Characterization of *Aeromonas sobria* TAP13 pili: a possible new colonization factor

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Pili of *Aeromonas sobria* TAP13 were purified and characterized. The molecular mass of the pilin was estimated to be about 23 kDa by SDS-PAGE. The TAP13 pili were immunologically different from *A. sobria* Ae1 pili and *A. hydrophila* Ae6 W 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 TAP13 and its purified pili did not agglutinate human, rabbit or sheep erythrocytes. However, they adhered to rabbit intestine. Organisms pretreated with the Fab fraction of an antipilus antibody failed to adhere to rabbit intestine, and organisms did not adhere to intestine pretreated with purified pili. These results suggest that the pili are a colonization factor of *A. sobria* TAP13 for the rabbit intestine.

Introduction

Aeromonas species are important human enteropathogens (Daily *et al.*, 1981; Moyer, 1987; Janda, 1991). The generally accepted mechanisms by which enteropathogens cause diarrhoeal diseases are initial adhesion to the intestinal epithelium, colonization, and secretion of toxins. Various factors associated with aeromonad virulence, such as enterotoxins, haemolysins, adhesive ability, and invasive ability, have been reported (Cahill, 1990; Pazzaglia *et al.*, 1990; Gray *et al.*, 1990; Ascencio *et al.*, 1991); however, their pathogenic role needs further assessment. Especially, details of the adherence mechanism(s), including identification of colonization factors and their receptors, are not yet fully understood. Various bacterial pili have been shown to be colonization factors (Krogfelt, 1991); therefore, studies on identification of colonization factors have initially focused upon pili. Among *Aeromonas* spp., one type of *A. sobria* pili and five types of *A. hydrophila* pili have been purified so far (Sato *et al.*, 1989; Honma & Nakasone, 1990; Ho *et al.*, 1990; Hokama *et al.*, 1990; Hokama & Iwanaga, 1991). These studies have shown that *A. sobria* Ae1 pili and *A. hydrophila* Ae6 W pili are colonization factors for the intestine as well as haemagglutinins, and demonstrated the possible presence of other colonization factors in

Aeromonas spp. This paper describes the purification and characterization of a type of *A. sobria* pili different from *Aeromonas* pili reported previously, including the adhesive properties of the organism and the isolated pili.

Methods

Bacterial strains. *A. sobria* TAP13, isolated from a patient with diarrhoea in Thailand, was used. *A. hydrophila* Ae6 and *A. sobria* Ae1, reported previously (Hokama *et al.*, 1990; Hokama & Iwanaga, 1991), were used for comparison. Other organisms stocked in our laboratory were used when necessary.

Culture conditions. The organisms, subcultured on a nutrient agar plate, were inoculated into 40 ml tryptic soy broth (TSB, Difco) in a 100 ml Erlenmeyer flask and cultured without shaking at 37 °C for 12 h. A 5 ml portion of this culture was inoculated into 500 ml of fresh TSB in a 3 litre Erlenmeyer flask and subsequently cultured at 37 °C for 12 h with reciprocal shaking.

Purification of pili. The organisms harvested from 2 litres of culture were suspended in 60 ml 50 mM-Tris/HCl buffer, pH 7.4. This suspension was agitated in a Biomixer (Nihon Seiki) for 10 min and was then stirred with a magnetic stirrer for 2 h to detach pili from the cells. After centrifugation at 26000 *g* for 30 min, the supernatant was filtered through a 0.45 µm pore-size membrane. The filtrate was mixed with solid ammonium sulphate (20% saturation). After 1 h incubation at 4 °C, the mixture was centrifuged at 26000 *g* for 30 min. The pellet was suspended in Tris/HCl buffer, and the suspension (0.5 ml) was layered on top of a 9.0 ml sucrose gradient prepared by layering 1.5 ml of 60, 50, 40, 30, 20 and 10% (w/w) sucrose in Tris/HCl buffer. This gradient was centrifuged at 152000 *g* for 16 h at 4 °C. Fractions of 0.2 ml were taken from the top to the bottom; each fraction was

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examined for purity by SDS-PAGE and electron microscopy. Fractions with pili were pooled, concentrated, and dialysed against Tris/HCl buffer. This preparation was regarded as purified pili. For comparison, *A. hydrophila* Ae6 W pili and *A. sobria* Ae1 pili were purified as reported previously (Hokama *et al.*, 1990; Hokama & Iwanaga, 1991).

Electrophoresis. SDS-PAGE was performed by the method of Laemmli (1970). Proteins solubilized in sample buffer were stacked in 4% (w/v) acrylamide and separated in 13% acrylamide.

Electron microscopy. Cultured 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 rabbit intestinal villi were observed with a Hitachi S450 scanning electron microscope. The samples were prepared as described previously (Nakasone & Iwanaga, 1987).

Immunological studies. Western blotting was performed as described by Towbin *et al.* (1979) to determine immunological cross-reactivity among TAP13 pili, Ae1 pili and Ae6 W pili. The agglutination titres of the nonimmunized rabbit serum and the antipilus antiserum were less than $\times 2$ and $\times 1024$, respectively, as examined by the test-tube dilution method; a 1:400 dilution of each serum was used in the reaction to blotted proteins. The specificity of the antiserum was also confirmed by electron microscopic immunogold labelling. Briefly, organisms on Formvar grids were reacted with a 1:100 dilution of the antipilus antiserum, and then exposed to a 1:20 dilution of colloidal gold particles (15 nm) conjugated to protein A (E-Y Labs). The grids were negatively stained with 4% uranyl acetate.

The adhesive properties of purified pili to the rabbit intestinal epithelium were examined immunohistochemically as described previously (Hokama *et al.*, 1990). Briefly, pieces of formalin-fixed intestine were incubated in the purified pilus suspension ($300 \mu\text{g ml}^{-1}$) at 30 °C for 30 min, and then washed vigorously in Krebs-Tris-Ringer 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). As a control, the intestinal sample was incubated in KRT buffer without the pili, and then all intestinal pieces were treated equally. The tissues were prepared for routine histological examination. Immunohistochemical reactions with antipilus rabbit serum, horseradish-peroxidase-conjugated anti-rabbit IgG goat IgG (Organon Teknika), and diaminobenzidine (Sigma) for enzymic colour development were done on glass slides by routine procedures.

The distribution of the pilus-related antigen in 98 other *Aeromonas* strains was examined by the test-tube agglutination test with antipilus antiserum. The strains were cultured in TSB for 12 h at 37 °C with shaking, and resuspended in 10 mM-phosphate-buffered saline (PBS; pH 7.4). A sample of 50 μl of bacterial suspension (about 10^9 cells ml^{-1}) was mixed with 50 μl of a 1:50 dilution of the antiserum in a test tube. The mixture was incubated for 12 h at room temperature. Agglutination was determined visually, using the bacterial suspension plus PBS alone as a control.

Haemagglutination test. The haemagglutinating activities of TAP13 cells and the purified pili were examined with human group A, rabbit and sheep erythrocytes. Equal volumes (25 μl) of 2% (v/v) washed erythrocytes and bacterial cells (about 10^9 cells ml^{-1} in KRT buffer) or purified pili (300 $\mu\text{g ml}^{-1}$ in KRT buffer) were mixed on a glass cavity slide. Agglutination was judged visually within 1 min.

Adhesion test. The ability of the organisms to adhere to rabbit intestinal epithelium was examined by the MASK method (Nakasone & Iwanaga, 1987), which we have used elsewhere (Nakasone & Iwanaga, 1990; Hokama & Iwanaga, 1991). Briefly, formalin-fixed intestine was incubated in the bacterial suspension (about 10^9 cells ml^{-1} in KRT buffer) 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 10 randomly selected scanning electron microscope fields at $\times 4000$ magnification. The adhesion index was expressed as the mean number of adherent organisms per observation field of the intestinal epithelium.

Adhesion inhibition test. Two kinds of pretreatment were performed before the adhesion test as described previously (Nakasone & Iwanaga, 1990; Hokama *et al.*, 1990). Briefly, in the first method, the organisms (about 10^9 cells ml^{-1}) were preincubated with the Fab fraction of either nonimmunized rabbit IgG or antipilus antibody at 30 °C for 30 min before they were reacted with the intestinal epithelium. The Fab fractions were used instead of intact antibody because intact antibody agglutinates the organisms. In the second method, intestinal epithelium pretreated in the purified pilus suspension at 30 °C for 30 min was exposed to the intact organisms.

Preparation of antibody. Antipilus antisera were prepared by multiple subcutaneous injections of approximately 0.1 mg of purified pili emulsified with Freund's adjuvant (Difco) into a Japanese White rabbit and by boosting every two weeks. Several days after each boost, blood was collected and the sera were pooled. Nonimmunized sera were obtained before the first injection. An IgG fraction of the sera was obtained by salting out the pooled sera with 33% saturated ammonium sulphate. The Fab fraction of the IgG was prepared by papain (Sigma) digestion and SP-Sephadex C-50 column chromatography (Pharmacia).

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

Protein assay. The protein content was assayed by the Lowry method, with bovine serum albumin as the standard.

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

Results

Morphological findings

A. sobria TAP13 cells had a small number of long, flexible, wavy pili (designated TAP13 pili) with a diameter of about 7 nm (Fig. 1). TAP13 pili were morphologically indistinguishable from *A. hydrophila* Ae6 W pili (Hokama *et al.*, 1990) and *A. sobria* Ae1 pili (Hokama & Iwanaga, 1991).

Purification of the pili

The salted-out precipitates contained mainly pili and flagella. The flagella and other contaminants were removed by sucrose density-gradient ultracentrifugation. Fig. 2 shows the electron microscopic appearance of negatively stained, purified TAP13 pili. The purified pili had the same appearance as the pili seen on the cells. The molecular mass of the TAP13 pilin was estimated to be about 23 kDa, identical to that of Ae1 pilin and larger than that of Ae6 W pilin (21 kDa) (Fig. 3a).

Fig. 1. Transmission electron micrograph of an *A. sobria* TAP13 cell, negatively stained with 4% uranyl acetate. Wavy, flexible pili are seen. P, pilus; F, flagellum. Bar, 500 nm.

Fig. 2. Transmission electron micrograph of purified pili of *A. sobria* TAP13, negatively stained with 4% uranyl acetate. Wavy, flexible pili with a diameter of about 7 nm are seen. Bar, 200 nm.

Immunological studies

Western blotting showed that TAP13 pilin, but not Ae1 or Ae6 W pilin, reacted with anti-TAP13 pilus antibody

(Fig. 3b). Nonimmunized serum, anti-Ae1 pilus antibody and anti-Ae6 W pilus antibody did not react with TAP13 pilin (data not shown). Specificity of anti-pilus antisera was also confirmed by immunogold staining, which showed gold particles only on the pili, not on the flagella or the bacterial surface (Fig. 4). Pili adherent to the rabbit intestinal epithelium were detected by using enzyme-labelled antibody (Fig. 5). The presence of pili was shown by brown colour development along the epithelial surface (Fig. 5b), which was absent on the control tissue (Fig. 5a).

The distribution of TAP13 pilus-related antigen in various strains of *Aeromonas* spp. is shown in Table 1. Eleven *Aeromonas* strains out of 98 examined (11%) agglutinated with anti-TAP13 pilus antiserum. Clinical and environmental isolates of *A. sobria* showed positive agglutination in 3 out of 16 strains (16%) and 0 out of 26 strains, respectively. The difference between the two detection rates was significant at $P = 0.035$ (chi-square test). Among the 47 *A. hydrophila* strains, 15% of both clinical and environmental isolates showed agglutination.

Haemagglutination

TAP13 cells and purified pili did not agglutinate human group A, rabbit or sheep erythrocytes.

Fig. 3. (a) SDS-PAGE profile of purified *Aeromonas* pili. Lanes: A, molecular mass markers (Pharmacia); B, purified pili of *A. sobria* TAP13; C, purified pili of *A. sobria* Ae1; D, purified W pili of *A. hydrophila* Ae6. (b) Western blot of the proteins in the SDS-PAGE gel treated with anti-TAP13 pilus antibody. Only TAP13 pilin reacted.

Fig. 4. Transmission electron micrograph of *A. sobria* TAP13 labelled by the immunogold technique. Cells were incubated with rabbit anti-TAP13 pilus antiserum and then with protein A conjugated with 15 nm colloidal gold particles. Labelled cells were negatively stained with 4% uranyl acetate. The gold particles bound specifically along the length of the pili. Note that pili reacting with the antibody appear distended. P, pilus; F, flagellum. Bar, 500 nm.

Adhesion and adhesion inhibition test

TAP13 cells had an adhesion index of approximately 70 for rabbit intestine as determined by the MASK method (Nakasone & Iwanaga, 1987). Adhesion of the bacteria to rabbit intestinal villi is shown in Fig. 6. The adhesion index was markedly lowered by pretreating the intestine with purified pili ($P < 0.001$; Cochran-Cox test) or by pretreating the organisms with the Fab fraction of the

anti-pilus antibody ($P < 0.001$). The Fab fraction of nonimmunized rabbit IgG did not inhibit bacterial adhesion (Table 2).

Amino acid sequence

The N-terminal amino acid sequence of TAP13 pilin is shown in Table 3. Comparing it with the sequences of Ae6 W pilin and Ae1 pilin previously reported (Hokama

Fig. 5. Immunohistochemical detection of purified pili of *A. sobria* TAP13 adhering to rabbit intestinal villi. (a) Control intestine (only the pili were removed from the reaction procedure). The section with stained with Mayer's haematoxylin. (b) Adherence of TAP13 pili to the rabbit intestine detected by enzymic colouration along the villus surface. Bars, 30 μ m.

Table 1. Distribution of strains immunologically cross-reactive with anti-TAP13-pilus antibody

Taxon	No. examined	No. positive (%)
<i>A. sobria</i>		
Clinical	19	3 (16)*
Environmental	26	0 (0)
<i>A. hydrophila</i>		
Clinical	20	3 (15)
Environmental	27	4 (15)
<i>A. caviae</i>		
Clinical	6	1 (17)
Total	98	11 (11)

* $P = 0.035$ compared to the detection rate in *A. sobria* environmental isolates (statistics by chi-square test).

& Iwanaga, 1991), there is 90% homology in the first 10 residues. The N-terminal amino acid is tyrosine in TAP13 pilin, whereas it is methionine in the two previously reported pilins.

Fig. 6. Scanning electron micrograph of *A. sobria* TAP13 cells adhering to formalin-fixed rabbit intestinal villi. Bar, 10 μ m.

Table 2. Adhesion and adhesion inhibition tests of *A. sobria* TAP13 cells to the rabbit intestinal epithelium

Pretreatment of the intestine or organisms with:	Adhesion index*
Control†	71 $\pm$ 7
Purified pili (600 μ g ml ⁻¹)	19 $\pm$ 3‡
Anti-pilus Fab (3 mg ml ⁻¹)	11 $\pm$ 2‡
Nonimmunized Fab (3 mg ml ⁻¹)	79 $\pm$ 8

* Values are means $\pm$ SEM for 10 determinations. The data are representative of three separate experiments.

† For the control, no pretreatment was performed before the reaction of the organisms and the rabbit intestine.

‡ $P < 0.001$ compared with the control (statistics by Cochran-Cox test).

Table 3. N-terminal amino acid sequence of *A. sobria* TAP13 pilin

The sequences of *A. sobria* Ae1 pilin and *A. hydrophila* Ae6 W pilin (Hokama & Iwanaga, 1990) are presented for comparison. Residues are presented in the one-letter code.

Pilin	Sequence
<i>A. sobria</i> TAP13	YTLIE LVIVI IILGI LA
<i>A. sobria</i> Ae1	MTLIE LVIVI
<i>A. hydrophila</i> Ae6 W	MTLIE LVIVI

Discussion

Various studies have been carried out to identify colonization factors produced by *Aeromonas* spp., and pilus and non-pilus colonization factors and haemagglutinins have been reported (Atkinson *et al.*, 1987; Nandapalan & Chang, 1989; Ho *et al.*, 1990; Hokama *et al.*, 1990; Hokama & Iwanaga, 1991). Among six *Aeromonas* pili purified so far, we proved that *A. sobria* Ae1 pili and *A. hydrophila* Ae6 W pili were colonization factors for the intestines as well as being haemagglutinins. Ho *et al.* (1990) reported that *A. hydrophila* AH26 flexible pili acted as a haemagglutinin. These pili are classified into two morphological types, straight and flexible. The flexible pili (Ho *et al.*, 1990; Hokama *et al.*, 1990; Hokama & Iwanaga, 1991) were haemagglutinative but the straight ones (Sato *et al.*, 1989; Honma & Nakasone, 1990; Ho *et al.*, 1990) were not. The TAP13 pili studied here are unique, in being morphologically flexible but not haemagglutinative, yet nevertheless adhering to rabbit intestinal epithelium.

TAP13 pili were shown to be a colonization factor of *A. sobria* TAP13 by immunohistochemical study and adhesion inhibition tests. However, 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 have been due to insufficient amounts of these blocking agents being used. Another possibility is the presence of non-pilus colonization factors within this organism. Even if the organism had a non-pilus colonization factor, it would not be a haemagglutinin, because whole cells of TAP13 did not agglutinate erythrocytes.

The haemagglutinating ability of bacteria and isolated pili has been used as a useful screening model for detecting their adhesive ability (Atkinson & Trust, 1980). Although most bacterial pilus colonization factors agglutinate erythrocytes, non-haemagglutinative colonization factors have occasionally been reported. Nakasone & Iwanaga (1990) reported a non-haemagglutinative pilus colonization factor isolated from a *Vibrio parahaemolyticus* strain, Teppema *et al.* (1987) reported one from a *V. cholerae* O1 strain, and Reyes *et al.* (1983) showed some *V. parahaemolyticus* strains in this category. In *Aeromonas* spp., Nishikawa *et al.* (1991) reported that a non-haemagglutinative *A. sobria* strain adhered to INT407 cells. Carrello *et al.* (1988) found that some *Aeromonas* strains adhered to HEp-2 cells without agglutinating human group O erythrocytes and that some of them possessed (flexible) type L pili. Moreover, preliminary work has shown that most non-haemagglutinative *Aeromonas* isolates stocked in our laboratory have adhesive ability (data not shown). Therefore, it is not

surprising that non-haemagglutinative *A. sobria* TAP13 pili play a role in intestinal adhesion. The relationship between haemagglutinating pattern and adhesion index requires further investigation.

Among 98 strains of *Aeromonas* spp. stocked in our laboratory, 52 reacted immunologically with either anti-TAP13, anti-Ae1 or anti-Ae6 W pilus antibody. Whether all these 52 strains have the respective pili has not yet been determined. The other 46 strains, immunologically not reactive to the above antibodies, were also adhesive to the rabbit intestine (data not shown). Therefore, colonization factors for many *Aeromonas* strains have yet to be identified. Further work on the identification of *Aeromonas* pilus and non-pilus colonization factors is required.

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