tr>
<td>Xf</td>
<td>Bacteriophage isolated from X. campestris pv. oryzae</td>
<td>13</td>
</tr>
</tbody>
</table>

electroporation (21). X. campestris pv. citri was made competent and transformed with plasmid DNA by electroporation as described previously (25).

Polyacrylamide gel electrophoresis and immunoblotting. Proteins of purified phage particles or the soluble fractions of bacteria containing plasmids were separated by electrophoresis on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels. Gels were either stained with Coomassie brilliant blue R250 or used for immunoblotting, which was performed as described by Towbin et al. (24).

Phage infectivity assay. One milliliter of culture was centrifuged, and the supernatant was filtered through a 0.45-μm-pore-size filter to remove any residual bacteria. The filtrate was serially diluted in LB medium, and 10 μl of each dilution was mixed with 100 μl of mid-log-phase culture of X. campestris and 3 ml of soft agar (0.75%) in LB broth. The mixture was poured and spread evenly onto an agar plate. The plate was scored for plaque formation after incubation at 28°C for 18 h.

Detection of kanamycin-resistant transducing particles. E. coli cells transformed with RF DNA of Cf-Km, which is a recombinant Cf phage with a kanamycin resistance gene inserted into the A protein gene, were plated on LB agar containing kanamycin (30 μg/ml). A kanamycin-resistant colony was picked and grown on LB agar containing kanamycin. One milliliter of the culture was centrifuged and filtered as described above. A 10-fold serial dilution of the filtrate was made, and 10 μl of each dilution was mixed with 100 μl of X. campestris pv. citri cells. Cells were spread on LB agar plates containing kanamycin to detect kanamycin-resistant colonies, which indicate the presence of infective Cf-Km phage particles.

RESULTS

Expression of the A protein of Cf. The gene encoding the A protein of phage Cf was cloned into an E. coli-X. campestris shuttle vector, pHC8 (4). A DNA fragment containing the A protein gene of Cf was isolated as a 1.5-kb NalIV fragment from pCFFE2501, which contains the entire Cf genome (23). This 1.5-kb NalIV fragment was inserted into the blunt-ended XbaI site of pHC8. Two recombinant plasmids, named pCFA1 and pCFA2 (Fig. 1), with their inserts in opposite orientations were obtained. These two plasmids were introduced into E. coli HB101 and X. campestris pv. citri XW47 to express the A protein of Cf. The cell lysates of E. coli and X. campestris pv. citri containing pCFA1 or pCFA2 were examined by immunoblotting with antibody against purified Cf particles. This antibody has been shown to react with both proteins A and B of Cf (26).

The 50-kDa protein band, which reacted with the antibody, was detected in lysates of E. coli cells containing either pCFA1 or pCFA2 (Fig. 2). The intensities of the 50-kDa protein bands from the two different cell lysates were about the same (Fig. 2, lanes 3 and 6 [pCFA1] and 4 and 7 [pCFA2]). The positive control lane, which contained lysate of Cf phage particles, also showed the 50-kDa protein band (lane 1). No reactions with the antisera were seen in lysates of host cells or host cells containing the vector pHC8 (lanes 2 and 5). These results indicated that the plasmids (pCFA1 and pCFA2) directed the expression of the A protein of Cf equally well in E. coli and X. campestris pv. citri.

Requirement of the A protein for Cf infectivity. To determine whether the A protein is essential for the infectivity of Cf, the A protein gene was mutated by inserting a kanamycin resistance gene into it. The kanamycin resistance gene was isolated as a 1.6-kb HinIII fragment from pUC4-KIXX (Pharmacia, Piscataway, N.J.) and inserted into the HinIII site in the A protein gene of the native Cf RF DNA genome. The RF DNA of the recombinant Cf phage (designated Cf-Km) was transformed into X. campestris pv. citri by electroporation. Cells were spread on LB agar plates containing kanamycin to detect kanamycin-resistant colonies, which indicate the presence of infective Cf-Km phage particles (referred to as Km′-transducing particles). No kanamycin-resistant colonies were observed, indicating that no infective Cf-Km phage particles were produced and that the A protein is essential for production of infectious particles. The culture supernatants of X. campestris pv. citri containing RF DNA of Cf-Km either alone or with transformed or transformed DNA produced the following amounts (in CFU per milliliter) of Km′-transducing particles: <10^2 for the nontransformed or -transformed organisms, (1.5 ± 0.3) × 10^5 for the organisms transfected with RF DNA of Cf, and (1.5 ± 0.1) × 10^5 for the organisms transformed with pCFA1. The CFU of Km′-transducing particles per milliliter are expressed as <10^5 for X. campestris pv. citri containing RF DNA of Cf-Km alone, since only 10 μl of the culture supernatant was assayed.

To determine whether this mutation can be complemented, the RF DNAs of both Cf-Km and wild-type Cf were cointroduced into X. campestris pv. citri. Under these conditions, the A protein produced by wild-type Cf may be incorporated into Cf-Km phage particles to generate infective Cf-Km phage. This infective Cf-Km phage will be able to infect X. campestris pv. citri and will confer upon the host a kanamycin resistance phenotype so that it will grow as a colony on agar plates containing kanamycin. However, X. campestris pv. citri infected by this infective Cf-Km phage will not be able to produce additional infective Cf-Km phage, since no functional A protein is produced.

The transformants containing RF DNAs of both Cf-Km and wild-type Cf were grown, and the culture supernatants were assayed for infective Cf-Km phage as described above. A substantial amount (~10^5 CFU/ml) of infective Cf-Km phage particles in the culture supernatant was detected. The same complementation experiment was performed with pCFA1 to supply the A protein. Plasmid DNA of pCFA1 and RF DNA of Cf-Km were cotransformed into X. campestris pv. citri, and the transformants were found to generate approximately the same amount of infective Cf-Km phage particles as those complemented with wild-type Cf. In both experiments, the control, which was RF DNA of Cf-Km introduced into X. campestris pv. citri alone, yielded no infective Cf-Km phage particles in any of the 10-μl aliquots of culture supernatants that were assayed.
Incorporation of the A protein of Cf into Xf virions. Experiments were then performed to determine whether the A protein of Cf could be incorporated into the viral particles of Xf and whether Xf with the A protein of Cf would infect *X. campestris* pv. *citri*. Single-stranded DNA of the Xf genome was introduced by electroporation into *X. campestris* pv. *citri* or *X. campestris* pv. *oryzae* containing pCFA1. Under these conditions, the A protein of Cf produced from pCFA1 may be incorporated into Xf virions. The transformed cells were grown in LB medium, and the clarified culture supernatant was plated separately on the lawns of three different hosts: *X. campestris* pv. *citri*, *X. campestris* pv. *oryzae*, and *X. campestris* pv. *citri* containing pCFA1.

Plating the phages on *X. campestris* pv. *citri* serves as a negative control, since Xf or Xf containing the A protein of Cf (mixed-phenotype Xf) does not produce plaques in this host. Plating the phages on *X. campestris* pv. *oryzae* allows detection of Xf phage produced by the introduced single-stranded Xf DNA genome, whereas plating the phages on *X. campestris* pv. *citri* containing pCFA1 allows detection of Xf phage that contains the A protein of Cf. The presence of pCFA1 in the host cell *X. campestris* pv. *citri* is essential for this assay because the mixed-phenotype Xf phage has the typical Xf genome, which requires a constant supply of the A protein of Cf to produce plaques on *X. campestris* pv. *citri*. As additional controls, the single-stranded Xf or Cf DNA genome was introduced separately into *X. campestris* pv. *citri* and *X. campestris* pv. *oryzae* and the culture supernatants of the transfectants were also assayed for infective bacteriophages on the three different hosts as mentioned above.

The results of these experiments are summarized in Table 2. Introduction of the Xf DNA genome into the permissive host, *X. campestris* pv. *oryzae*, produced 3.1 × 10^8 PFU of Xf progeny per ml. However, only 1.4 × 10^4 PFU (4 orders of mag-
DISCUSSION

Several new findings with regard to the Cf phage were obtained in this study. The observation that inactivation of the A protein gene abolished the infectivity of Cf suggests that the A protein is essential. The finding that the A protein of Cf altered the host specificity of Xf when it was incorporated into Xf viral particles suggests that the A protein is responsible for the host specificity of Cf. These results indicate that the A protein of Cf is functionally analogous to gIIp of Ff phages. gIIp is involved in the adsorption step of infection and controls the host specificities of Ff phages (2, 10, 11, 17, 18). The ability of the A protein of Cf to change the host specificity of Xf indicates that the A proteins of Cf and Xf are interchangeable and that phenotypic mixing can occur between these two phages.

The possibility of interchanging the A proteins of Cf and Xf suggests that these two phages may be related. It may be possible to create hybrid phages that contain a heterologous A protein gene, e.g., Cf with the A protein gene of Xf. The hybrid phages would allow confirmation of the mixing of phenotypes between Cf and Xf and further investigation of the interaction between phages and their receptors. Such experiments will be possible when the nucleotide sequence of the Xf genome becomes available. The availability of the Xf genomic sequence will also enable determination of the relatedness between Cf and Xf.

Although we have demonstrated that the A protein of Cf is responsible for host specificity, it is unknown whether this is its sole function. gIIp of Ff phages has been shown to have multiple functions. It recognizes receptors and attaches the phage to receptors on the surfaces of host cells (2, 3, 18, 22). It is also involved in the penetration of the phage DNA into host cells (7, 20, 22). There is also evidence suggesting that gIIIp is involved in viral assembly and maintenance of the structural integrity of phage particles (15, 19).

The observation that Cf and Xf replicate more efficiently (4 orders of magnitude higher) in permissive than in nonpermissive hosts when their genomes are introduced into host cells suggests that host factors are involved in phage replication.

<table>
<thead>
<tr>
<th>Culture supernatanta</th>
<th>Amt of progeny phage (PFU/ml)b produced on:</th>
<th>Phage typec</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X. campestris pv. oryzae</td>
<td>X. campestris pv. citri</td>
</tr>
<tr>
<td>Xf DNA-X. campestris pv. citri</td>
<td>(1.4 ± 0.1) × 10⁴</td>
<td>&lt;10⁷</td>
</tr>
<tr>
<td>Xf DNA-X. campestris pv. oryzae</td>
<td>(3.1 ± 0.2) × 10⁴</td>
<td>&lt;10⁷</td>
</tr>
<tr>
<td>Cf DNA-X. campestris pv. citri</td>
<td>&lt;10⁷</td>
<td>(4.3 ± 0.5) × 10⁴</td>
</tr>
<tr>
<td>Cf DNA-X. campestris pv. oryzae</td>
<td>&lt;10⁷</td>
<td>(1.7 ± 0.1) × 10⁴</td>
</tr>
<tr>
<td>Xf DNA-pCFA1-X. campestris pv. citri</td>
<td>(1.6 ± 0.3) × 10⁴</td>
<td>&lt;10⁷</td>
</tr>
<tr>
<td>Xf DNA-pCFA1-X. campestris pv. oryzae</td>
<td>(7.0 ± 0.4) × 10⁸</td>
<td>&lt;10⁷</td>
</tr>
</tbody>
</table>

a Single-stranded DNA of phage Cf or Xf was electroporated into X. campestris pathovars alone or with pCFA1.
b Values are means of three experiments.
c Phage types that were released into culture supernatants.
would be very interesting to identify the host factors that are involved in phage replication. The determination of which steps in the phage replication cycle are affected and how the host factors affect those steps will further our knowledge of the biology of Cf and Xf phages.

ACKNOWLEDGMENTS

We thank Chao-Hung Lee for valuable discussions and for critically editing the manuscript.

This work was supported by a grant from the National Science Council, Taipei, Taiwan, Republic of China (NSC 84-2311-B-030-001).

REFERENCES