A Filamentous Phage of *Vibrio parahaemolyticus* O3:K6 Isolated in Laos

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Received October 15, 1998; in revised form, January 8, 1999. Accepted January 12, 1999

Abstract: A filamentous phage, ‘Ivpf5,’ of *Vibrio parahaemolyticus* O3:K6 strain LVP5 was isolated and characterized. The host range was not restricted to serotype O3:K6, but 7 of 99 *V.* *parahaemolyticus* strains with a variety of serotypes were susceptible to the phage. The phage was inactivated by heating at 80°C for 10 min and by treating with chloroform. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the phage exhibited a 3.8 kDa protein. The amino-terminal amino acid sequence of the coat protein was determined as AEGGAADPFEAIDLLGVATL. The phage genome consisted of a single-stranded DNA molecule. The activity of the phages was inhibited by anti-Na2 pil antibody.

Key words: Filamentous phage, *Vibrio parahaemolyticus*

*V. parahaemolyticus* is a Gram-negative halophilic bacterium widely distributed in coastal waters and a causative agent of food-bone gastroenteritis. Almost all strains isolated from diarrheal patients produce thermostable direct hemolysin (TDH) and/or TDH-related hemolysin (TRH). These toxins are believed to be important virulent factors for the disease (12). Recently, gastroenteritis due to *V. parahaemolyticus* O3:K6 has been prevailing in Southeast Asia (14).

In 1997, an outbreak of the illness emerged in Vientiane, Laos. The isolates exhibited serotype O3:K6 and had the *tdh* gene but not *trh*. We found a filamentous phage in an isolate from this outbreak. Since the filamentous phage of *V. cholerae* is thought to be a virulence transfer factor from non-toxigenic strains to toxigenic strains (3, 4, 6, 8, 19), it is possible that the filamentous phage of *V. parahaemolyticus* may also be involved in virulence transfer. In this study, we presented an outline of the filamentous phage of *V. parahaemolyticus* O3:K6 strain LVP5 isolated from a patient in Laos in 1997.

The organisms were cultured in heart infusion broth supplemented with 3% (w/v) NaCl (3% NaCl-HIB) at 37°C for 15 hr with shaking. The culture supernatant obtained by centrifugation at 15,000×g for 30 min was filtered through a 0.45 µm pore-size membrane. The filtrate was supplemented with 40% saturated ammonium sulfate. The salted out precipitate was collected by centrifugation at 10,000×g for 30 min. The sediment was suspended in 50 mM Tris-HCl, pH 8.0, and fractionated by stepwise sucrose gradient centrifugation (10 to 60%), as previously reported (9), to purify the phage. The purified phage was negatively stained by 4% uranyl acetate on a carbon-coated formvar grid, and observed with a Hitachi H7500 electron microscope. The phage lvpf5 consisted of flexible filaments approximately 7 nm wide, and usually about 1 µm length; but occasionally, very long phages were observed (Fig. 1). SDS-polyacrylamide gel electrophoresis (PAGE) (13) of the whole phage revealed a protein band with the molecular mass of 3.8 kDa (Fig. 2). The N-terminal amino acid sequence of the lvpf5 major coat protein was analyzed by automated Edman degradation on a Applied Biosystems 4773A protein sequencer. The N-terminal amino acid sequence of the 3.8 kDa protein was AEGGAADPFEAIDLLGVATL.

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fore, the number of phage particles is not evaluated by plaque-forming unit (pfu). The phage lvpf5 was inactivated by heating at 80°C for 10 min or by treating with chloroform. These physicochemical properties of lvpf5 are similar to that of other filamentous phages reported (3, 5, 18).

To investigate the host range of the phage, other strains stocked in our laboratory including clinical and environmental isolates were used. The tdh or trh genes of the strains were detected by PCR according to the method of Tada et al (17). Seven strains out of 99 examined developed a confluent plaque by spotting the phage (Table 1). Six of 62 tdh-positive strains (9.7%) and one of 29 tdh-negative strains (3.4%) were sensitive to the phage. The serotypes of the sensitive strains were 01:NT, K38, K56, O2:K3, O4:K11, and K12. These results indicate that the phage sensitivity of the strains is not related to the presence of tox genes and the bacterial serotypes.

A pili (fimbriae), as a receptor of the filamentous...
phage, has been reported (7, 16, 19). Two types of V. parahaemolyticus pili have been isolated from strains Na2 (10) and Ha7 (11) by us. If a pilus is the lvpf5 phage receptor, anti-pili serum is supposed to inhibit phage infection. To confirm this hypothesis, anti-Na2 pili and anti-Ha7 pili serum were each incubated with the indicator strain. The serum, diluted serially using as two-fold dilution with 10 mm PBS, was incubated with Ha8904 at 37 °C for 30 min. After the organisms were spread on 3% NaCl-HIB, the phage was spotted on the organisms. The plaque formation was inhibited by anti-Na2 pili serum but not by anti-Ha7 pili serum or preimmune serum (Fig. 4). The result indicates that the Na2-type pili may be the phage receptor of the lvpf5 phage for the indicator strain.

The phage genome was extracted from the purified phage by the phenol-chloroform method (15). The phage genome was resistant to RNase but sensitive to DNase I and Mung bean nuclease (data not shown). This suggests that the phage genome is single-stranded DNA. The RF of the phage was isolated from the lysogenic Ha8904 cell by the method of Birnboim and Doly (1). The RF DNA of the lvpf5 phage was digested with various restriction enzymes (Fig. 5). The molecular size of the lvpf5 RF DNA was estimated to be about 8.5 kb by the restriction cleavage patterns. Figure 6 shows the restriction map of lvpf5 constructed by Hind III, EcoR I, Kpn I and Pst I digestion fragments. The restriction patterns of lvpf5 phage were similar to those of vf33 phage (18).

Recently, Chang et al reported the sequence of the vf33 gene (2). We looked up the homology of the N-terminal amino acid sequence of the lvpf5 major coat protein in DDBJ with accession number AB012574. Seventeen residues of 20 were identical (85% homology) to the vf33 product (major coat protein of vf33). The similarity of the N-terminal amino acid sequences between lvpf5 and vf33 that suggests that both phages have similar major coat proteins. Taniguchi et al reported the biological properties of vf33 in 1984 (18). At least two differences between lvpf5 and vf33 were presented: 1) lvpf5 did not make a single plaque even though 0.02% Tween 80 was contained on soft agar; and 2) organisms with various serotypes were sensitive to lvpf5 phage but only K38 to the vf33 phage. These differences suggest lvpf5 may be a novel filamentous phage of V. parahaemolyticus.

References

