INFECTION AND IMMUNITY, Jun. 1990, p. 1640–1646
0019-9567/90/061640-07$02.00/0

Vol. 58, No. 6

Pili of *Vibrio cholerae* Non-O1

NOBORU NAKASONE[1]* AND MASAAKI IWANAGA[1,2]

Department of Bacteriology[1] and Research Institute of Comprehensive Medicine,[2] University of the Ryukyus School of Medicine, 207 Uehara, Nishihara, Okinawa 903-01, Japan

Received 20 July 1989/Accepted 15 February 1990

Pili of *Vibrio cholerae* non-O1 strain S7 were purified and characterized. The pili of S7 were morphologically, electrophoretically, and immunologically (as far as polyclonal antibody was used) indistinguishable from the 16-kilodalton pili of *V. cholerae* O1 strain 82P7. The purified pili and organisms had D-mannose- and L-fucose-resistant hemagglutinin. The hemagglutinating activity of the purified pili was inhibited by the Fab fraction of antipilus antibody, but the hemagglutinating activity of live organisms was not inhibited completely. The purified pili or Fab fraction of antipilus antibody did not inhibit the adhesion of *V. cholerae* non-O1 to rabbit intestines. Therefore, the pili were not regarded as a colonization factor of *V. cholerae* non-O1. A total of 148 *V. cholerae* non-O1 and O1 clinical isolates were screened for the presence of S7 pili by using an agglutination test with anti-S7 pilus serum; 12 of 49 *V. cholerae* non-O1 strains and 25 of 99 *V. cholerae* O1 strains were positive for agglutination. These agglutination reactions were not correlated with adhesion of the organisms to intestines.

Vibrio cholerae non-O1 is recognized as a causative agent of diarrheal disease in humans (13). Colonization of the small intestine by this pathogen is the initial step in the infectious process (5). However, little is known about colonization by *V. cholerae* non-O1. In contrast, marked advances in the study of the toxins produced by *V. cholerae* non-O1 have occurred. A cholera-toxin-like enterotoxin (23), an El Tor-like hemolysin (22), a heat-stable enterotoxin similar to the heat-stable enterotoxin of enterotoxigenic *Escherichia coli* (7), and a heat-stable hemolysin similar to the thermostable direct hemolysin of *Vibrio parahaemolyticus* (7) have been described, and these compounds are suspected to be involved in the pathogenicity of *V. cholerae* non-O1.

The pili of many pathogenic bacteria have been identified as colonization factors (3, 4, 11, 18, 19). Pili of *V. cholerae* O1 have been purified and characterized by Taylor et al. (20), Ehara et al. (2), and Iwanaga et al. (9). The protein subunit of the pili described by Taylor et al. had a molecular weight of 20,500, and the pili were clearly identified as a colonization factor (20.5-kilodalton [kDa] pili). The pili described by Ehara et al. and Iwanaga et al. had a molecular weight of 16,000, but they have not yet been proven to be a colonization factor (16-kDa pili). These 20.5- and 16-kDa pili are morphologically different from each other.

Honda et al. found pili on the cell surface of *V. cholerae* non-O1 which had high hydrophobicity and suspected that these pili are a colonization factor (8). These pili had an appearance similar to that of the 16-kDa pili of *V. cholerae* O1. In this paper we describe the purification and characterization of *V. cholerae* non-O1 pili and compare them with the 16-kDa pili of *V. cholerae* O1.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *V. cholerae* non-O1 strain S7, which was isolated from a patient with cholera-like symptoms in Sudan in 1968, was used. *V. cholerae* O1 strain 82P7 (El Tor, Ogawa), which was isolated from a cholera patient in The Philippines in 1982, was used for the comparative study. Other *V. cholerae* non-O1 and O1 clinical isolates stocked in our laboratory were also used, as necessary. The organisms, which were grown on plate agar medium, were inoculated into 40-ml portions of heart infusion broth (Eiken Co., Tokyo, Japan) in 100-ml Erlenmeyer flasks and cultured with no shaking at 37°C for 15 h. A 5-ml portion of each culture was transferred to 400 ml of fresh heart infusion broth in a 3-liter Erlenmeyer flask, and the organisms were cultured at 37°C for 4 h with reciprocal shaking.

Purification of pili. The cell pellet collected from each culture fluid (1,600 ml) was suspended in 30 ml of 0.05 M Tris hydrochloride (pH 8.0) and vigorously agitated in a Biomixer (Nihon Seiki Co., Tokyo, Japan) at dial setting 60 for 5 min to detach the pili from the cells. The agitated suspension was centrifuged at 10,000 × *g* for 20 min, and then the supernatant was subjected to high-speed centrifugation at 67,400 × *g* (30,000 rpm; Hitachi model RP50-2 rotor) for 30 min. The supernatant was filtered through a 0.45-μm-pore-size membrane, and the filtrate was dialyzed against 0.1 M acetate buffer (pH 4.0) for a few hours until acid precipitation was observed. The precipitate was collected by centrifugation at 10,000 × *g* for 20 min and was suspended in 5 M urea in 0.05 M Tris hydrochloride (pH 8.0), and the resulting preparation was incubated for 2 h at 37°C. After incubation, 0.1 volume of a saturated ammonium sulfate solution was added, and the solution was incubated at 37°C for 30 min. Finally, the preparation was centrifuged at 12,000 × *g* for 30 min. The sediment was dialyzed against the Tris buffer, and this preparation was regarded as the purified pilus preparation. The pili obtained from 1,600 ml of culture were suspended in 0.3 ml of 0.05 Tris hydrochloride buffer (pH 8.0) and stored in a freezer.

Electron microscopy. The organisms grown in heart infusion broth were placed directly on a carbon-coated Formvar grid which was negatively stained with 4% (wt/vol) uranyl acetate. The preparations were observed with a JEM model 2000EX transmission electron microscope. Electron microscopy of the purified pili was also performed. To examine the binding of antibodies to the pili, bacteria on Formvar grids were incubated in 1% bovine serum albumin for 30 min and then incubated in a drop of antipilus serum for 30 min at room temperature. The grids were then washed several

* Corresponding author.

times in normal saline solution and reacted with gold colloid (diameter, 15 nm)-conjugated protein A (E-Y LABS, Inc., San Mateo, Calif.) diluted 1:5. The grids were then washed several times and finally stained with uranyl acetate as described above.

For scanning electron microscopy, an intestine reacted with the organisms was fixed with 2.5% glutaraldehyde and 2% tannic acid in KRT buffer (128 mM NaCl, 5.1 mM KCl, 1.34 mM $MgSO_4 \cdot 7H_2O$, 2.7 mM $CaCl_2$, 10 mM Tris hydrochloride; pH 8.0) (10) for 2 h at room temperature and then postfixed with 1% osmium tetroxide for 2 h at room temperature. The fixed samples were dehydrated by stepwise replacement of the water with acetone. The specimens were then dried in a Hitachi model CPDIIB critical-point drying apparatus and coated with gold-palladium. They were then observed by using a Hitachi model S450 scanning electron microscope.

Electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was carried out as described by Laemmli (12), with a 12% gel. Samples were heated at 100°C for 5 min in the presence of 1% (wt/vol) SDS. Polyacrylamide gel isoelectric focusing (7%) with a pH gradient ranging from 3.5 to 10 was carried out as described by O'Farrell (16). Cytochrome *c* and its acetylated derivatives (Oriental Yeast Co., Tokyo, Japan) were used as pI marker proteins. The isoelectric focusing point was determined from the standard curve obtained with marker proteins or with the actual pH values on gels.

Adhesion and adhesion inhibition tests. The adhesion test was carried out by using the MASK method (14). Briefly, bacteria cultured in heart infusion broth and harvested by centrifugation were suspended to a density of ca. 10^9 cells per ml in KRT buffer (pH 8.0), and a piece of Formalin-fixed rabbit intestine was immersed in the bacterial suspension. This was followed by incubation at 30°C for 10 min. After being washed with KRT buffer, the intestine was processed for scanning electron microscopy as described above. The procedure used for the inhibition test was the same as that used for the adhesion test except that we used intestine treated with purified pili or organisms treated with antibody (Fab fraction). In the first method, intestine pretreated in the pilus suspension (0.5 mg/ml) was exposed to the intact organisms at 30°C for 30 min. In the second method, organisms (10^9 cells per ml) suspended in KRT buffer containing 2.5 mg of the Fab fraction of antipilus antibody per ml were used.

Hemagglutination test. Human group A erythrocytes were used for hemagglutination. A bacterial suspension (10^9 cells per ml in KRT buffer) and purified pili (500 μg/ml in KRT buffer) were used as agglutinins. Both agglutinins were serially diluted twofold in 96-well microdilution plates (Becton Dickinson and Co., Paramus, N.J.). An equal volume (25 μl) of 1% washed erythrocytes was then added. The plates were incubated at room temperature for 1 h and then at 4°C overnight. The hemagglutination titer was defined as the reciprocal of the highest dilution at which hemagglutination was observed. Hemagglutination inhibition by D-mannose and L-fucose was examined in the same way, except that the erythrocytes were suspended in buffer containing 1% (wt/vol) sugar. Hemagglutination inhibition by the Fab fraction of antipilus antibody was examined by using bacterial cells or purified pili suspended in buffer containing 2 mg of Fab per ml.

Antiserum. Antipilus antibody was obtained by immunizing rabbits which weighed 2 to 2.5 kg each. A 100-μg portion of purified pili (0.5 ml) mixed with the same volume of Freund complete adjuvant was injected subcutaneously at multiple sites, and booster injections were administered every week. Several days after each boosting, 10 to 20 ml of blood was removed from the ear vein of each rabbit.

Preparation of Fab fraction. Antipilus immunoglobulin was obtained from the sera by salting out with 33% saturated ammonium sulfate. The immunoglobulin was digested with papain (Sigma Chemical Co., St. Louis, Mo.), and Fab fragments were collected by using DEAE-Sephadex column chromatography.

Immunological examination. Western blotting (immunoblotting) and the immunoprecipitation test were carried out as described by Towbin et al. (21) and Ouchterlony (17), respectively. The pili were solubilized with 0.2% SDS for the precipitation test. The slide agglutination test to determine the distribution of immunologically cross-reactive strains was carried out by using organisms cultured on agar plates and antipilus sera. The ability of purified pili to adhere to the intestinal epithelium was examined by using an immunohistological technique after the intestine was incubated in the pilus suspension (15).

Protein assay. Protein content was assayed by the method of Bradford (1), with bovine serum albumin as the standard.

RESULTS

Morphological findings. *V. cholerae* non-O1 strain S7 cells had a small number of long, thin, curved pili (Fig. 1A). The diameter of these pili was 7 to 8 nm, and the length was up to 2 μm. These pili were morphologically indistinguishable from the 16-kDa pili of *V. cholerae* O1 (Fig. 1B). The purified pili had essentially the same appearance as the pili seen on the cells (Fig. 2).

Electrophoresis and immunological properties. Purified pili from *V. cholerae* non-O1 strain S7 produced a single band on SDS-PAGE gels. The molecular weight was estimated to be 16,000, which was the same as the molecular weight of the pili of *V. cholerae* O1 (Fig. 3A). In Western blotting, the protein bands of both types of pili reacted with the anti-S7 pilus antibody to the same extent (Fig. 3B). A single, fused precipitin line developed with the pili of *V. cholerae* non-O1 and the pili of *V. cholerae* O1 against each antiserum (Fig. 3C). In isoelectric focusing, both types of pili (from *V. cholerae* non-O1 and *V. cholerae* O1) focused at pH values ranging between 4.2 and 4.9. In immunoelectron microscopic observations, gold particles were observed only on the pili (pili reacted with the antibody looked fat), not on the flagella or bacterial surface (Fig. 4).

Hemagglutination test. The purified pili of *V. cholerae* non-O1 had almost the same hemagglutinating activity as the 16-kDa pili of *V. cholerae* O1, which was D-mannose and L-fucose resistant (Table 1). Agglutination by the purified pili was completely inhibited by the addition of Fab fragments of antipilus antibody. However, the Fab fragments did not completely inhibit agglutination by the live organisms.

Adhesion and adhesion inhibition tests. *V. cholerae* non-O1 strain S7 and O1 strain 82P7 adhered to villus surfaces with adhesion indices of more than 200 (Table 2). Adhesion was not blocked by pretreatment of the intestines with purified pili or by pretreatment of the organisms with antipilus Fab fractions by using homologous pili or antibody (Table 2). The purified pili of strains S7 and 82P7 did not adhere to the intestinal epithelium.

Distribution of strains that were immunologically cross-reactive with anti-S7 pilus antibody. A total of 148 clinical isolates of *V. cholerae* (49 non-O1 strains and 99 O1 strains)

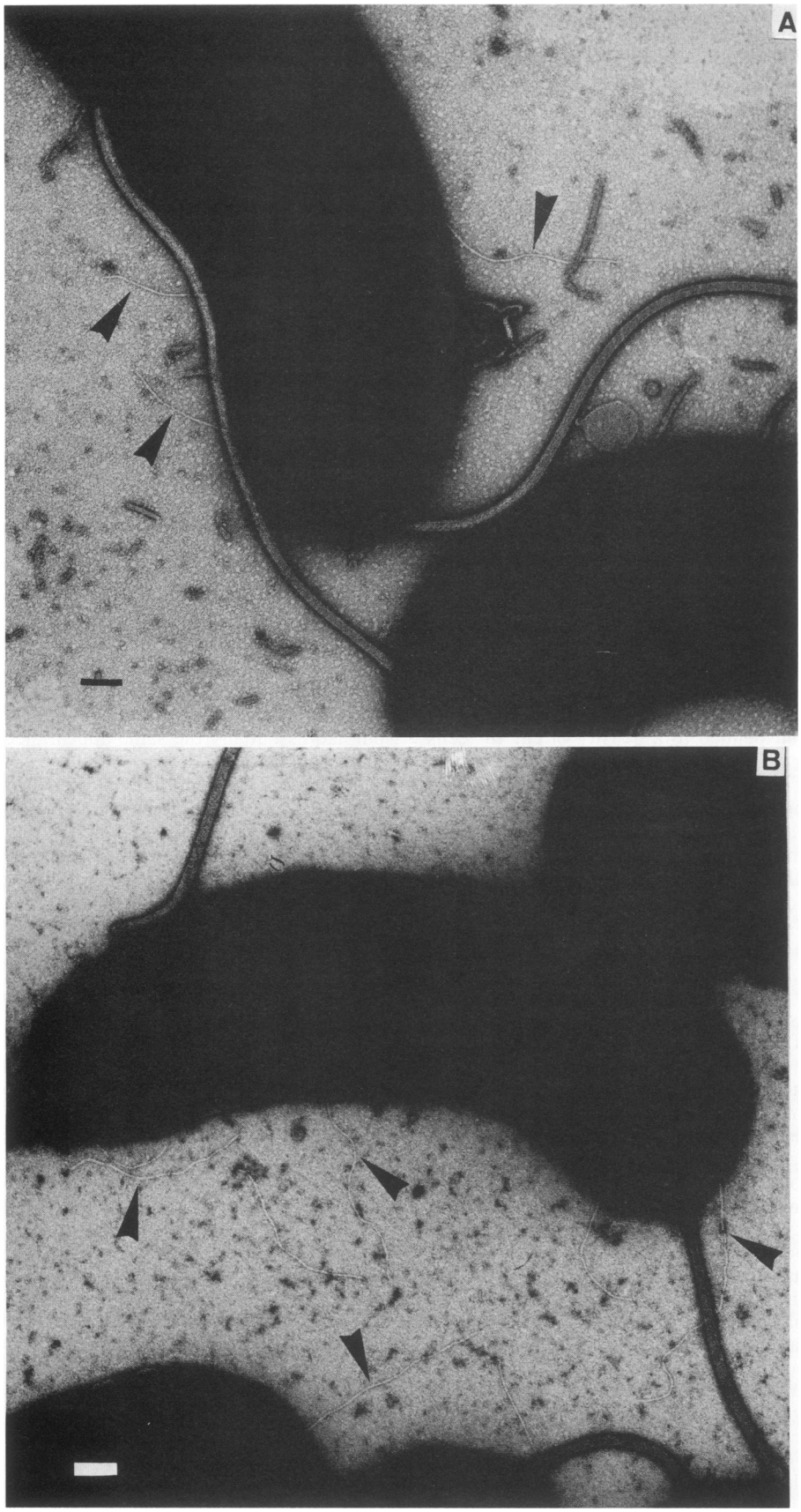

FIG. 1. Electron micrographs of negatively stained *V. cholerae* non-O1 strain S7 (A) and *V. cholerae* O1 strain 82P7 (B). Arrowheads indicate the pili. Bars = 100 nm.

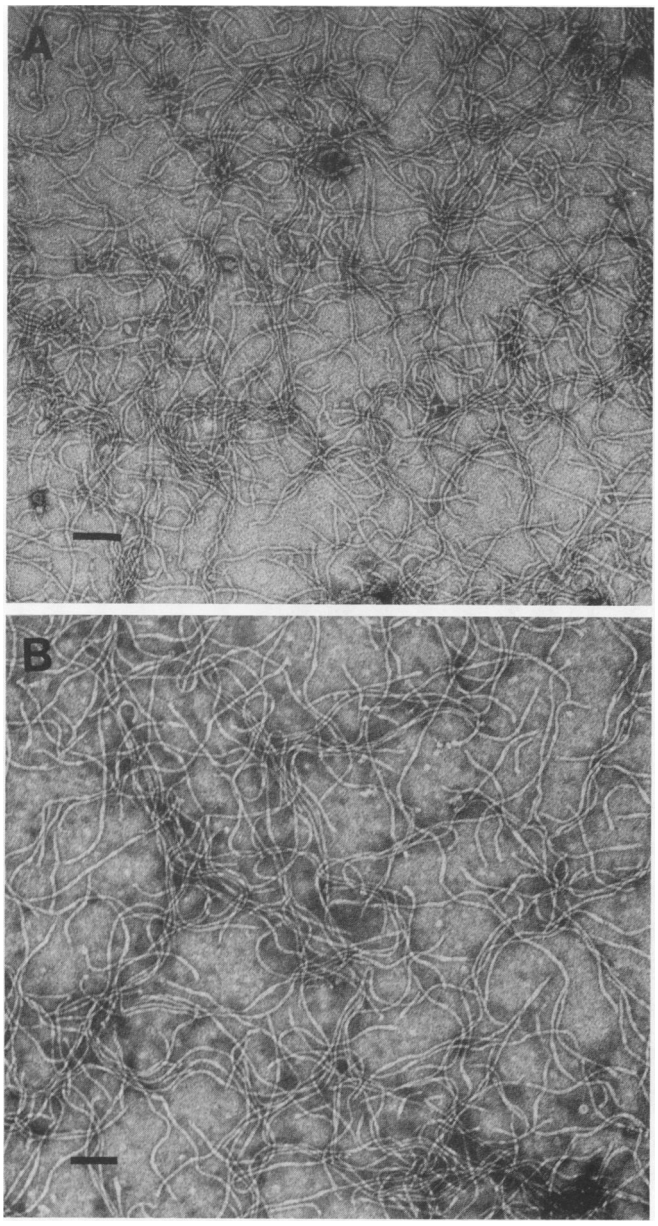

FIG. 2. Electron micrographs of purified pili from strains S7 (A) and 82P7 (B). Bars = 100 nm.

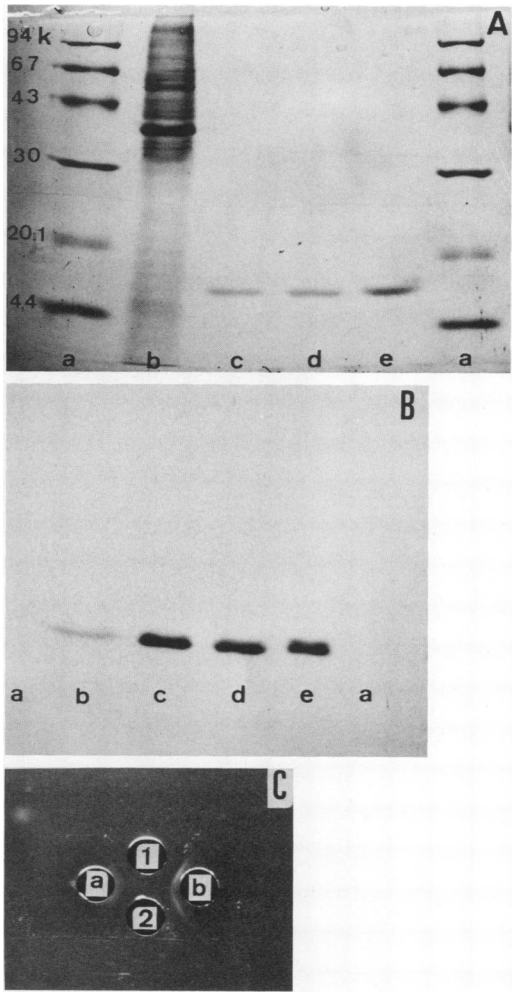

FIG. 3. SDS-PAGE profile (A), Western blot of the SDS-PAGE gel prepared with anti-S7 pilus antibody (B), and agar gel double immunodiffusion test of strain S7 and 82P7 pili with anti-S7 pilus and anti-82P7 pilus sera (C). (A and B) Lanes a, Molecular weight markers (Pharmacia Fine Chemicals, Piscataway, N.J.); lanes b, strain S7 whole cells; lanes c, purified pili of strain S7; lanes d, purified pili of strain 82P7; lanes e, mixed pili of strains S7 and 82P7. (C) Well a, Anti-S7 pilus serum; well b, anti-82P7 pilus serum; well 1, S7 pili; well 2, 82P7 pili. k, Kilodaltons.

were tested for agglutination against anti-S7 pilus antibody; 12 of 49 *V. cholerae* non-O1 strains (24%) and 25 of 99 *V. cholerae* O1 strains (25%) were positive for agglutination against this antibody (Table 3). The agglutination frequency for *V. cholerae* O1 El Tor strains was 36% (18 of 50 strains), and the agglutination frequency for classical strains was 14% (7 of 49 strains). This agglutination had nothing to do with the ability of these organisms to adhere to intestines (Table 4).

DISCUSSION

Bacterial pili have received much attention because of their adhesiveness to the epithelia of host organs. The relationship between pili and colonization factors of enteropathogens has been intensively investigated, especially for enterotoxigenic *E. coli*, and many kinds of pili have been identified as colonization factors (3, 4, 11, 18, 19).

The pili of potently pathogenic *V. cholerae* non-O1 strain S7 were purified and characterized in this study. The molecular weight of the purified pili was estimated to be about 16,000 by SDS-PAGE; this value was the same as that reported previously for *V. cholerae* O1 pili by Ehara et al. and Iwanaga et al. (2, 9). Tests for immuno-cross-reactivity between *V. cholerae* non-O1 strain S7 pili and *V. cholerae* O1 El Tor strain 82P7 pili proved that there is a very close relationship. In the agar gel double immunodiffusion test, strain S7 and 82P7 pilus proteins raised a single fused precipitin line against each antiserum. If the pili have some epitopes that are independent from each other along with the common epitopes, spur formation within the precipitin line should have been seen; however, no spur formation was observed. Therefore, the results obtained from immunoblotting and Ouchterlony tests suggested that the two types of

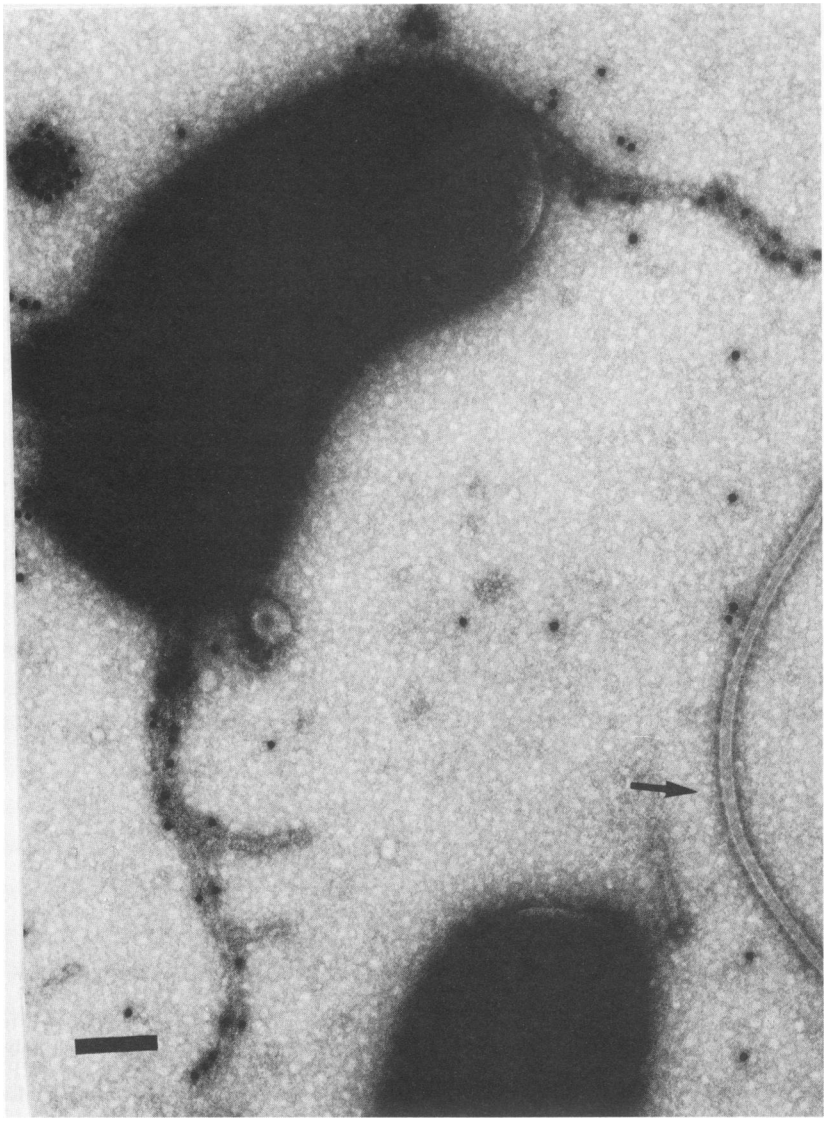

FIG. 4. Immunoelectron micrograph of *V. cholerae* non-O1. The gold particles were found on the pili but not on the flagella (arrow). Bar = 200 nm. Note that the pili reacted with antibody look fat.

pili share many cross-reactive epitopes. Both types of pili had hemagglutinating activity which was resistant to D-mannose and L-fucose; however, the pili did not block the adhesion of the organisms to intestines. On the basis of these results, the 16-kDa pili of strain S7 are most likely identical to the pili of strain 82P7.

The hemagglutinating activity of the purified pili was completely inhibited by the Fab fraction of the antipilus antibody; however, the Fab fraction did not completely inhibit the hemagglutinating activity of live organisms. This suggested that some bacterial components other than the 16-kDa pili may also be involved in cell-mediated hemagglutination.

The adhesion inhibition test in which we used intestines pretreated with purified pili or organisms pretreated with

TABLE 1. Titration of hemagglutination and hemagglutination inhibition tests of organisms and purified pili

Prepn	Titer			
	Control	Inhibitors		
		D-Mannose	L-Fucose	Fab
S7 cells	2^8	2^8	2^9	2^5
S7 pili	2^5	2^5	2^5	<2
82P7 cells	2^{12}	2^{13}	2^{11}	2^6
82P7 pili	2^7	2^7	2^7	<2

TABLE 2. Adhesion and adhesion inhibition tests

Strain	Adhesion index (mean ± SD) for[a]:		
	Control	Blocking test I	Blocking test II
S7	226 ± 97	194 ± 73	225 ± 56
82P7	320 ± 59	307 ± 79	313 ± 85

[a] In blocking test I the intestines were pretreated with homologous purified pili. In blocking test II bacteria were pretreated with the Fab fraction of homologous antipilus antibody.

TABLE 3. Distribution of strains immunologically cross-reactive with anti-S7 pilus antibody

Taxon	No. of strains		
	Examined	With agglutination	With no agglutination
V. cholerae non-O1	49	12	37
V. cholerae O1			
El Tor	50	18	32
Classical	49	7	42

antipilus Fab revealed no inhibition, and the purified pili did not adhere to the intestines (data not shown). On the basis of these results, the strain S7 16-kDa pili are probably not a colonization factor. It has been reported previously that the 16-kDa pili of *V. cholerae* O1 strain 82P7 are also probably not a colonization factor (9). Yamamoto and Yokota reported that a mucus coat is a primary adherence target for *V. cholerae* non-O1 strains (24). They concluded however that the bacterial pili are not related to adherence because the markedly piliated bacteria had low levels of adherence. These results suggest that purified pili may not adhere to mucus, although this suggestion has to be confirmed experimentally.

Although homology between the 16-kDa major pilus subunits of strains S7 and 82P7 is discussed above, we have to keep in mind the question of whether the minor ancillary adhesin proteins are present on the pili. These minor proteins could be lost during the pilus purification procedure. However, we believe that the purified pili in this study exhibited the native characteristic, since they had the ability to adhere to erythrocytes. The 16-kDa pili of strains S7 and 82P7 were purified by using the same method and exhibited the same characteristics as long as we examined them. Therefore, the 16-kDa pili are probably homologous. If the 16-kDa pili of *V. cholerae* are not responsible for colonization, we need to pay attention to the other components of the cells. We are now directing our attention to the outer membrane proteins, because we have found that the outer membrane proteins of adhesive *V. cholerae* are slightly different from those of a nonadhesive strain, as determined by SDS-PAGE (data not shown).

Honda et al. described the pili of *V. cholerae* non-O1 cultured on CFA agar (8). These pili are morphologically similar to the strain S7 16-kDa pili. Although Honda et al. speculated that the pili are a colonization factor because of their high hydrophobicity, adhesion of the organisms was not studied.

Two kinds of pili from *V. cholerae* O1 have been purified so far (2, 9, 20). The pili described by Taylor et al. (20.5-kDa pili) appeared to be straight and fascicular and were clearly confirmed to be a colonization factor (20). The 16-kDa pili described by Ehara et al. were thin and wavy, and their biological role was not clarified (2). We also purified 16-kDa pili from adhesive *V. cholerae* O1 strain 82P7 and nonadhesive strain C128 (9). The pili of strains 82P7 and C128 were identical as far as we could determine.

Yamamoto and Yokota reported that the production of pili in *V. cholerae* non-O1 is not related to cell adherence to intestines (24). Our results suggested that *V. cholerae* non-O1 pili do not play a role in colonization. The colonization factor of *V. cholerae* non-O1 may be in components other than the pili.

TABLE 4. Relationship between *V. cholerae* non-O1 agglutination with anti-S7 pilus serum and adhesion to intestines

Agglutination reaction	No. (%) of strains			
	Total	With adhesion level of[a]:		
		+/−	+	++
Positive	12	2 (17)	5 (42)	5 (42)
Negative	37	6 (16)	18 (49)	13 (35)

[a] +/−, Adhesion index of 0 to 10; +, adhesion index of 11 to 100; ++, adhesion index of more than 100. Adhesion indices were determined by the MASK method (14).

ACKNOWLEDGMENTS

This study was supported by a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan and by the Ohyama Health Foundation.

LITERATURE CITED

1. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
2. **Ehara, M., M. Ishibashi, Y. Ichinose, M. Iwanaga, S. Shimotori, and T. Naito.** 1987. Purification and partial characterization of pili of *Vibrio cholerae* O1. Vaccine **5**:283–288.
3. **Evans, D. G., and D. J. Evans, Jr.** 1978. New surface-associated heat-labile colonization factor antigen (CFA/II) produced by enterotoxigenic *Escherichia coli* of serogroups O6 and O8. Infect. Immun. **21**:638–647.
4. **Evans, D. G., R. P. Silver, D. J. Evans, D. G. Chase, and S. L. Gorbach.** 1975. Plasmid-controlled colonization factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. Infect. Immun. **12**:656–667.
5. **Finkelstein, R. A.** 1984. Cholera, p. 107–136. *In* R. Germanier (ed.), Bacterial vaccine. Academic Press, Inc., Orlando, Fla.
6. **Finkelstein, R. A., and L. F. Hanne.** 1982. Purification and characterization of the soluble hemagglutinin (cholera lectin) produced by *Vibrio cholerae*. Infect. Immun. **36**:1199–1208.
7. **Honda, T., M. Arita, T. Takeda, M. Yoh, and T. Miwatani.** 1985. Non-O1 *Vibrio cholerae* produces two newly identified toxins related to *Vibrio parahaemolyticus* hemolysin and *Escherichia coli* heat-stable enterotoxin. Lancet **ii**:163–164.
8. **Honda, T., K. Kasemsuksakul, T. Oguchi, M. Kohda, and T. Miwatani.** 1988. Production and partial characterization of pili on non-O1 *Vibrio cholerae*. J. Infect. Dis. **157**:217–218.
9. **Iwanaga, M., N. Nakasone, and M. Ehara.** 1987. Pili of *Vibrio cholerae* O1 biotype El Tor; a comparative study on adhesive and non-adhesive strains. Microbiol. Immunol. **33**:1–9.
10. **Jones, G. W., G. D. Abrams, and R. Freter.** 1976. Adhesive properties of *Vibrio cholerae*: adhesion to isolated rabbit brush border membranes and hemagglutinating activity. Infect. Immun. **14**:232–239.
11. **Jones, G. W., and J. M. Rutter.** 1974. The association of K88 antigen with haemagglutinating activity in porcine strains of *Escherichia coli*. J. Gen. Microbiol. **84**:135–144.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
13. **Morris, J. G., Jr., and R. E. Black.** 1985. Cholera and other vibrioses in the United States. N. Engl. J. Med. **312**:343–350.
14. **Nakasone, N., and M. Iwanaga.** 1987. Quantitative evaluation of colonizing ability of *Vibrio cholerae* O1. Microbiol. Immunol. **31**:753–761.
15. **Nakasone, N., and M. Iwanaga.** 1990. Pili of *Vibrio parahaemolyticus* strain as a possible colonization factor. Infect. Immun. **58**:61–69.
16. **O'Farrell, P. H.** 1975. High-resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
17. **Ouchterlony, O.** 1969. Antigen-antibody reactions in gel. Acta Pathol. Microbiol. Scand. **26**:507–515.
18. **Punsalang, A. P., Jr., and W. D. Sawyer.** 1973. Role of pili in the

virulence of *Neisseria gonorrhoeae*. Infect. Immun. **8**:255–263.

19. **Sato, Y., K. Izumiya, M. A. Oda, and H. Sato.** 1979. Biological significance of *Bordetella pertussis* fimbriae or hemagglutinin: a possible role of the fimbriae or hemagglutinin for pathogenesis and anti-bacterial immunity, p. 51–57. *In* C. R. Manclark and J. C. Hill (ed.), International symposium on pertussis. U.S. Department of Health, Education, and Welfare, Washington, D.C.
20. **Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalanos.** 1987. Use of PhoA gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. USA **84**:2833–2837.
21. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
22. **Yamamoto, K., M. Al-Omani, T. Honda, T. Takeda, and T. Miwatani.** 1984. Non-O1 *Vibrio cholerae* hemolysin: purification, partial characterization, and immunological relatedness to El Tor hemolysin. Infect. Immun. **45**:192–196.
23. **Yamamoto, K., Y. Takeda, T. Miwatani, and J. P. Craig.** 1983. Evidence that non-O1 *Vibrio cholerae* produces enterotoxin that is similar but not identical to cholera enterotoxin. Infect. Immun. **41**:896–901.
24. **Yamamoto, T., and T. Yokota.** 1988. *Vibrio cholerae* non-O1 production of cell-associated hemagglutinins and in vitro adherence to mucus coat and epithelial surfaces of the villi and lymphoid follicles of human small intestines treated with Formalin. J. Clin. Microbiol. **26**:2018–2024.