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Purification and Expression of the Gene III Protein from Filamentous Phage ϕLf

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The gene III protein (pIII) from ϕLf, a filamentous phage of *Xanthomonas campestris* pv. *campestris,* was purified by gel filtration with FPLC. The *gIII* coding region was amplified by PCR, which was then cloned into pUC18 and expressed in *Escherichia coli.* The size of both pIII, purified from phage particle and expressed in *E. coli,* is similar to the value deduced from the nucleotide sequence as shown by Western blot analysis. This is different from the case in Ff phages (f1, fd, and M13), in which the size of pIII observed in SDS-polyacrylamide gel electrophoresis is substantially larger than the deduced value. Upon infection of *X. c.* pv. *vesicatoria* carrying cloned ϕLf *gIII* with ϕXv, a filamentous phage of pv. *vesicatoria,* the progeny particles in supernatant were able to infect both pv. *campestris* carrying cloned ϕLf *gIII* and pv. *vesicatoria,* indicating that a mixture of authentic ϕXv and chimeric phage consisting of ϕXv DNA and ϕLf pIII was produced. These results suggest pIII to be the adsorption protein required for host recognition. © 1998 Academic Press

ϕLf is a filamentous phage which specifically infects the Gram-negative plant pathogen *Xanthomonas campestris* pv. *campestris* (1). This phage is similar to other filamentous phages in morphology, using RF (replicative form) as the intermediate for DNA replication, and propagating without lysis of the host (2). However, it differs from other filamentous phages in i) possessing a mechanism to integrate its RF DNA into the host chromosome (3, 4), ii) having a replication initiation protein (pII) that possesses sequence domains conserved to the superfamily I Rep proteins of the rolling circle replicating replicons which does not include the proteins of other filamentous phages (5), and iii) having the origin for viral strand replication being contained within the pII coding region instead of the intergenic region (6). The complete nucleotide sequence of ϕLf has been determined and ten genes have been found on the viral strand (7). These genes having an organization similar to the order of Ff phages (f1, fd and M13) genes, *gII-gX-gV-gVII-gIX-gVIII-gIII-gVI-gI-gXI,* code for proteins pII, pX, pV, pVII, pIX, pVIII, pIII, pVI, pI and pXI, respectively. Although most of these proteins have been characterized (5, 8-10, unpublished results), the minor coat proteins pVII, pIX and pIII remain unknown.

In Ff phages, the four minor coat proteins (pIII, pVI, pVII and pIX) are present at three to five copies each in the phage particle with pIII and pVI locating at one end and pVII and pIX at the other (2). pIII mediates phage adsorption to pilus and is also necessary for phage uncoating and DNA penetration which requires the function of the host proteins TolQ, R and A (11–15). In addition to the Ff *gIII,* the nucleotide sequence has been determined for the *gIII* of filamentous phages Ike, I2-2 and Pf1 (16–18). Their deduced amino acid sequences show the following similarities: each protein possesses a signal peptide at N-terminus, which is followed by a repeated glycine-rich region(s), and a C-terminal hydrophobic sequence for anchoring the protein into the host membrane. In ϕLf, ORF367, able to code for 367 amino acids with a calculated MW of 36,710, has been identified as *gIII* based on i) the similarity in gene organization to the Ff *gIII,* locating between *gVIII* and *gVI,* and ii) the presence of all the conserved amino acid sequence domains common to the pIIIs of filamentous phages (9). In this study, we purified pIII from the ϕLf particle, used the purified protein to raise antibody, and expressed the pIII protein in *Escherichia coli.* By Western blot analysis, the gene-protein relationship as well as the presence of this protein in ϕLf particle were demonstrated. Furthermore, our pre-

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Abbreviations used: aa, amino acid; Ap^R, ampicillin-resistance; Ff, F-specific filamentous phage; FPLC, fast protein liquid chromatography; kDa, kilodalton; MOI, multiplicity of infection; ORF, open reading frame; PCR, polymerase chain reaction; PFU, plaque forming unit; RF, replicative form; SDS, sodium dodecyl sulfate; ssDNA, single-stranded DNA; Tc^R, tetracycline-resistance.

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liminary results showed that chimeric phage can be produced by the DNA of ϕXv, a filamentous phage specifically infecting *X. c.* pv. *vesicatoria,* and the ϕLf pIII provided *in trans.* The chimera thus produced was able to infect *X. c.* pv. *campestris.* Based on these observations, we suggest pIII to be the adsorption protein required for the determination of host specificity.

MATERIALS AND METHODS

Phages, bacterial strains, and growth conditions. Filamentous phages ϕLf and ϕXv have been described previously (1, 19). P20H was a non-mucoid mutant isolated from *X. c.* pv. *campestris* strain 11 by nitrous acid mutagenesis (20). *X. c.* pv. *vesicatoria* strain 36, the pathogen causing spot disease in pepper and tomato, was a gift from Dr. S. -T. Hsu, National Chung Hsing University (21). *E. coli* DH5α (Bethesda Research Laboratories) was the host for gene cloning and expression of the cloned ϕLf pIII. LB broth and L agar (22) were used for growing *E. coli* (37°C) and *X. campestris* strains (28°C).

Phage techniques. Double layer bioassay (23) was performed to determine the phage titer on LB plate, in which the top layer contained 0.75% agar. Spot test was done by dropping 5 μl of a phage suspension onto the freshly poured top agar containing indicator cells. The ϕLf particles were purified by ultracentrifugation, then the banded phage was collected, dialyzed and lyophilized as described previously (10).

DNA techniques. Restriction endonucleases, T4 DNA ligase, Klenow enzyme, S1 nuclease and other enzymes were purchased from New England BioLabs (Beverly, MA) and used following the instructions provided by the supplier. DNA techniques described by Sambrook et al. (24) were used for plasmid extraction, preparation of RF DNAs of ϕLf and M13, gene cloning, Southern hybridization and transformation of *E. coli. X. c.* pv. *campestris* was transformed by electroporation (25). The dideoxy chain termination method described by Sanger et al. (26) was used for DNA sequence determination on single-stranded templates.

PCR amplification. PCR was performed in GeneAmp PCR system 2400 (Perkin-Elmer), using synthesized oligonucleotides UP28, 5′-CTCCCCGGGAAGCTTCTTCGGCTAATGG-3′ with a *Sma*I site, and DOWN27, GCGTCTAGATCAGGTCACAGCAATGCG-3′ with an *Xba*I site, as the primers. Using these primers and the ϕLf RF DNA as the template, the DNA amplified was a 1,147-bp fragment encompassing the region from -43 to the termination codon of *gIII* (9). PCR reactions were performed for 25 cycles in a volume of 100 μl containing 100 ng DNA template, *Taq* polymerase (2.5 U), 20 mM $MgCl_2$, 0.1 mM dNTP mix, 20 μM of each primer, and the reaction buffer from Promega (Madison, WI). Each cycle consisted of incubations for 1 min at 94°C, 2 min at 37°C, and 3 min at 72°C. After the last cycle, the reaction mixture was incubated at 72°C for 10 min, then at 4°C to terminate the reaction. The resulting fragment, a 1,147-bp *Sma*I-*Xba*I fragment, was cloned into the compatible sites of pUC18 to generate pUF3.

Expression of gIII in E. coli. To express the cloned *gIII,* cells of *E. coli* DH5α containing pUF3 were grown till an A_{550} of 0.8 and induced by addition of IPTG at a final concentration of 0.5 mM. The culture was further grown for 3 hr and harvested by centrifugation. The pellet was stored in deep freezer until used.

Column chromatography. The ϕLf pIII was purified by gel filtration (Superose 12) with fast protein liquid chromatography (FPLC). The chromatographic instrument was a Pharmacia LKB FPLC system which consisted of a 2150 HPLC pump, a 2154 loop injection valve, a 2158 Uvicord SD monitor (set at 280 nm), a 3390A integrator (Hewlett-Packard), and a 2211 Super-Rac fraction collector. The lyophilized ϕLf particles were suspended in 10 mM sodium citrate (pH 5.4) containing 1% (w/v) sodium dodecyl sulfate (SDS). The sample (250 μl) was loaded onto a Superose 12 column (1 cm × 30 cm) and eluted with 5 mM citric acid (pH 3.0) containing 50 mM Na_2SO_4 and 1% SDS. Fractions containing pIII were pooled and then dialyzed exhaustively into water.

Gel electrophoresis of protein. SDS-polyacrylamide gel (12%) electrophoresis was performed by the method of Laemmli (27), using a Mini Protean II electrophoresis apparatus (Bio-Rad). The purified pIII or the cellular proteins were mixed with 2× cracking buffer (50 mM Tris-HCl, pH 6.8, containing 100 mM dithiothreitol, 2% SDS, 10% glycerol and 0.1% bromophenol blue) and heated in boiling water bath for 5 min prior to loading onto the wells. To visualize the coat proteins in the gel, the purified phage particles were treated with chloroform as described previously before the treatment with cracking buffer and loading the gel (10). Electrophoresis was run at 90 V, and the protein was detected by staining with Coomassie brilliant blue R250.

Immunoblotting. Antiserum was prepared following the method of Harlow and Lane (28) by immunizing the rabbit (New Zealand White Strain) with FPLC-purified ϕLf pIII. Two mg of the protein was delivered by intradermal injection each time for four consecutive injections at intervals of one week. For Western blot analysis, the method described by Sambrook et al. (24) was employed with some modifications. The proteins separated in SDS-polyacrylamide gel were electrophoretically transferred to nitrocellulose filter (Amersham) using a BioRad Mini-Protean II cell transfer apparatus (250 mA, 2 hr). The filter was treated with 30 ml of blocking buffer containing 1.5 g of nonfat dry milk, incubated with antiserum raised against the FPLC-purified pIII, washed, and probed with horseradish peroxidase-conjugated goat anti-rabbit IgG.

RESULTS AND DISCUSSION

Purification of the ϕLf pIII

We have previously demonstrated that upon electrophoretic separation of the ϕLf phage particles in SDS-polyacrylamide gel, three bands corresponding to molecular masses of 37, 10 and 4 kDa, respectively, can be observed after staining the proteins with Coomassie brilliant blue (Fig. 1, 10). Further analysis by Western blotting has shown that the 37- and 10-kDa bands are pIII and pVI, respectively, and the 4-kDa band presumably contains the major coat protein pVIII (4.1 kDa) at a larger amount and the two minor coat proteins, pVII (3.8 kDa) and pIX (4.3 kDa), at smaller amounts (10). pVIII, pVII and pIX with similar molecular sizes could not be resolved in SDS-polyacrylamide gel electrophoresis under the conditions we used (8, 10).

To purify the ϕLf pIII, the phage particles purified by ultracentrifugation (about 1×10^{13} PFU from the original lysate) were dissociated in 250 μl of the citrate buffer containing 1% SDS and passed through a FPLC gel filtration column (Superose 12). Two peaks were observed (Fig. 2A). The first peak was identified to be the phage ssDNA that could form an ethidium bromide-stained DNA band co-migrating in agarose gel (0.8% in TBE buffer) with the ssDNA prepared from the ϕLf particles. Like the ssDNA prepared from ϕLf, it was sensitive to S1 nuclease treatment and did not migrate in SDS-polyacrylamide gel (12%) electrophoresis. The second peak could form a ~4-kDa protein band in SDS-

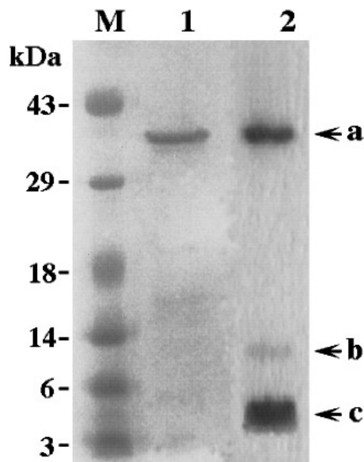


FIG. 1. SDS-polyacrylamide gel electrophoresis of the ϕLf pIII. Proteins were run in SDS-polyacrylamide gel and stained with Coomassie brilliant blue. Lanes: M, the protein molecular weight markers; 1, pIII purified by gel filtration with FPLC; 2, the ϕLf particles treated by chloroform extraction, as described by Liu et al. (10), which greatly reduced the amount of pVIII without affecting significantly the amounts of pIII and pVI. Protein bands: a, pIII; b, pVI; c, a mixture of pVII, pVIII, and pIX.

polyacrylamide gel which presumably contains pVIII, pVII and pIX (data not shown).

Since the molecular size deduced for pIII falls between the phage ssDNA and the ~4-kDa proteins, the eluates came between the two peaks from different runs were combined. This pooled protein solution was desalted by dialysis and concentrated by lyophilization and then subjected to a second FPLC chromatography in the same column and buffer system. As shown in Fig. 2B, two major and one minor peaks were obtained. The first and the second major peaks, containing phage ssDNA and the ~4-kDa proteins, correspond to the first and the second peaks, respectively, of the first chromatography. The protein in the minor peak formed a single band of 37 kDa in SDS-polyacrylamide gel electrophoresis which migrated at the same rate as the pIII from the ϕLf particle, indicating that it has been purified to apparent homogeneity (Fig. 1, lane 1).

Anti-ϕLf pIII serum was prepared by immunizing a rabbit with the FPLC-purified pIII as described in Materials and Methods. As shown in Fig. 3, this serum reacted strongly with the 37-kDa protein from the phage particle, indicating that this protein is indeed present in the phage particle. This coincides with our previous results of Western blot analysis, using the serum raised against the ϕLf phage particles, which showed that this protein is virion-associated (10).

Expression of the ϕLf pIII in E. coli

The ϕLf *gIII* resides between nt 3,119 and 4,222 counting from the unique *Pst*I site of the RF DNA (7, 9). To clone this gene, the region between nt 3,076 and 4,222 of the RF DNA was amplified by PCR as a 1,147-bp *Sma*I-*Xba*I fragment as described in Materials and Methods. The fragment was cloned into M13 for sequence determination to assure that no mutation had been caused. This *gIII*-containing fragment was then ligated with pUC18 which had been cut with *Xba*I plus *Sma*I. The resulting plasmid was designated as pUF3 (Fig. 4A) which carried the *gIII* being inserted at the same direction as the *lac* promoter and, therefore, the gene can be induced by addition of IPTG. *E. coli* DH5α(-pUF3) was then used for expression of the cloned *gIII* with *E. coli* DH5α(pUC18) as the control. No distinct band was observed in SDS-polyacrylamide gel upon electrophoretic separation of the cellular proteins from *E. coli* DH5α(pUF3), indicating that pIII was synthesized at a very small amount in *E. coli,* even though the gene was under the control of the strong *lac* promoter (data not shown). However, Western blot analysis of the same gel using the antiserum prepared against the FPLC-purified pIII detected a protein of 37

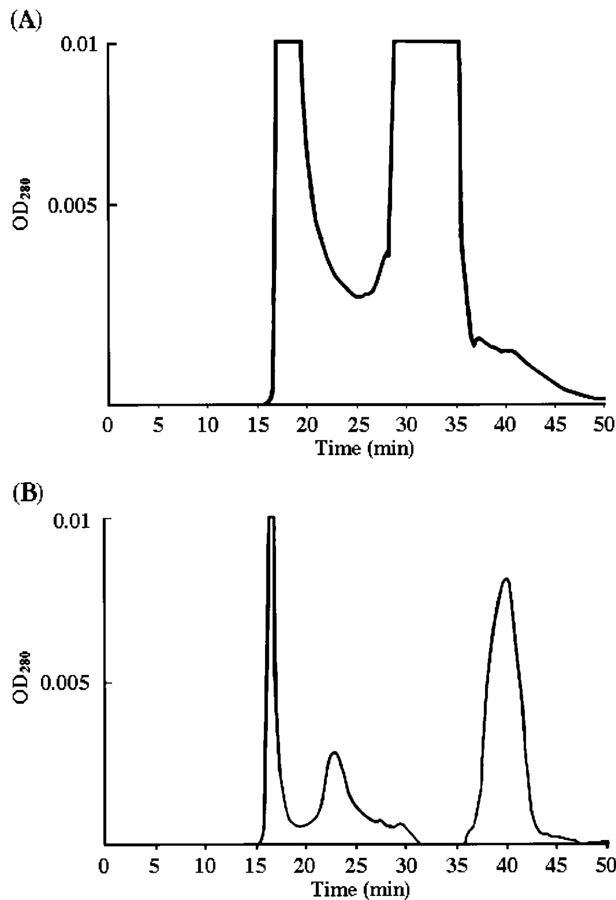


FIG. 2. Purification of the ϕLf pIII by FPLC. The ultracentrifuge-purified ϕLf particles were dissociated in the SDS-containing buffer, loaded onto the Superose 12 column, and then eluted at a flow rate of 0.4 ml/min. (A) Elution profile of the first run. (B) Elution profile of the second run by applying the eluates collected between the two peaks of the first run onto the same column.

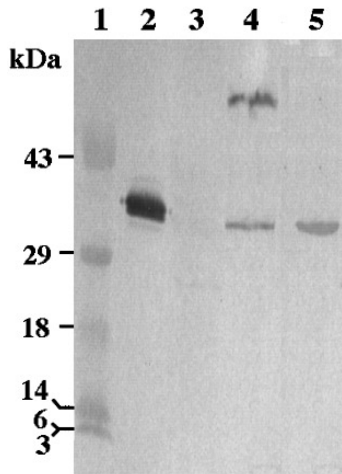


FIG. 3. Western blot analysis of the ϕLf pIII. Lanes: 1, prestained protein molecular weight markers; 2, FPLC-purified pIII; 3, cellular proteins of *E. coli* DH5α(pUC18); 4, cellular proteins of *E. coli* DH5α(pUF3); 5, proteins from the ϕLf particles after chloroform extraction as described previously (10).

kDa in *E. coli* DH5α(pUF3) (Fig 3, lane 4), but not in the control *E. coli* DH5α(pUC18) (Fig 3, lane 3). These results demonstrate the relationships between the gene and its product, and confirm our previous prediction that ORF367 is the ϕLf *gIII* (9).

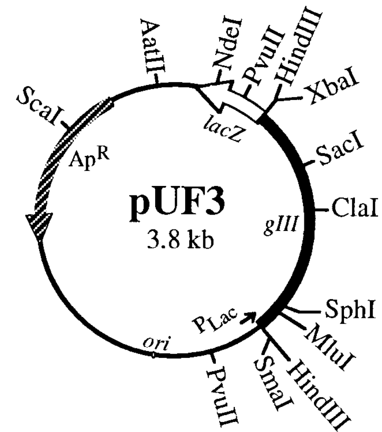
Molecular Size of the ϕLf pIII

In Ff phages, pIII is a protein of 424 amino acids with a deduced molecular weight of 42,675 (29). However, substantially higher values (59-70 kDa) have been observed for this protein in SDS-polyacrylamide gel (29-32). Different possibilities have been proposed to explain this discrepancy, including that impurity may be associated with the protein (32), and the mobility may be affected by the presence of unusual clustering of glycine and serine residues in the polypeptide (29). Long stretch of glycine-rich repeatings (70 amino acids) are also present in the ϕLf pIII, however, its molecular size was shown to be similar to that deduced from the nucleotide sequence (Fig. 1). Furthermore, in our Western blot analysis, no difference in mobility was observed with the pIII protein obtained from different sources, i.e. directly from the phage particle or expressed in *E. coli* (Fig. 3).

Chimera Formed by ϕLf pIII and ϕXv DNA Can Infect X. c. pv. campestris

ϕXv is a filamentous phage of *X. c.* pv. *vesicatoria* which was isolated in our laboratory and has been characterized by us (19). ϕXv and ϕLf each can only infect its own host but not other non-host *X. campestris* pathovars. However, their DNAs share strong homology as demonstrated by Southern hybridization, and their antisera can react with the particles of both phages. Furthermore, when RF or ssDNA from these phages were electroporated into the nonpermissive host, authentic phage particles can be produced (19). These findings have suggested that in different pathovars of *X. campestris,* the cellular machinery for phage DNA replication, morphogenesis and export are homologous, whereas some cell surface structures required for early steps of phage infection are different. Similar results have been obtained for filamentous phages Cf and Xf which specifically infect *X. c.* pv. *citri* and *X. oryzae* pv. *oryzae,* respectively, by electroporating the DNA of these phages into nonpermissive hosts (33). In addition, chimeric phage containing Xf DNA and the A pro-

(A)



(B)

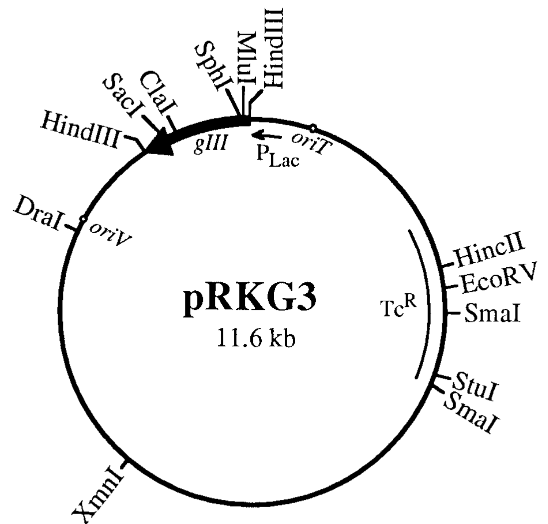


FIG. 4. The ϕLf pIII-expression plasmids pUF3 (A) and pRKG3 (B). The PCR fragment containing the ϕLf *gIII* (1,147-bp *Sma*I-*Xba*I fragment) was cloned into the compatible sites of pUC18, forming pUF3. pRKG3 was constructed by cloning the ϕLf *gIII* from pUF3 into the *Hin*dIII site of pRK415 (33), a 10.5-kb broad host range vector derived from RK2. Abbreviations: *ori,* ColE1 origin of replication; *oriT,* origin for conjugal transfer; *oriV,* origin for plasmid replication; *lacZ,* the region coding for α fragment of β-galactosidase; P_{Lac}, *lac* promoter; Ap^R, ampicillin resistance; Tc^R, tetracycline resistance.

tein (adsorption protein) from Cf able to infect *X. c.* pv. *citri* (the nonpermissive host of Xf) was produced by electroporation of Xf DNA into *X. c.* pv. *citri* containing cloned A protein gene of Cf; therefore, A protein was suggested to be the adsorption protein involved in the determination of host specificity (34).

In this study, we tested whether the ϕLf pIII can be incorporated into ϕXv to form chimeric phage particles, containing ϕXv DNA and ϕLf pIII, which can infect *X. c.* pv. *campestris.* For such a test, the ϕLf *gIII* was cloned from pUF3 into the RK2-derived broad host range vector pRK415 (35) to form pRKG3 (Fig. 4B), then the *X. c.* pv. *vesicatoria* strain 36 (5×10^8 cells/ml) containing pRKG3 was infected with ϕXv at an MOI of 10. The supernatant taken from this infected culture was able to form clearing zones after it was spotted on a lawn of *X. c.* pv. *campestris* P20H containing pRKG3 or *X. c.* pv. *vesicatoria* 36. In contrast, no clearing zone was observed in the control using P20H as the indicator, due to the reason that only authentic ϕXv was produced in P20H without pRKG3 and consequently no clearing zone was formed since no subsequent infection by the progeny could happen. These results indicate that a mixture of authentic ϕXv and chimeric phage containing ϕXv DNA and the ϕLf pIII had been produced during the ϕXv propagation with the cloned ϕLf *gIII* being provided *in trans.* In addition, these data, although remain preliminary, suggest pIII to be the determinant specifying the host specificity of *Xanthomonas* filamentous phages.

A drawback encountered in this experiment was that we were unable to differentiate the chimeric phage from the authentic ϕXv by Southern hybridization due to the high DNA homology between ϕXv and ϕLf, or by Western blotting due to the cross-reactivity between the anti-ϕLf particle and anti-ϕLf pIII sera and the ϕXv coat proteins.

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