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Purification and Characterization of Pili Isolated from *Vibrio parahaemolyticus* Na2

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Pili from *Vibrio parahaemolyticus* Na2 isolated from a patient with diarrhea were purified and characterized. The organisms were hemagglutinative, but the purified pili were not. Na2 pili were physicochemically and immunologically quite different from the previously described *V. parahaemolyticus* Ha7 pili. Nevertheless, there was a high degree of homology between their N-terminal amino acid sequences.

Vibrio parahaemolyticus is a halophilic, gram-negative marine bacterium. It causes acute gastroenteritis in humans via contaminated seafood (3). It is a generally accepted theory that the mechanism whereby enteropathogens cause diarrheal diseases is by adhesion to the intestinal epithelium, colonization, and secretion of toxins. A variety of bacterial pili have been identified as colonization factors; therefore, purification of bacterial pili would be a first step toward identifying a colonization factor. There have been many reports on adherence of *V. parahaemolyticus* (1, 6, 7, 9, 10), and a certain pilus (the Ha7 pilus) has been proved to be a colonization factor of *V. parahaemolyticus* Ha7 in the rabbit intestine (9). However, the Ha7 pilus is unique to strain Ha7, and the purification method is not available for other *V. parahaemolyticus* strains. By changing the purification procedure, we finally succeeded in purifying pili from strain Na2 (a clinical isolate with a positive Kanagawa phenomenon). The Na2 pili were morphologically similar to, but physicochemically and immunologically quite different from, Ha7 pili.

V. parahaemolyticus Na2 was subcultured on a nutrient agar plate supplemented with 3% NaCl. A colony was inoculated into heart infusion broth (Eiken Co., Tokyo, Japan) and precultured overnight at 25 to 30°C without agitation. Five-milliliter portions of this preculture were transferred into 400 ml of heart infusion broth supplemented with 3% NaCl in 3-liter Erlenmeyer flasks, and the cultures were incubated at 37°C for 4 h with shaking. The pili were purified by the following procedures. The harvested organisms were suspended in 3% NaCl in water, agitated in a biomixer to detach pili, and centrifuged at 12,000 × *g* for 20 min (the pellet was discarded). Polyethylene glycol 6000 (PEG) (Wako Chemical Co., Tokyo, Japan) was added to the supernatant at a concentration of 1% (wt/vol), and the mixture was subjected to higher-speed centrifugation (70,000 × *g*) for 30 min (the pellet was discarded). More PEG was added to make the final concentration 1.5%, and the solution was incubated at 4°C overnight. The sample was centrifuged at 12,000 × *g* for 30 min (the supernatant was discarded), and the pellet was suspended in 5 M urea in 50 mM Tris hydrochloride (pH 8.0). After a 1-h incubation at 37°C, 7 volumes of the sample and 3 volumes of saturated ammonium sulfate were mixed to salt out the pili, which were collected by centrifugation at 12,000 × *g* for 30 min. Sedimented pili suspended in Tris buffer and dialyzed were regarded as purified. The pili of strain Ha7 were purified as described previously (9).

Piliation of strain Na2 organisms is shown in Fig. 1, revealing flexible pili with a diameter of 7 nm. Throughout the process of purifying pili, samples from each step were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2) and electron microscopy. The 1% PEG precipitate contained mainly flagella (Fig. 2, lane b), and that of 1.5% PEG contained mostly pili but also a substantial number of flagella (Fig. 2, lane c). Flagella were eliminated by the treatment with urea and ammonium sulfate, resulting in purified pili (Fig. 2, lane d). There was a clear distinction between the mobility of Na2 pili and that previously reported for Ha7 pili (Fig. 2, lanes e and f). The sample used for lane d (purified pili) was negatively stained and is presented in Fig. 3 as purified Na2 pili.

Physicochemically, the molecular mass of the pilin estimated by SDS-PAGE was 18 kDa, and the isoelectric point was between pH 4.7 and 4.9. The specific gravity of the pili as determined by CsCl density gradient centrifugation was 1.363.

Immunologically, there was no cross-reactivity between Na2 pili and Ha7 pili in Western immunoblotting and the double gel-immunodiffusion test.

V. parahaemolyticus Na2 agglutinated rabbit, human, and sheep erythrocytes, and the hemagglutination was not inhibited by D-mannose and L-fucose. However, the purified pili had no hemagglutinating activity. This is different from Ha7 pili, which were hemagglutinative and which were proved to be a colonization factor for the rabbit intestine (9). Both strains Na2 and Ha7 virtually did not adhere to the human intestine when examined by using Formalin-fixed intestine (8). Since the two strains were isolated as the causative organisms from diarrheal patients, potent adhesion of the strains to the human intestine was expected, but no such experimental evidence was obtained. This method of examining the adhesive properties of bacterial strains should be reconsidered, especially for the human intestine.

In spite of clear distinctions in physicochemical and immunological properties between Na2 and Ha7 pili, a high degree of homology was observed in the amino-terminal amino acid sequences. The sequences were determined through residue 18. Although the first 2 residues of Ha7 pilin were not identified, only 3 residues of 16 overlapped amino acids were different (Fig. 4). This homology suggests that

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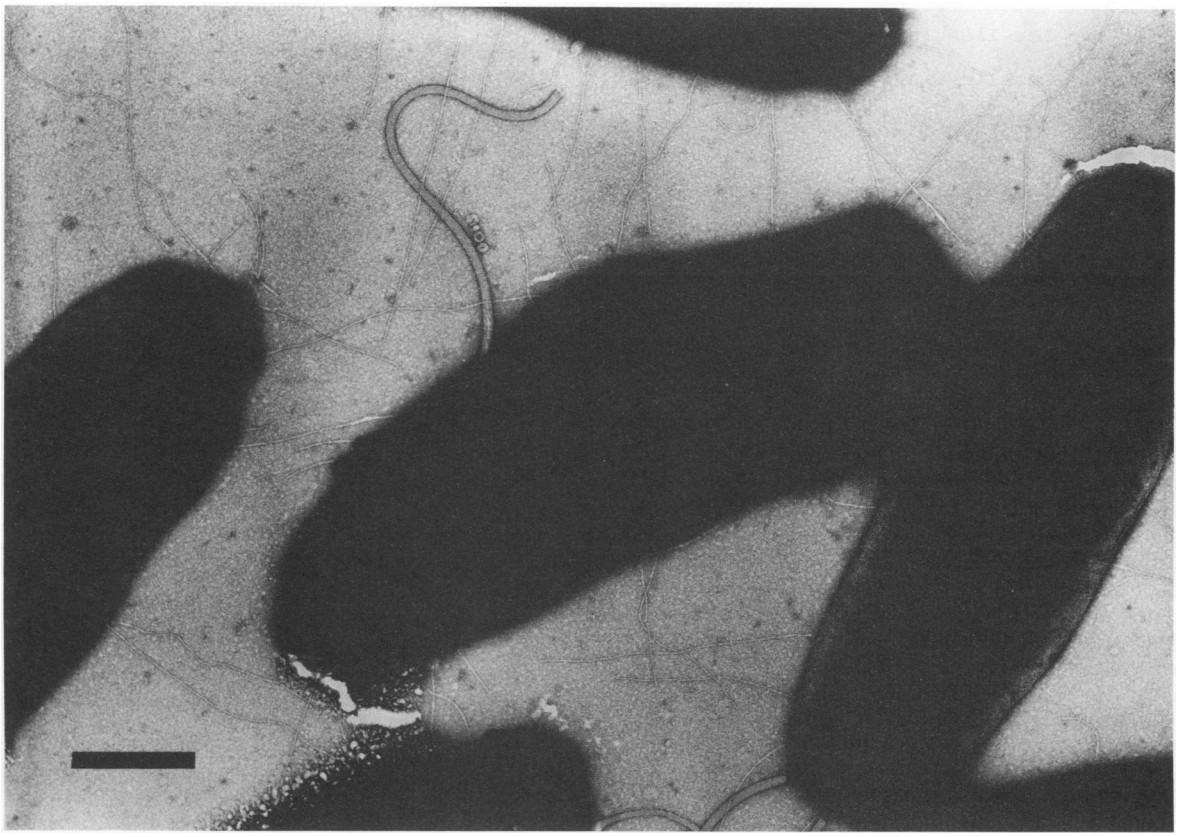

FIG. 1. Piliation of *V. parahaemolyticus* Na2. Bar, 0.5 μm.

both pili are encoded by a family of genes coding for similar but not identical polypeptides. The speculation can be made that a certain gene of the gene family is expressed, depending on a variety of cell conditions, while the other genes are silent. Rearrangement from a silent site to an expression site would then lead to the appearance of variant pili.

Three kinds of pili have been purified from *V. parahaemolyticus* (4, 9). Although the three pili are morphologically similar, they were characterized as different from one another. The previously described Ha7 pilus is seen in only strain Ha7, as far as we could discern (9). The distribution of

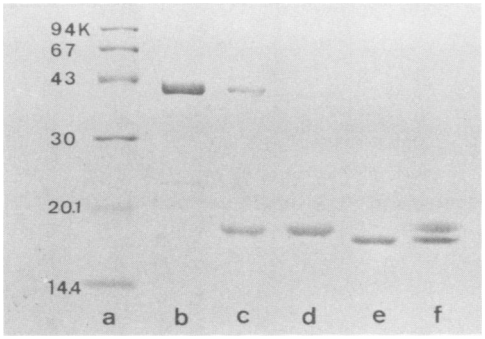

FIG. 2. SDS-PAGE profile of proteins. Lanes: a, molecular weight markers; b, pellet obtained with 1% PEG; c, pellet obtained with 1.5% PEG; d, purified Na2 pili; e, Ha7 pili for comparison; f, mixture of Na2 and Ha7 pili.

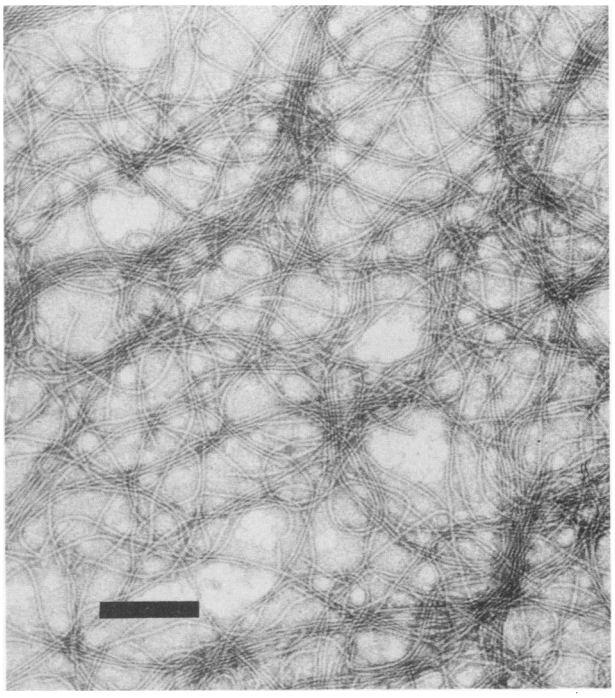

FIG. 3. Electron micrograph of purified Na2 pili. Bar, 0.2 μm.

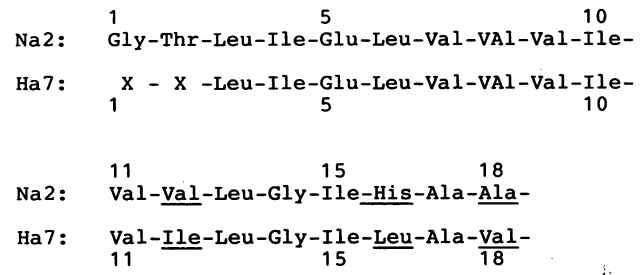

FIG. 4. N-terminal amino acid sequences of Na2 and Ha7 pilins. The first two residues of Ha7 pilin were not identified. Residue differences in the overlapped 16-amino-acid sequence are underlined.

Na2 pilus-related antigens in 16 strains, including the strain Na2, was examined. Three strains of 16 revealed a positive reaction when examined by slide agglutination of the organisms and by Western blotting of whole-cell lysates against anti-Na2 pilus antibody (data not shown). The three strains belonged to serogroup O4:K12. This finding suggests that the Na2 pilus may be specific for organisms of serotype O4:K12. Anti-O4 and anti-K12 sera did not react with Na2 pili. The pili described by Honda et al. appear to be widely distributed in strains of *V. parahaemolyticus* (4), and the molecular mass of the pilin is less than 10 kDa (personal communication), which is quite different from those of Na2 pilin (18 kDa) and Ha7 pilin (17 kDa). *V. parahaemolyticus* pili may thus be characterized by great variety.

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