***Aeromonas trota* strains, which agglutinate with *Vibrio cholerae* O139 Bengal antiserum, possess a serologically distinct fimbrial colonization factor**

Noboru Nakasone,¹ Masaaki Iwanaga,¹ Tetsu Yamashiro,²
Kazutoshi Nakashima¹ and M. John Albert³

Author for correspondence: Noboru Nakasone. Tel: +81 98 095 3331 ext 2287. Fax: +81 98 895 2951.

^{1,2} Department of Bacteriology¹, and Research Center of Comprehensive Medicine², Faculty of Medicine, University of the Ryukyus, Okinawa 903-01, Japan

³ International Centre for Diarrhoeal Disease Research, Bangladesh GPO Box 128, Dhaka 1000, Bangladesh

Pili of *Aeromonas trota* strain 1220, which agglutinates with *Vibrio cholerae* O139 Bengal antiserum, were purified and characterized. The molecular mass of the subunit protein was estimated to be 20 kDa and the pI was 5.4. The pili were immunologically unrelated to the other *Aeromonas* pili reported so far. However, the N-terminal amino acid sequence of the subunit pilin was similar to those of the pilins from other *Aeromonas* pili reported previously. Neither *A. trota* cells nor pili purified from strain 1220 agglutinated human and rabbit erythrocytes, but both adhered to the rabbit intestine. Bacterial cells pretreated with antipilus antibody (Fab portion) failed to adhere to the rabbit intestine. Moreover, bacteria did not adhere to the rabbit intestine pretreated with the purified pili. This pilus antigen was not detected in *V. cholerae* O139 Bengal and other *Aeromonas* spp. These findings suggest that the pilus of the *A. trota* strain is a novel colonization factor of *Aeromonas* spp.

Keywords: *Aeromonas trota*, pili, colonization factor

INTRODUCTION

Aeromonas spp. have been recognized as important human enteropathogens (Moyer, 1987; Janda, 1991), and a variety of haemagglutinins or colonization factors of these organisms have been reported (Ho *et al.*, 1990; Quinn *et al.*, 1993; Hokama *et al.*, 1990; Hokama & Iwanaga, 1991, 1992; Iwanaga & Hokama, 1992). *Vibrio cholerae* O139 Bengal is a new aetiological agent of cholera which caused large epidemics in the Indian subcontinent in 1992–1993 and spread into neighbouring countries (Albert *et al.*, 1993; Chongsa-nguan *et al.*, 1993). During the epidemic caused by the new cholera organism in Bangladesh, an attempt was made to identify *Aeromonas* spp. cross-reacting with *V. cholerae* O139 and several strains of *Aeromonas sobria* were found to agglutinate with *V. cholerae* O139 antiserum. The cross-reacting antigens resided in the LPS as demonstrated by the reactivity of the purified LPS with antisera in Western blots (Ansaruzzaman *et al.*, 1994). However, the cross-reacting *A. sobria* strains were subsequently identified as *Aeromonas trota* in the laboratory of Dr T. Shimada, National Institute of Health, Tokyo, Japan, by standard tests (Carnahan *et al.*, 1991). Strain 1220 is one of the six isolates which agglutinated with the *V. cholerae* O139 antiserum.

Ansaruzzaman *et al.* (1994) examined the virulence properties of the *Aeromonas* isolates and found that all strains produced a cytotoxin for HeLa cells and adhered to HEP-2 cells. We were interested in characterizing the colonization factor of these unique strains because colonization by pathogens is the first step of the infectious disease process. In this communication, we report the characterization of pili (fimbriae) isolated from one strain.

METHODS

Bacterial strains. *A. trota* strain 1220, isolated from surface water in Bangladesh, was used for the purification of pili. This strain is one of the six isolates which agglutinated with *V. cholerae* O139 antiserum (Ansaruzzaman *et al.*, 1994). The identification characteristics of the isolates included negative reactions for aesculin hydrolysis, arabinose fermentation and the Voges–Proskauer test, positive reactions for cellobiose fermentation, lysine decarboxylation and citrate utilization, and susceptibility to ampicillin (Carnahan *et al.*, 1991). *A. sobria* strains Ae1, Ae24 and TAP13, *A. hydrophila* strain Ae6 and other *Aeromonas* spp. reported previously (Hokama & Iwanaga, 1991, 1992; Iwanaga & Hokama, 1992; Hokama *et al.*, 1990), were used for comparison. *V. cholerae* O1 (El Tor 21, Classical 21), *V. cholerae* O139 and other serotypes of *V. cholerae*, isolated from diarrhoeal patients, were also used when necessary.

Growth conditions. The organisms, subcultured on a nutrient (0.5%, w/v, meat extract; 1%, w/v, peptone; 0.5%, w/v, NaCl) agar plate, were inoculated into 500 ml Trypticase Soy Broth (Difco) in a 3 l Erlenmeyer flask and incubated at 30 °C for 12 h with reciprocal shaking.

Purification of pili. The pili were purified according to the method of Honma & Nakasone (1990) with a slight modification. Briefly, a heavy suspension of the harvested cells in 50 mM Tris/HCl buffer, pH 8.0, was agitated in a biomixer (Nihon Seiki) for 5 min, and centrifuged at 26 000 *g* for 30 min. NaCl and PEG 6000 (Wako Chemical) were added to the supernatant fraction (final concentrations of 5.8%, w/v, and 2%, w/v, respectively). The mixture was incubated for 30 min with stirring, and then centrifuged at 15 000 *g* for 30 min. PEG was further added to the supernatant fraction to make the final concentration 6% (w/v), and the mixture was incubated overnight at 4 °C. Pili-rich material which precipitated was collected by centrifugation at 15 000 *g* for 30 min. The pili were purified by 60–10% sucrose density gradient centrifugation (15 200 *g*, 2 h).

Protein assay. The protein content was assayed using the Bio-Rad protein kit with BSA as the standard.

Electrophoresis. SDS-PAGE was performed by the method of Laemmli (1970) with 12% (w/v) polyacrylamide gels. The isoelectric point of the purified pili was determined by the method of O'Farrell (1975) with prestained pI markers as standards (Oriental Kobo).

Preparation of antibody. Antipilus antiserum was prepared by immunizing Japanese White rabbits. Two doses, each containing 100 µg purified pili protein, were given as multiple subcutaneous injections 2 weeks apart. The first dose was emulsified with Freund's complete adjuvant, the booster dose with incomplete adjuvant. The Fab fraction of antibody was prepared as described previously (Iwanaga & Hokama, 1992).

Haemagglutination test. The haemagglutinating activities of *A. trota* (strain 1220) cells and of the purified pili were examined with human group A and rabbit erythrocytes (Iwanaga & Hokama, 1992).

Adhesion test. The ability of the organisms to adhere to rabbit intestinal epithelium was examined by the MASK method (Nakasone & Iwanaga, 1987). Briefly, formalin-fixed intestine was incubated in the bacterial suspension [about 10^9 cells ml⁻¹ in Krebs-Tris-Ringer's buffer (KRT: 128 mM NaCl, 5.1 mM KCl, 1.34 mM MgSO₄·7H₂O), 2.7 mM CaCl₂, 10 mM Tris/HCl buffer, pH 7.4)] for 10 min at 30 °C, then vigorously washed with KRT buffer. The sample was prepared for scanning electron microscopy. Adherent organisms were counted in 30 randomly selected scanning electron microscope fields at ×4000 magnification. The adhesion index was expressed as the mean of the count per field.

Fig. 1. Transmission electron micrographs of (a) *A. trota* strain 1220 cells showing flexible pili (P) and a flagellum (F) (bar, 500 nm) and (b) purified pili from *A. trota* strain 1220 (bar, 200 nm). (c) Transmission electron micrograph of *A. trota* strain 1220 examined by using the immunogold-labelled Protein A. Cells were incubated with rabbit anti-*A. trota* pilus antiserum and then with Protein A conjugated with 15 nm colloidal gold particles. The gold particles bound specifically along the length of the pili. Note that pili reacting with the antibody appear distended. Bar, 500 nm. Preparations were negatively stained with 4% uranyl acetate.

Adhesion inhibition test. Two kinds of pretreatment were performed before the adhesion test. Briefly, in the first method, the organisms were treated with the Fab fraction of either IgG from nonimmunized rabbits or antipilus antibody at 30 °C for 30 min and then the adhesion test was performed using the intact rabbit intestine. In the second method, intestinal epithelium pretreated in the purified pilus suspension at 30 °C for 30 min to block the receptor was exposed to the bacterial cells (Iwanaga & Hokama, 1992).

Immunological techniques. Western blotting was performed as described by Towbin *et al.* (1979). A 1:200 dilution of antipilus serum was used as the first antibody. Electron microscopic immunogold labelling to confirm the specificity of the antiserum and immunohistochemical examination to detect the adhered pili on the surface of the rabbit intestinal epithelium were performed as described previously by Iwanaga & Hokama (1992). Slide agglutination tests were carried out using agar-grown organisms and antipilus serum to study the distribution of the pilus-associated antigen.

Electron microscopy. *A. trota* cells and purified pili were negatively stained with 4% (w/v) uranyl acetate on carbon-coated Formvar grids and observed with a JEM 2000EX transmission electron microscope. Organisms adherent to the rabbit intestinal villi were observed with a Hitachi S450 scanning electron microscope. The samples were prepared as described previously by Nakasone & Iwanaga (1987).

Amino acid sequence. The N-terminal amino acid sequence of the strain 1220 pili was analysed by automated Edman degradation on a Shimadzu PSQ-1 protein sequencer.

RESULTS

On electron microscopy, *A. trota* strain 1220 was found to have long, flexible pili with a diameter of about 7 nm (Fig. 1a). In the pili purification protocol used, flagella and cell debris were precipitated with 2% (w/v) PEG. In the precipitates obtained with 6% (w/v) PEG, the pili were almost pure. Finally, after sucrose density gradient centrifugation, about 250 µg purified pili was obtained from 2 l culture. Electron microscopy of the negatively stained preparations demonstrated that the purified pili

were of flexible appearance with a diameter of about 7 nm as seen on the cell surface (Fig. 1b).

The subunit protein of the pili (pilin) migrated as a single molecular species in SDS-PAGE. The molecular mass was estimated to be about 20 kDa. Isoelectric focusing revealed the pI to be about 5.4. The N-terminal amino acid sequence of the *A. trota* pilin was highly similar to those of other *Aeromonas* pilins reported previously (Table 1).

The agglutination titre of the antipilus antibody obtained by immunizing rabbits was 1:2048 in a serial test-tube dilution method using live organisms. The antibody was highly specific for the pilin because the antiserum reacted only with the 20 kDa protein of whole-cell lysate as examined by Western blotting. Also in the immunogold electron microscopy analysis, the antibody bound to the pili on bacteria but not to flagella or outer-membrane debris (Fig. 1c).

The distribution of the *A. trota* strain 1220 pilus-associated antigen in other organisms was examined by agglutination and by Western blotting of whole-cell lysates. None of the 103 *Aeromonas* strains (47 *A. hydrophila*, 45 *A. sobria* and 11 *A. caviae*), and none of the 64 *V. cholerae* strains (42 O1, 8 O139 and 14 strains of other serovars) possessed this pili-associated antigen. However, all six strains of *A. trota* which cross-reacted with the *V. cholerae* O139 antiserum were positive for this pili-associated antigen. The other *Aeromonas* pilus antisera which we have previously reported (Hokama & Iwanaga, 1991, 1992; Iwanaga & Hokama, 1992; Hokama *et al.*, 1990) did not react with the 1220 pili.

A. trota cells and purified pili did not agglutinate human group A and rabbit erythrocytes. The live cells of *A. trota* adhered strongly to the rabbit intestine (Fig. 2). The purified pili also adhered to the rabbit intestine as examined by an enzyme-labelled antibody technique (data not shown). Pili-like structures were observed on the organisms attaching to the rabbit intestinal epithelial

Table 1. N-terminal amino acid sequences and molecular masses of *Aeromonas* and other bacterial pilins

The sequences and molecular mass of the pilins of *A. sobria* Ae1 (Hokama & Iwanaga, 1991), TAP13 (Iwanaga & Hokama, 1992) and Ae24 (Hokama & Iwanaga, 1992), *A. hydrophila* Ae6W (Hokama & Iwanaga, 1991), *V. cholerae* TCP (Taylor *et al.*, 1987), *Neisseria gonorrhoeae* (Hermanson *et al.*, 1978) and *Pseudomonas aeruginosa* (Sastray *et al.*, 1983) are presented for comparison. Residues are presented in the one-letter code.

Pilin	N-terminal amino acid sequence	Molecular mass (kDa)
<i>A. trota</i> strain 1220	YTLIELVIVIIIILGILAVTAAPKFL	20
<i>A. sobria</i> Ae1	MTLIELVIVI	23
<i>A. sobria</i> TAP13	YTLIELVIVIIIILGILA	23
<i>A. sobria</i> Ae24	YTLIELVIVIIIILGILADDA	19
<i>A. hydrophila</i> Ae6W	MTLIELVIVI	21
<i>V. cholerae</i> TCP	MeFTLLEVIIVLLGIMGVVSAGVV	20.5
<i>N. gonorrhoeae</i>	MeFTLIELMIVIAIVGILAAIALPAQ	18
<i>P. aeruginosa</i>	MeFTLIELMIVVAIIGILAAIAIPQ	18

Fig. 2. Scanning electron micrograph of *A. trota* 1220 cells adhering to rabbit intestinal epithelium. Preparations were formalin-fixed. Bar, 50 μm .

Table 2. Adhesion and adhesion-inhibition tests of *A. trota* (strain 1220) cells to the rabbit intestinal epithelium

Pretreatment of the intestine or the organisms with:	Adhesion index*
Pili ($\mu\text{g ml}^{-1}$)	
0	119 $\pm$ 60
85	92 $\pm$ 39
170	47 $\pm$ 36
255	53 $\pm$ 41
350	58 $\pm$ 43
Fab portion of antipilus antibody (mg ml^{-1})	
0	131 $\pm$ 55
0.5	104 $\pm$ 37
1	24 $\pm$ 21
3	25 $\pm$ 25
Fab portion of control antibody (mg ml^{-1})	
3	131 $\pm$ 61

* Numbers of adherent organisms per field (mean $\pm$ SD of 30 fields). Control antibody was obtained from preimmune sera of rabbits.

surface (Fig. 2). The adhesion index of this organism was about 130.

The adhesion-inhibition test with pretreatment of the intestinal epithelium with purified pili to block the receptors revealed dose-dependent inhibition; up to a 50% decrease in the adherence of organisms was observed (Table 2). Pretreatment of the *A. trota* cells with antipilus antibody also blocked the adherence of the organisms in a dose-dependent manner; up to an 80% decrease in adherence was observed (Table 2). Both blocking tests

failed to completely inhibit adhesion. The Fab portion of IgG antibody from preimmune sera of rabbits failed to inhibit adhesion.

DISCUSSION

The pili purified from *A. trota* (strain 1220) were identified as a colonizing factor of the strain, but they had no relation to *V. cholerae* O139 components. Previously, we examined the properties of adhesion of *V. cholerae* and *Aeromonas* species to the human intestine and the rabbit intestine and found that they adhered equally well to both intestines (Yamamoto *et al.*, 1988; Hokama & Iwanaga, 1991). In the present study, we have used the rabbit intestine and found that it was a satisfactory system for *A. trota* attachment studies. It is noteworthy that the flexible pili of *Aeromonas* species were all identified as colonization factors (Hokama *et al.*, 1990; Hokama & Iwanaga, 1991; Iwanaga & Hokama, 1992), but the flexible pili of *V. cholerae* were not (Iwanaga *et al.*, 1989; Nakasone & Iwanaga, 1993). Although the N-terminal amino acid sequences of the pilin subunits of *Aeromonas* flexible pili are highly similar, the N-terminal residue is a methylated phenylalanine in type 4 pilin in *V. cholerae*, but it is methionine or tyrosine in *Aeromonas* spp. A unique flexible pilus reported by Ho *et al.* (1990) has a different amino acid sequence. The flexible pili of *Aeromonas* so far reported are distributed in 10–30% of the organisms belonging to the same genus (Hokama *et al.*, 1990; Hokama & Iwanaga, 1991, 1992; Iwanaga & Hokama, 1992). However, the pili of *A. trota* (strain 1220) were found only in the six strains that shared somatic antigen with *V. cholerae* O139. Since *Aeromonas* species have a variety of haemagglutinins (Atkinson *et al.*, 1987) and colonization factors as described above, it may be difficult to develop vaccines using these factors.

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