Purification and characterization of a pilus of a Vibrio cholerae strain: a possible colonization factor.

T Yamashiro and M Iwanaga

Purification and Characterization of a Pilus of a Vibrio cholerae Strain: A Possible Colonization Factor

TETSU YAMASHIRO1* AND MASAAKI IWANAGA1,2
Research Center of Comprehensive Medicine1 and Department of Bacteriology,2 Faculty of Medicine, University of the Ryukyus, 207 Uehara Nishihara, Okinawa 903-01, Japan

Received 21 May 1996/Returned for modification 9 July 1996/Accepted 3 September 1996

A new flexible type of pilus was purified from Vibrio cholerae non-O1, non-O139 strain NAGV14 and characterized. The molecular mass of the pilin was estimated to be 20 kDa, and the antigenicity differed from that of known pili such as toxin-coregulated pili, mannose-sensitive hemagglutinating pili, V10 pili, and AI-1841 pili. The NAGV14 pilus was regarded as a colonization factor because the purified pili adhered to rabbit intestine and adhesion was inhibited by treating the organisms with the Fab fraction of an antipilus antibody. An intestinal receptor blockade using purified pili failed to inhibit adhesion of the organisms. The NAGV14 pilus adhered to the surface of live V. cholerae. An antigen cross-reacting with the NAGV14 pilus was widely and specifically distributed among V. cholerae strains irrespective of serotype and biotype. The amino acid sequence of the pilin was homologous with that of MSHA. The NAGV14 pilus did not agglutinate human and rabbit erythrocytes.

The first step in infection by an enteropathogenic bacterium is attachment to and colonization of the mucosal surface of the intestine. The bacterial element facilitating the attachment to the mucosal surface, adhesin, has been an important target for investigations of pathogenic mechanisms and vaccine development (1, 6).

There have been many reports that bacterial surface appendages, especially pili, are intimately involved in bacterial adherence to and colonization of the intestinal surface. For example, colonization factor antigens of enterotoxigenic Escherichia coli (3, 4), the bundle-forming pilus of enteropathogenic E. coli (13), and a variety of flexible pili in Aeromonas spp. (8, 18) have been identified.

Several types of Vibrio cholerae pili have been isolated, purified, and characterized in efforts to identify a colonization factor. In the infant mouse model, toxin-coregulated pili (TCP; bundle-forming straight type 4 pili) are reportedly involved in colonizing the intestine by the organisms (28) and to be postinfection protective antigens for cholera caused by the classical V. cholerae biotype (20, 27). Mannose-sensitive hemagglutinin (MSHA) pili (flexible type 4 pili) induce protective immunity in experimental cholera induced by an El Tor biotype (20, 21). Core-encoded pili (Cep) (23) and accessory colonization factor (Acf) (22), which are thought to form a pilus structure, are also potential colonization factors. In addition to pili, outer membrane protein OmpU has been reported to be an adhesin (25).

Considering these reports, the colonization factor of V. cholerae is not likely a single protein. Therefore, a cholera vaccine using a single colonization factor protein has yet to be developed. An effective cholera vaccine requires an antigen which not only can induce protective immunity but also is widely distributed among V. cholerae strains irrespective of serotype and biotype. When a commonly distributed colonization factor is obtained, it will significantly contribute to the development of an effective cholera vaccine.

In this report, we describe novel flexible pili of V. cholerae as a colonization factor which is commonly shared by V. cholerae strains.

MATERIALS AND METHODS

Bacterial strains and cultures. Pili were collected from V. cholerae NAGV14 (non-O1, non-O139), isolated from sewage in Thailand in 1985. Other strains stocked in our laboratory were also used to detect antigens that cross-react with NAGV14 pili. NAGV14 was cultured in 100 ml of heart infusion broth (HIB) (Eiken Co., Tokyo, Japan) in a Roux bottle with a large surface/volume ratio, and other strains were cultured in 10 ml of HIB in 100-ml Erlenmeyer flasks at 30°C for 18 h.

Purification of pili. Organisms grown in 24 bottles of HIB were harvested by centrifugation. The pellet was suspended in 30 ml of 0.05 M Tris-HCl (pH 8.0), agitated in a biomixer (model DX-5; Nihon Seiki Ltd., Tokyo, Japan) to detach pili, centrifuged at 8,000 × g for 20 min, then centrifuged at 87,700 × g for 30 min. The supernatant was filtered through a 0.45-μm-pore-size membrane, and the filtrate was 5% saturated with ammonium sulfate. The precipitate was then removed by centrifugation at 16,000 × g for 30 min and 30% saturated with ammonium sulfate. The precipitate was then removed by centrifugation at 16,000 × g for 30 min and 30% saturated with ammonium sulfate. The precipitate was collected by centrifugation at 16,000 × g for 30 min and suspended in an appropriate amount of Tris buffer.

The suspension was dialyzed against the same buffer and fractionated by sucrose density gradient (10 to 60% stepwise gradient) centrifugation (152,000 × g for 18 h). The pellets containing pili were pooled and dialyzed against Tris buffer; the products were regarded as purified pili. Other pili of V. cholerae (TCP [28], MSHA pili [9], V10 pili [31], and AI-1841 pili [30]) for comparative studies were prepared as described elsewhere.

Electrophoresis and Western blotting (immunoblotting). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (14). Isoelectric focusing was carried out as described by O’Farrell (19). Western blotting was performed as described by Towbin et al. (29).

Electron microscopy. Organisms and the purified pili were negatively stained with 4% uranyl acetate on carbon-coated Formvar grids and observed in a JEM 2000 EX transmission electron microscope. Organisms were stabilized on the Formvar grids with 1:40-diluted anti-NAGV14 pilus serum and 1:10-diluted gold-conjugated goat anti-rabbit immunoglobulin G (E-Y Laboratories Inc., San Mateo, Calif.) and then negatively stained. Scanning electron microscopy was performed to detect the adherent organisms on the surface of the intestine as described previously (17).

Cell agglutination test. The distribution of the pilus-associated antigen among several V. cholerae strains was examined in U-bottom microdilution plates. The organisms cultured in HIB were collected and resuspended in physiological saline with 1.5% (ca. 5.0 × 10^9 CFU/ml). Equal volumes of bacterial suspension and 1:20-diluted anti-NAGV14 pilus serum (25 μl of each) were added to the wells, gently shaken on a microvibrator for 1 min, and then incubated for 1 h at room temperature followed by 4°C overnight. Hemagglutinating activities of the organisms and purified NAGV14 pili were examined by using human type A and rabbit erythrocytes. Serial twofold dilutions of bacterial cells (ca. 10^9 cells per ml) were used.
and purified pili (ca. 200 μg/ml) in Krebs-Ringer-Tris (KRT) buffer (128 mM NaCl, 5.1 mM KCl, 1.34 mM MgSO₄, 2.7 mM CaCl₂, 10 mM Tris-hydrochloride [pH 7.4]) were prepared in U-bottom microdilution plates. An equal volume (25 μl) of 2% washed erythrocyte suspension was added to each well, gently mixed, and left at room temperature for 1 h. The hemagglutination titer was defined as the reciprocal of the highest dilution at which hemagglutination was observed.

**Adhesion test.** Adhesion of NAGV14 to the rabbit intestine was examined by the MASK method (16). Briefly, formalin-fixed rabbit intestine was incubated in the bacterial suspension (ca. 3 x 10⁸/ml) for 10 min at 30°C and then vigorously washed with KRT buffer. The sample was prepared for scanning electron microscopy. The adhesion index was expressed as the average number of adherent organisms in one field of the intestinal epithelium at a magnification of x4,000. Adhesion of the purified pili to the rabbit intestinal epithelium was examined immunohistochemically. Briefly, a piece of formalin-fixed intestine was incubated in the pilus-KRT buffer suspension (ca. 400 μg/ml) at 30°C for 30 min and then washed vigorously in KRT buffer to remove nonadherent pili. A control specimen was incubated in KRT buffer without pili. The specimens were processed by routine histological means and prepared on glass slides. All immunohistochemical reactions were performed on sliced specimens on glass slides. At first, the specimens were exposed to hydrogen peroxide and bovine serum albumin to block endogenous peroxidase and nonspecific reactions. The specimens were then treated with antipilus rabbit sera, biotinylated anti-rabbit immunoglobulin, streptavidin-conjugated horseradish peroxidase, and finally diaminobenzidine for color development.

**Adhesion inhibition test.** Two inhibition tests were performed. In the first, the organisms were treated with the Fab fraction of antipilus antibody before reaction with the intestinal epithelium (pili block). The Fab fraction was used instead of whole antibody to avoid cell agglutination. In the second procedure, intestinal epithelium was treated with the purified pili to block the receptors before testing the adhesion of the organisms.

**Preparation of antisera.** A rabbit was immunized subcutaneously with 100 μg of purified pili emulsified in Freund’s complete adjuvant and then boosted every 2 weeks.

**Amino acid sequence.** The N-terminal amino acid sequence of the subunit...
protein of the pilus was determined by means of automated Edman degradation on a Shimadzu PSQ-1 protein sequencer (Shimadzu Co., Kyoto, Japan).

RESULTS

Purification of pili. A few long, flexible pili were expressed on the surface of NAGV14 cells together with a short, rigid pilus (Fig. 1A). After removal of various structural proteins such as flagella and outer membrane debris from the crude pilus sample by salting out with 5% saturated ammonium sulfate, the flexible pili were concentrated in the sediment of 30% saturated ammonium sulfate. The concentrated pili were fractionated by sucrose density gradient centrifugation.

FIG. 3. Immunoelectron micrograph of V. cholerae non-O1, non-O139 (NAGV14). The gold particles bound specifically to the pili. The pili reacting with the antibody appear enlarged. P, pili; F, flagella. Bar, 500 nm.

FIG. 4. Scanning electron micrograph of V. cholerae non-O1, non-O139 NAGV14 cells adhering to rabbit intestinal epithelium. An aggregative adherence was frequently observed (arrows).
and recovered from fractions containing 20 to 40% sucrose. The purified flexible pili were named NAGV14 pili (Fig. 1B).

Electrophoresis and immunological tests. The SDS-PAGE profile of the purified NAGV14 pili revealed a single molecular species of about 20 kDa (Fig. 2A). In Western blotting using anti-NAGV14 pilus serum, only NAGV14 pili, not TCP, MSHA pili, V10 pili, and Al-1841 pili, reacted (Fig. 2B). The isoelectric focusing point of the NAGV14 pilus protein ranged from 4.8 to 5.1. Immunogold staining with anti-NAGV14 pili and cultured cells revealed gold particles specifically on the flexible pili (Fig. 3).

Adhesion and adhesion inhibition tests. The organisms adhered to the intestinal epithelium with an adhesion index of approximately 300 by the MASK method. An aggregative adherence was frequently observed (Fig. 4). The purified pili also adhered to the intestine, as immunohistochemical analysis showed brown color development along the brush border of the epithelium (Fig. 5B). Adhesion inhibition by blocking pili with antipilus Fab was dose dependent. The adhesion index was definitely lowered by treating the organisms with Fab, but adhesion was not completely inhibited even at an Fab concentration of 8 mg/ml. Anti-NAGV14 Fab did not inhibit the adherence of *V. cholerae* O1 El Tor (strain 82P7). The receptor block with the purified pili failed to inhibit cell adhesion. On the contrary, the adhesion index increased, although not statistically significantly, after treatment of the intestine with the purified pili (Table 1).

Distribution of NAGV14 pilus antigen. All 165 *V. cholerae* strains, regardless of serotype and biotype, had a 20-kDa protein reacting to anti-NAGV14 pilus serum as examined by Western blotting. *Aeromonas* spp., *Vibrio parahaemolyticus*, and *E. coli* did not possess the antigen cross-reacting with NAGV14 pili. The antiserum agglutinated 57% of *V. cholerae* O1 strains (6 of 23 classical and 22 of 26 El Tor strains), 97% (32 of 33) of O139 strains, and 76% (37 of 49) of non-O1 strains (Table 2).

Hemagglutination test. NAGV14 cells agglutinated human type A and rabbit erythrocytes, but the purified pili did not.

Amino acid sequence. The N-terminal amino acid sequence of the NAGV14 pilus protein was determined up to residue 48. The first 36 residues were identical to those of MSHA pilin of *V. cholerae*, although residues 37, 40, 43, 44, and 47 were different from those of MSHA pilin (Fig. 6). Considering the fact that the adhesion was not inhibited by blocking the epithelial receptor with the purified pili, the interaction between purified pili and organisms was additionally examined. NAGV14 live organisms or human erythrocytes
were added to the pilus suspension and incubated at 37°C for 30 min. Then the mixtures were centrifuged, and the supernatants were examined by SDS-PAGE to detect pil. The pil remained in the supernatant of the mixture with erythrocytes but disappeared from the supernatant of the mixture with live organisms (Fig. 7).

**DISCUSSION**

Colonization of the intestine is a phenomenon in which organisms enter the intestine, adhere to the epithelium, proliferate, and remain there (1). Adhesion of the organisms to the intestinal epithelium mediated by pil can be proven by confirming that (i) adhesion is inhibited by treating the organisms with the Fab fraction of antipilus antibody (pilus block), (ii) adhesion is inhibited by treating the organisms with the Fab fraction of antipilus antibody (pilus block), and (iii) purified pil adhere to the intestinal epithelium.

Among the various pil of *V. cholerae*, TCP and MSHA pil are thought to be colonization factors of the organisms, but whether they mediate adhesion has yet to be elucidated on the basis of the above criteria. We have purified and characterized a variety of pil from *Vibrio* and *Aeromonas* spp., and all flexible pil of *Aeromonas* spp. mediated adhesion of the organisms to the intestinal epithelium (7, 8, 18). In contrast, all *V. cholerae* pil that we isolated were not involved in adhesion (9, 17, 31).

The NAGV14 pilus described here was the first *V. cholerae* pilus to be identified as a mediator of the organisms. However, the reason why blocking of the epithelial receptor with purified pil failed to inhibit adhesion should be explained. According to the absorption tests, the purified NAGV14 pil adhered to *V. cholerae* organisms but not to erythrocytes. Therefore, the pil that bound to the receptors simultaneously attached to bacterial surfaces and helped them remain on the intestinal surface. Thus, the pil that adhered to the epithelial receptors also adhered to the organisms; therefore, the receptor block apparently failed to inhibit adhesion of the organisms. Besides being a colonization factor, the NAGV14 pil may also play a role in bacterial aggregation, like the rope-like structure resembling bundle-forming pil of enteropathogenic *E. coli* that links those organisms (5). Blocking the pil with antipilus Fab did not completely inhibit adhesion, suggesting that the amount of Fab was not sufficient to block all adhesives sites of the pil or that there is another adhesion factor.

All *V. cholerae* strains examined were positive for a 20-kDa protein cross-reacting with NAGV14 pilus antigen, as determined by Western blotting, whereas *Aeromonas* spp. and *E. coli* were not. Among members of the genus *Vibrio*, *V. vulnificus* and *V. alginolyticus* had a cross-reactive antigen in the same analysis, but the molecular weight was lower than that of the NAGV14 pil (data not shown). Thus, the 20-kDa protein cross-reacting with the NAGV14 pil is likely to be common among and specific to *V. cholerae* strains. The cell agglu-
tination of most *V. cholerae* strains with the anti-NAGV4 pilus serum was generally much weaker than that of NAGV4 cells, and there were many agglutination-negative strains, especially among classical *V. cholerae* O1 strains. The adhesion of a *V. cholerae* O1 El Tor strain (82P7) to the intestinal epithelium was not inhibited by anti-NAGV4 pilus antibody (Fab). Expression of NAGV4 pilin in the other strains of *V. cholerae* has yet to be elucidated.

The originally described type 4 pilins from microorganisms like *Pseudomonas aeruginosa* (11) and Neisseria gonorrhoeae (15) have almost complete identity at the amino-terminal amino acids. In contrast, *V. cholerae* Tcp (24) and *E. coli* bundle-forming pilin (2) are more divergent. One recent approach used by several workers has been to classify the original type 4 pilins as type 4A and the divergent ones as type 4B (24, 26). In this classification scheme, NAGV4 pilin would be classified as type 4B.

We consider that NAGV4 pilin are a novel type of pilin different from MSHA pilin because they differ in molecular weight and antigenicity from MSHA, the structural subunit of MSHA pilin (12). Furthermore, NAGV4 pilin lack hemagglutinating activity, which is inherent to MSHA pilin, and are involved in receptor binding of *V. cholerae* (12). The N-terminal amino acid sequences have been determined for several of the flexible pilins of *V. cholerae*, and they all seem to have the characteristics of type 4 pilin (10, 12, 31). The type 4 pilus protein is reported to possess a conservative N-terminal amino acid region (26). Therefore, it is not strange that MSHA, a type 4 pilus protein, shares the conservative region with NAGV4 pilin, particularly in the N-terminal amino acid region. Differences between the amino acid sequences at and after residue 37 may contribute to differences in antigenicity and hemagglutinating properties between the two types of pili. Further analysis of the sequence is needed.

Jonson et al. determined the DNA sequences of four open reading frames encoding type 4 pilin-like proteins, one of which was MSHA (12). The amino acid sequence of the NAGV4 pilus subunit protein was not deduced from the sequences of three other open reading frames.

REFERENCES


Editor: P. E. Orndorff