CHARACTERIZATION OF THE PILI ISOLATED FROM *VIBRIO PARAHAEMOLYTICUS* O3:K6

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Abstract. Pilus of *Vibrio parahaemolyticus* O3:K6 strain LVP9 belonging to the newly identified clone was purified and characterized. The molecular mass of the pilin was estimated to be about 18 kDa by SDS-PAGE, and the isoelectric point of the pilin was 5.0 ± 0.2. The LVP9 pili were antigenically different from the other *V. parahaemolyticus* Na2 pili and Ha7 pili as previously reported, nevertheless all three had indistinguishable morphology and shared a high degree of homology in their N-terminal amino acid sequences. Strain LVP9 and its purified pili did not agglutinate human and rabbit erythrocytes. The LVP9 organisms and the purified pili were adhesive to the rabbit intestine. The adhesion was inhibited by pretreatment of the rabbit intestine with the purified pili or by pretreatment of the organisms with the Fab fractions of anti-pilus antibody. These results indicate that the LVP9 pilus is an adherent factor to the rabbit intestine.

INTRODUCTION

V. parahaemolyticus, a gram-negative halophilic bacterium, is a causative agent of seafood-borne gastroenteritis. The gastroenteritis caused by *V. parahaemolyticus* is closely associated with the ingestion of contaminated seafood, and the illness has rarely been reported in inland areas where seafood is not readily available. Thermostable direct hemolysin (TDH), and/or TDH-related hemolysin (TRH) produced by the organisms are thought to be important virulent factors (Nishibuchi and Kaper, 1995).

The number of reported clinical cases (gastroenteritis) caused by *V. parahaemolyticus* O3:K6 is increasing in Southeast Asia since 1996 (Okuda *et al*, 1997). Although the disease is not transmissible from human to human, it is rapidly spreading through Southeast Asian countries. *V. parahaemolyticus* O3:K6 caused epidemics in an inland country, Lao PDR, in 1997. The isolates were positive for the *tdh* gene, but negative for the *trh* gene and urease (Yamashiro *et al*, 1998). Some strains examined were found to produce pili. Since a variety of pili have been recognized as one of adherent factors of pathogenic bacteria (Gabriel and Hultgren, 1999), the adhesive property may be associated with communicability of the organisms. In the present study, we characterize the pili of *V. parahaemolyticus* O3:K6.

MATERIALS AND METHODS

Bacterial strains

V. parahaemolyticus LVP9 (serotype O3:K6, *tdh* gene positive, *trh* gene and urease negative), isolated from a diarrheal patient in Vientiane, Lao PDR in 1997, was used for the purification of pili. This strain did not produce a filamentous phage, nevertheless some strains of *V. parahaemolyticus* serotype O3:K6 produced the filamentous phage, which was morphologically similar to a pili, have been reported (Nakasone *et al*, 1999). The organisms were cultured in a heart infusion broth supplemented with 3% NaCl at room temperature (25ºC) overnight stationary. Other *V. parahaemolytus* strains stocked in our laboratory, including clinical and environmental isolates, were used when necessary.

Purification of pili

The cultured bacterial cells were harvested by centrifugation and suspended in a small amount of 3%(w/v) NaCl solution. This heavy suspension was agitated in a biomixer to detach pili, and centrifuged at 20,000*g* for 30 minutes. The supernatant was filtered through a 0.45 μm-pore size membrane and the filtrate was salted out with 40% (w/v) saturated ammonium sulfate. The precipitate was suspended in urea-Tris buffer (5 M urea, 50 mM Tris-HCl, pH 8.0) and incubated for 1 hour at room temperature. It was salted out with 30% (w/v) saturated ammonium sulfate, and the precipitate was collected by centrifugation at 10,000*g* for 30 minutes, and resuspended in Tris-HCl buffer (pH 8.0). The suspension was applied to sucrose density gradient [10-60% (w/v) in Tris buffer] centrifugation at 150,000*g* for 1.5 hours. The 0.25 ml-fractions were collected from the top of the centrifuge tube. Each fraction was examined by SDS-PAGE and electron microscopy. Fractions rich in pili were

pooled and dialyzed against Tris buffer. This preparation was regarded as purified pili. For comparison, pili were also prepared from *V. parahaemolyticus* Ha7 (O2:K3) and Na2 (O4:K12) as reported previously (Nakasone and Iwanaga, 1990, 1991, 1992).

Protein assay

The protein content was assayed by using the Bio-Rad protein assay kit with bovine serum albumin as standard.

Electrophoresis

SDS-PAGE was performed by the method described by Laemmli (1970) using 12%(w/v) gel. The isoelectric point of the pilin protein was determined by the method of O'Farrell (1975) using prestained pI markers (Oriental Kobo) as standards.

Electron microscopy

The samples were negatively stained with 4%(w/v) uranyl acetate on carbon coated Formvar grids and observed with a Hitachi H-7500 transmission electron microscope. Organisms adherent to the rabbit intestinal villi were observed with a Hitachi S-2380N scanning electron microscope. The samples were prepared as described previously (Nakasone and Iwanaga, 1987).

Preparation of antibody

A rabbit was immunized by subcutaneous injection with 100 μg of purified pili emulsified in Freund's complete adjuvant and then boosted in every 2 weeks. Specificity of anti-sera was confirmed by Western blotting. The Fab fraction of the antibody was prepared by digesting the IgG fraction of the antisera with papain, and chromatography using SP Sephadex C50 column.

Immunological studies

Western blotting against the purified pili was performed as described by Towbin *et al* (1979). Immunogold staining of the organisms was conducted by using 1: 100 (v/v)-diluted anti-pilus serum and 1:10(v/v)-diluted gold-conjugated goat anti-rabbit immunoglobulin IgG (E-Y Laboratories Inc. CA, USA). The adhesive property of purified pili to the intestinal epithelium was examined by using an immunohistological technique as described previously (Nakasone and Iwanaga, 1990). Briefly, two pieces of the intestine (one treated with purified pili and the other not) were prepared. Paraffin-embedded specimens were sliced and placed on a slide glass. After the paraffin was removed with xylene, the specimen was exposed first to antipilus antibody, then to peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG), and finally to diaminobenzidine tetra-hydrochloride or hydrogen peroxide, depending on the routine technique.

Hemagglutination

The hemagglutination abilities of the purified pili or the organisms were examined using a suspension of human (group A) and rabbit erythrocytes in Krebs-Tris Ringer's buffer (KRT) (128 mM NaCl, 5.1 mM KCl, 1.34 mM $MgSO_4$, $7H_2O$, 2.7 mM $CaCl_2$, 10 mM Tris/HCl buffer pH 8.0) as previously reported (Nakasone and Iwanaga, 1990).

Adhesion and adhesion inhibition test

The adhesive ability of the organisms to the formalin-fixed rabbit intestinal epithelia, under three different conditions, was examined by the MASK method (Nakasone and Iwanaga, 1987). First, the organisms were suspended in KRT buffer, and reacted with the rabbit intestine. Second, the organisms were suspended in the Fab fraction of the anti-pilus antibody or the preimmune antibody, and reacted with the rabbit intestine. Third, a piece of the rabbit intestine was immersed in the suspension of purified pili, and reacted with the intact organisms. The sample was prepared for scanning electron microscopy. The number of adherent organisms was counted in 30 randomly selected scanning electron microscope fields at x 4000 magnification. The adhesion index was expressed as the mean of the number of organisms observed per field.

Amino acid sequence

The N-terminal amino acid sequence of the subunit protein of the pilus was determined by automated Edman degradation on an Applied Biosystems 473A protein sequencer.

Statistical analysis

Data analysis was performed by the chi-square test and the Cochran-Cox test.

RESULTS

Purification of the pili

A long flexible pili with a diameter of about 7 nm was observed on the surface of LVP9 cells

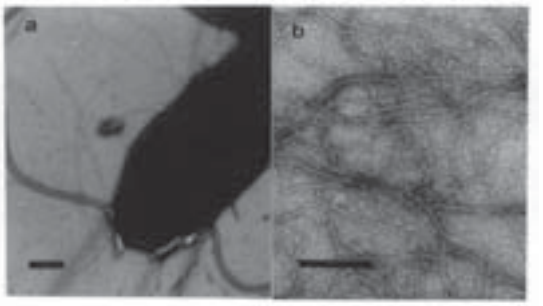

Fig 1–Electron micrograph of negatively stained *V. parahaemolyticus* LVP9 (a) and the purified pili (b). Bar, 200 nm.

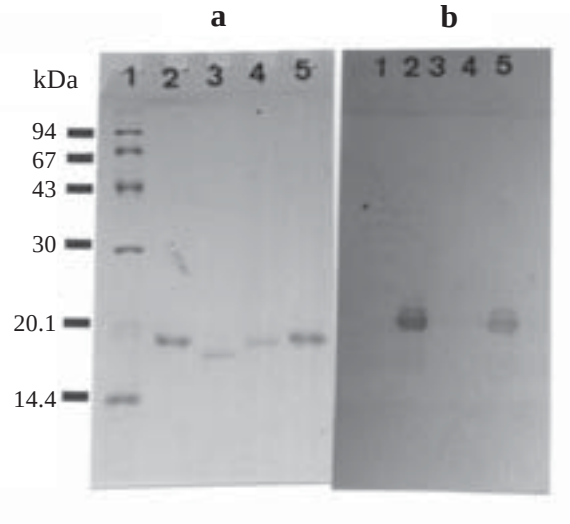

Fig 2–(a) SDS-PAGE profile of *V. parahaemolyticus* LVP9, Ha7 and Na2 pili. Lane 1, molecular weight markers (Pharmacia); lane 2, purified LVP9 pili; lane 3, purified Ha7 pili; lane 4, purified Na2 pili; lane 5, mixture with purified LVP9 and Na2 pili. (LVP9 pili was mixed with Na2 pili to confirm the similar molecular weight of them).
(b) Western blotting of anti-LVP9 pilus antiserum.

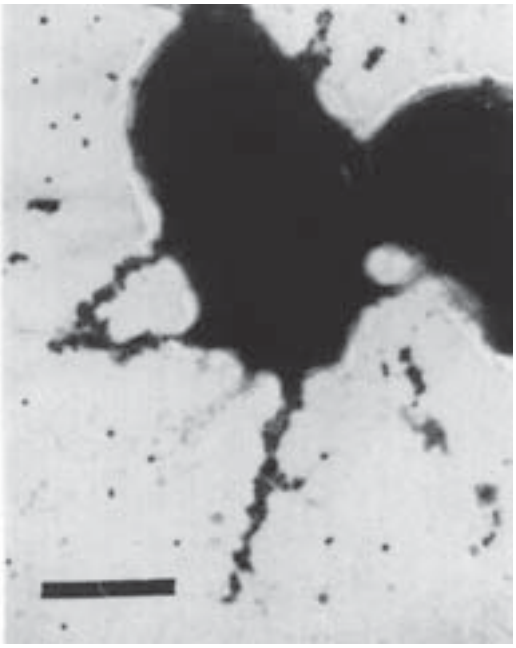

Fig 3–Immunoelectron micrograph of *V. parahaemolyticus* LVP9. The gold particles bound specifically to the pili. The pili reacting with the antibody appear enlarged. Bar, 500 nm.

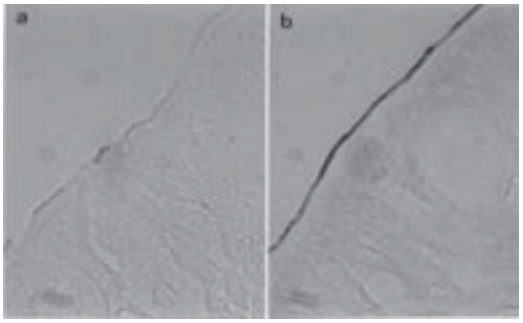

Fig 4–Immunohistochemical findings. (a) Control (untreated) intestine; (b) intestine treated with purified LVP9 pili.

(Fig 1a). The salted-out precipitates contained pili, flagella and small amount of outermembranes. The contaminants were solubilized with 5 M urea, and separated from the pili by sucrose density-gradient ultracentrifugation. The purified LVP9 pili were negatively stained with uranyl acetate (Fig 1b). The purified pili had the same appearance as the pili seen on the cells, and morphologically indistinguishable from *V. parahaemolyticus* Ha7 pili (Nakasone and Iwanaga, 1990) and Na2 pili (Nakasone and Iwanaga, 1991).

Characterization of LVP9 pili

The molecular mass of the LVP9 pilin was estimated to be about 18 kDa in SDS-PAGE, identical to that of Na2 pilin and larger than that of Ha7 pilin (17 kDa). (Fig 2a). Isoelectric point of the pilin was 5.0±0.2. The N-terminal amino acid sequence was determined as XXLIELVVVIVIL GILAVTA (Table 1). The residues were different

Table 1
Molecular masses, pl and N-terminal amino acid sequences of LVP9, Ha7 and Na2 pilins.

Pili	Serotype of bacterial strains	MW (kDa)	N-terminal amino acid sequence	pl	Reference
LVP9	03:K6	18	XXLIELVVVIVILGILAVTA	4.8-5.2	This study
Ha 7	02:K3	17	XXLIELVVVIVILGILAV	ND	Nakasone and Iwanaga, 1990
Na 2	04:K12	18	GTLIELVVVIVVLGIHAA	4.7-4.9	Nakasone and Iwanaga, 1991

The data of *V. parahaemolyticus* Ha7 pili and Na2 pili are presented for comparison.
X: unidentified amino acid residue; ND: not down

from those of the Na2 pilin by three residues, but identical to those of the Ha7 pilin.

Immunological studies

Western blotting of whole cell lysate showed that anti-LVP pilus antiserum reacted with only LVP9 pilin, but not LPS or flagella. Nonimmunized serum did not react with LVP9 pili (data not shown). Anti-LVP9 pilus antiserum did not react with Ha7 pili or Na2 pili (Fig 2b). Anti-Ha7 pilus antiserum was weakly cross-reactive to LVP9 pilin and anti-Na2 pilus antiserum was not cross-reactive to LVP9 and Ha7 pilin. In immunoelectron microscopy, gold particles were observed only on the pili, neither on the flagella nor on the bacterial surface (Fig 3).

Since all O3:K6 isolates examined (36 strains) reacted with anti-LVP9 pilus antiserum, LVP9 pilus-associated antigen was examined in *V. parahaemolyticus* strains other than serotype O3:K6 by Western blotting of whole cell lysate (Table 2). Eleven strains out of 97 examined (11%) reacted with anti-LVP9 pilus antiserum. Clinical and environmental (seawater) isolates of *V. parahaemolyticus* showed positive reactions in 5 out of 70 and 6 out of 27 strains, respectively. The serotypes of the positive strains were O1:K1, O3:K31, K37, O4:K8, K42, and O5:K15. The differences in the detection rates between clinical and environmental isolates was not significant (chi-square test).

Hemagglutination

LVP9 cells and purified pili did not agglutinate human or rabbit erythrocytes.

Adhesive properties

LVP9 organisms adhered to the rabbit intestine (Table 3). The purified pili also adhered to the rabbit intestine, as immunohistochemical analysis showed brown color development along the brush border of the epithelium (Fig 4). The adhesion of the organisms to the intestine was inhibited by pretreating the organisms with the Fab fraction of anti-LVP9 pilus antibody ($p<0.001$; Cochran-Cox test) or by pretreating the intestine with purified pili ($p<0.001$). The Fab fraction of nonimmunized rabbit IgG did not inhibit bacterial adhesion (Table 3).

Table 2
Distribution of strains immunologically cross-reactive with anti-LVP9 pilus antibody in non-03:K6 serotype*.

Genotype		No. of strains	
tdh	*trh*	Examined	Positive (serotype)
+	-	62	1 (O4:K8)
-	+	8	4 (O1:K1)
-	-	27	6 (O3:K31, O3:K37, O4:K42, O5:K15)

*All of O3:K6 isolates examined (36 strains) were reacted to anti-LVP9 pilus antibody. Whole cell lysates were used as an antigen.

DISCUSSION

Previously we reported a filamentous phage of a *V. parahaemolyticus* O3:K6. There were some example that the filamentous phages were mistaken for pili, and vice versa (Karaolis *et al*, 1999; Nakasone *et al*, 1998; Yamashiro *et al*, 1994). Therefore, we have to mention at first that the newly identified pilus in the present study was not a filamentous phage. The findings presented here (amino-terminal amino acid sequence of the subunit protein was highly homologous to that of type IV pili, mo-

Table 3
Adhesion inhibition tests with purified pili or Fab fraction of anti pilus antibody.

Treatment with	Adhesion index (mean ± SD)*	% inhibition	Probability
Pili (μg/ml)			
0	44.8 ± 25.7	0	
200	8.9 ± 10.9	80	<0.001
Anti pilus Fab (myml)			
0	80.5 ± 38.6	0	
2	20.3 ± 19.2	75	<0.001
Control Fab (mg/ml)			
2	65.5 ± 34.6	19	NS

*Numbers of adherent organisms per field (mean±SD of 30 fields). The data are representative of three separate experiments. NS: not significant.

lecular weight was that of ordinary pilin, culture supernatant did not produce plaque on the lawn of indicator strains, and morphological characteristics of filamentous phage, blunt in one end and tapering in another end, were not observed) are enough to say that the filamentous structure mentioned in this study is a pilus and not a phage.

In contrast to already known *V. parahaemolyticus*, the antigen of the newly identified pili was detected in *V. parahaemolyticus* strains with a variety of serotypes.

Although the pili presented here were shown to be a colonization factor as examined by using rabbit intestine, the bacterial adhesion to rabbit intestine was not completely inhibited by blocking pilus function with anti-pilus Fab or by blocking the epithelial receptors with the purified pili. This incomplete inhibition may be due to insufficient amounts of these blocking agents being used, or the presence of non-pilus colonization factors within this organism. An isogenic mutant lacking the pili would clarify this hypothesis.

The whole cells and purified pili adhered to the rabbit intestine without agglutination of human and rabbit erythrocytes, probably because the receptors of enterocytes and erythrocytes are different. However, it is no wonder because this kind of pili, adhesive to the intestinal epithelium but not to erythrocytes, is well documented (Nakasone and Iwanaga, 1992; Yamashiro and Iwanaga, 1996). *Escherichia coli* expressing CFA/I agglutinate erythrocytes but purified CFA/I pili do not, because the adhesive property of CFA/I pili is monovalent (Evans *et al*, 1979). From this viewpoint, LVP9 pili are totally not adhesive to erythrocytes, because the pili rich organisms did not agglutinate erythrocytes. This fact was confirmed by adsorption test in the mixture of erythrocytes and pili (data not shown). Similar results in other *V. parahaemolyticus* pili have been reported (Nakasone and Iwanaga, 1990, 1991).

V. parahaemolyticus is usually not adhesive to the human intestine and so is the O3:K6 strains used in this study (Nakasone and Iwanaga, 1992; Yamamoto and Yokota, 1989), nevertheless serotype O3:K6 has an epidemic potential. Although novel pili of *V. parahaemolyticus* were found in the present study, other factor(s) than pili of this newly recognized O3:K6 strain may contribute to the epidemic potential.

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