The Pili of *Aeromonas hydrophila*: Identification of an Environmentally Regulated "Mini Pilin"

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Summary

Ultrastructural studies of *Aeromonas hydrophila* strain AH26 revealed two distinctive pilus types: "straight" pili appear as brittle, rod-like filaments, whereas "flexible" pili are supple and curvilinear. Straight pili are produced constitutively under all tested conditions of growth. In contrast, the expression of flexible pili is regulated by physical and chemical variables, being produced at 22 vs. 37°C, in a liquid vs. a solid medium, and when the availability of free-iron is reduced by the presence of deferoxamine mesylate. Both pilus proteins were purified and biochemically and functionally characterized. The major repeating subunit of the straight pilus is a 17,000-mol wt polypeptide with amino acid sequence homology with *Escherichia coli* type 1 and Pap pili. The flexible pilus filament is a homopolymer composed of a novel 46 amino acid polypeptide. Resistance of the flexible pilus filament to disaggregation using various chemical treatments was demonstrated; its stability as a polymer and its apparent mechanical strength seem to be conferred by a 20 amino acid hydrophobic, COOH-terminal domain. Purified straight pili lack hemagglutinating function. In contrast, purified flexible pili cause the agglutinin of human, guinea pig, ovine, bovine, and avian erythrocytes, although this property could only be demonstrated in the presence of divalent cations and was most evident at 4 vs. 22°C. Taken together, these results suggest that the pathogenic and ecological roles of the flexible pilus are related to this species' existence as a free-living organism in aquatic environments and its ability to cause infections, both in cold-blooded vertebrates and the human intestine.

Aeromonas hydrophila is a Gram-negative rod belonging to the family *Vibrionaceae*. It is a normal inhabitant of lakes and other aquatic environments, and a well-established cause of epizootic infections in fish, amphibians, and reptiles (1, 2). Rare septicemic infections in immunosuppressed humans have also occurred, and it has been increasingly recognized as a cause of sporadic intestinal infections ranging from dysentery to cholera-like syndromes (3, 4).

Previous studies by other laboratories have identified several potential virulence determinants of *A. hydrophila*, including α and β hemolysins (5), a surface-exposed outer membrane protein layer (6, 7), extracellular proteases (8, 9), and enterotoxins (10, 11). Electronmicroscopy studies of the organism have led to the discovery of pilus-like appendages, and in some strains their presence appears to be correlated with the capacity to agglutinate human erythrocytes; nonpilus hemagglutinins have also been detected (12, 13). More recently, ultrastructural examination of *A. hydrophila* and the related species *A. sobria* have revealed the existence of two types of pilus filaments that could be distinguished by morphologic criteria (13, 14). Strains expressing one of these pilus types adhere to the HEp-2 cell line, raising the possibility that the colonization of host epithelial surfaces might be mediated by this pilus type.

The most intriguing aspect of this organism's biology is its capacity to infect both cold-blooded vertebrates and mammals, and its ability to exist as a free-living form in water. This led us to examine a putative virulence determinant of the organism and how its expression is regulated by physical and chemical signals that typify these diverse environmental niches. We discovered that two distinctive pilus types could be expressed by the same *A. hydrophila* strain and that one of these was biochemically and functionally unique. Regulation of its expression by temperature and iron availability was demonstrated, leading to a proposal for its possible pathogenic role.

Materials and Methods

Bacteria, Media, and Reagents. *A. hydrophila* strain AH26, which had been isolated from the stool specimen of a patient with watery diarrhea, fever, and abdominal pain, was kindly provided by Dr.

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Michael Janda, Microbial Diseases Laboratory, California Department of Health Services, Berkeley, CA. The isolate was maintained as a stock culture at −70°C.

Trypticase soy agar (TSA)[1] or tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI) was used for all bacterial cultures. Plate or static broth cultures were obtained by growing the bacteria either at room temperature or at 37°C in a humidified atmosphere containing 5% CO_2 or at 22–30°C in an incubator. For growth of the organism under iron limitation, the iron chelator deferoxamine mesylate (Sigma Chemical Co., St. Louis, MO) at a concentration of 50, 100, and 200 μM was used to supplement the media described above.

SDS, acrylamide, 2-ME, bromophenol blue, urea, and nitrocellulose membranes were obtained from Bio-Rad Laboratories (Richmond, CA); tris(hydroxymethyl)-aminomethane base (Tris base), ammonium sulfate, magnesium chloride, BSA, phosphotungstic acid, Amido Black, Tween 20, D-mannose, D-fucose, D-galactose, CFA, IFA, and octyl glucoside were from Sigma Chemical Co.; molecular weight standards were from Pharmacia Fine Chemicals (Piscataway, NJ); guinea pig, sheep, horse, and chicken and calf RBC were from Colorado Serum Company (Denver, CO.); goat anti-rabbit IgG conjugated with 10 nm gold particles and 200-mesh copper electronmicroscopy grids covered with formvar and carbon were from Ted Pella (Redding, CA); Coomassie brilliant blue (No. 250) was from Bethesda Research Laboratories (Bethesda, MD); ^{125}I protein A was from DuPont Co. (Wilmington, DE); carboxypeptidase Y was from Pierce Chemical Co. (Rockford, IL); protected amino acids and resins (substituted 0.09 meq/g) for peptide synthesis were from Milligen/Biosearch (Cambridge, MA); and piperidine, 1-hydroxybenzotriazole, and TFA were from Aldridge Chemical Co. (Milwaukee, WI). Human type O RBC were collected by venipuncture.

Propagation of A. hydrophila for Purification of Pili. A single colony of strain AH26 was inoculated into 100 ml of TSB and incubated at 37°C for 16 h. 1 ml of this broth culture was plated onto each of 100 TSA-containing petri plates (100 × 15 mm), and the plates were incubated at either 37°C or at room temperature for 24 h. The resulting confluent growth was harvested with a glass slide into 400 ml of 0.05 M Tris-base, pH 8.5 (Tris buffer). This bacterial suspension was homogenized for 30 min at 4,500 rpm in an Omnimixer (Sorvall, Newton, CT) or stirred gently using a stirring bar depending on which type of pili was desired. The bacteria were then removed by centrifugation (20,000 *g* for 30 min) from the pili-containing supernatant.

Purification of Pili. Pili were precipitated from the supernatant by using either ammonium sulfate or magnesium chloride. The former was accomplished by the drop-wise addition of a saturated ammonium sulfate solution to the pili-containing supernatant described above to a final saturation of 20%. After 16 h at 4°C, the precipitate was collected by centrifugation (20,000 *g* for 30 min). The resulting pellet was allowed to stand in 5 ml (in each tube) of Tris buffer at 4°C for 16 h. Insoluble contaminants were separated from the solubilized pilus filaments by centrifugation (20,000 *g* for 30 min). The purity of this pilus preparation was assessed by SDS-PAGE and electronmicroscopy. If necessary, further purification was achieved by successive cycles of ammonium sulfate precipitation.

Magnesium chloride precipitation of pili was performed by the drop-wise addition of a 1 M magnesium chloride solution to the pili-containing supernatant described above to a final magnesium chloride concentration of 0.1 M. The aggregated pilus filaments were collected by centrifugation (20,000 *g* for 30 min), and the pellet was then dissolved in Tris buffer. Finally, insoluble contaminants were removed by centrifugation, and the pili in the supernatant were analyzed as mentioned above.

Electronmicroscopy. A colony of bacteria from a TSA plate was suspended in PBS, pH 7.2, and the suspension (20 μl) was placed on a carbon-, formvar-coated 200-mesh copper grid for 2 min at room temperature and then gently removed by blotting the excess solution with a piece of tissue. The grid was allowed to air dry for 2 min. Bacteria were negatively stained by the addition of 15 μl of 1% (wt/vol) phosphotungstic acid, pH 7.4. The stain was removed after 2 min, and the air-dried grid was examined using a transmission electron microscope (201; Philips Electronics Instruments, Inc., Mahwah, NJ). The pilus suspensions were prepared for electronmicroscopy as described above, except that the grids were stained for 3 min.

SDS-PAGE. Electrophoresis was carried out with a 16–20% gradient gel according to the methods for gel separation and buffered previously described by Laemmli (15), but modified by the addition of 70 mM NaCl (final concentration) to the resolving gel (16). The pilus proteins (20 μg) were solubilized in the sample buffer and boiled for 10 min. Electrophoresis was carried out at constant current of 8 mA for ~24 h. The separated proteins were then visualized using Coomassie blue.

Purification of specific polypeptides was accomplished by excising selected Coomassie blue–stained bands from the SDS-polyacrylamide gel; the proteins were then freed from the gel by electroelution using the Elutrap Electro-Separation System (Schleicher & Schuell Inc., Keene, NH).

Performic Acid Oxidation of Pilus Protein. Performic acid was prepared by adding 1 ml of hydrogen peroxide to 9 ml 88% formic acid. The mixture was incubated at room temperature for 1 h and was then chilled on ice. An aliquot of 200 μl of this performic acid preparation was added to a freeze-dried sample of 30 μg pilus protein and was allowed to react at 4°C. After 4 h, the reaction was stopped by adding 2 ml of water to the reaction mixture. The sample was dried and then used for amino acid composition analysis and sequencing.

Amino Acid Sequence and Composition Analysis. Amino acid sequencing was performed by automated Edman degradation on sequences (890M; Beckman Instruments, Inc., Palo Alto, CA; and 470A; Applied Biosystems, Inc., Foster City, CA) in the presence of polybrene (17). The amino acid compositions of pili were obtained using Durrum D-500 and Beckman 6300 analyzers (Beckman Instruments, Inc.). Composition analysis was derived from proteins hydrolyzed in 6 N HCl in vacuo at 110°C for 24, 48, and 72 h. Cysteine and methionine residues were identified after performic acid oxidation (18) of the intact pilus filaments.

Carboxypeptidase Y Cleavage. Protein (300 μg) was suspended in 0.1 M pyridine acetate buffer, pH 5.5. The enzyme (3 μg) was reconstituted in water and added to the protein solution. The reaction was carried out at room temperature. Aliquots were collected at different time points: 0, 15, 30, 60, 90, 120, 180, and 240 min. The free amino acids in the aliquots were analyzed. A synthetic peptide with the same amino acid at the COOH terminus as the protein under examination was used as a positive control.

Synthesis of Peptides and Peptide-Carrier Conjugates. Two synthetic peptides were prepared for this study. In both cases, peptide synthesis was carried out on a peptide synthesizer (9050; Milligen/Biosearch) using N-α-fluorenylmethoxycarbonyl-protected amino acids activated as pentafluorophenyl esters (19). Synthesis was accomplished using successive cycles of deprotection with 20%

[1] *Abbreviations used in this paper:* TSA, trypticase soy agar; TSB, tryptic soy broth.

(vol/vol) piperidine and coupling in the presence of 1-hydroxybenzotrizole as described (19). Peptides were cleaved from 2 g of resin by acidolysis using 30 ml of a solution containing 95% TFA, 5% distilled water, and 1 mg of phenol for a period of 8 h. The solution was separated from the resin, evaporated, and extracted with 200 ml of cold ether. The precipitated peptide was dried, resuspended in 100 ml of 5% acetic acid, and freeze-dried. The peptide product was characterized by amino acid composition analysis as described above.

The 27 amino acid synthetic peptide was synthesized with COOH-terminal cysteine through which it was coupled to the carrier protein thyroglobulin. The procedure or the preparation of this conjugate has been described previously (18).

Preparation of Specific Antisera. Purified pili (150 μg) or 500 μg of the synthetic-thyroglobulin conjugate was emulsified with CFA and injected intramuscularly into female New Zealand White rabbits at multiple sites. 21 d later, the same dose prepared with IFA was given. Subsequent boosts were administered every 14 d. The rabbit was bled 14 d after each boost.

Immunogold Electronmicroscopy. The peptide antiserum a the goat anti-rabbit IgG conjugated to 10-nm gold particles were added to separate tubes of PBS containing 1% (wt/vol) BSA (PBS-BSA) in order to form dilutions of 1:50 and 1:10, respectively. A colony of bacteria from a TSA plate was suspended in PBS. The bacterial suspension (20 μl) was added to a carbon-, formvar-coated grid and allowed to incubate for 2 min, after which excess fluid was removed and the grid air dried for 2 min as described above. The diluted antiserum was then added to the grid; after 2.5 h at room temperature, the grid was washed four times with PBS-BSA and air dried for 2 min. Gold-conjugated goat anti-rabbit IgG (15 μl) was then added to the grid; after 1 h at room temperature, the grid was washed three times with PBS and air dried. The grid was finally stained with 1% (wt/vol) phosphotungstic acid for 6 min. The excess stain was removed and the grid examined under the electron microscope as described above. A similar procedure was used with the antiserum elicited to "straight pili," diluted 1:400.

Western Blot Analysis. Proteins were separated by SDS-PAGE and transblotted onto a nitrocellulose membrane at a constant current of 300 mA for 4 h as described (20). The nitrocellulose blot was stained by Amido Black in order to assess the efficiency of transblotting. Excess protein-binding sites were blocked by washing the blot in 50 ml PBS-BSA for 20 min at room temperature. It was

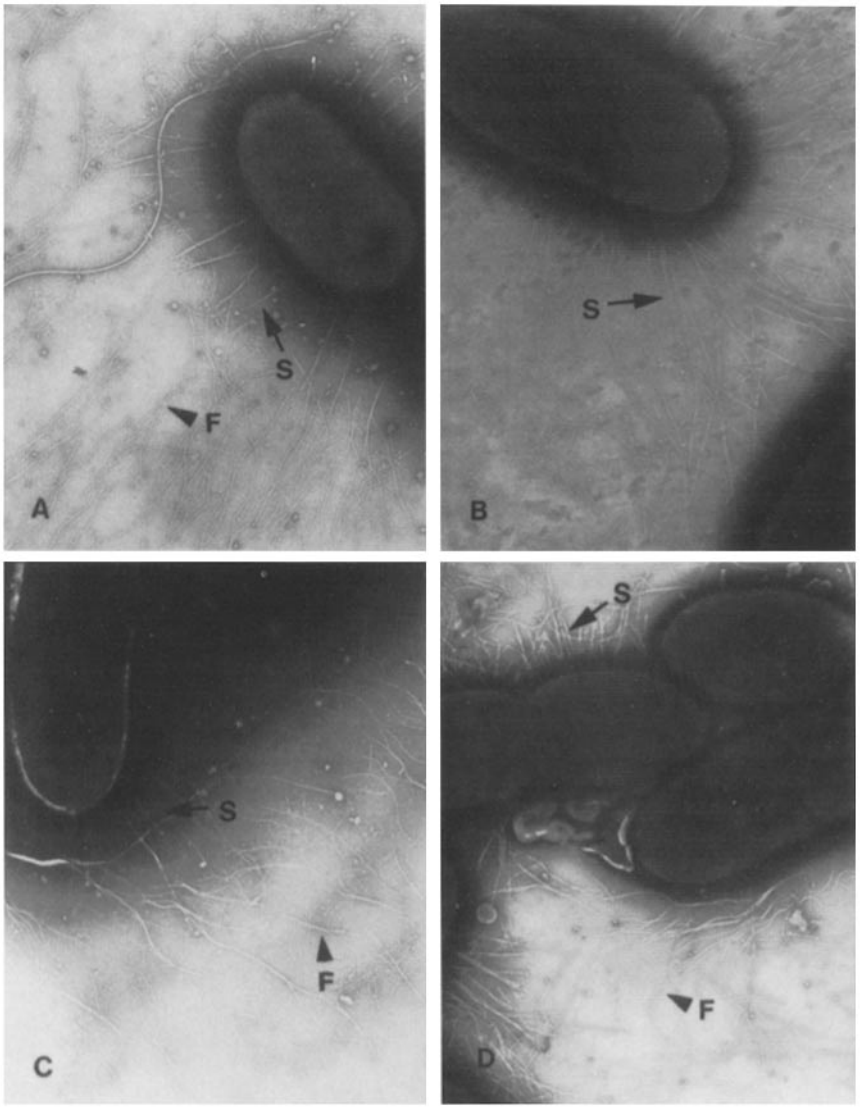

Figure 1. Ultra-structural characterization and differential expression of pili by *A. hydrophila* strain AH26. Strain AH26 was propagated for 16 h under different growth conditions, including temperature, a liquid vs. a solid medium, and in the presence or absence of available free-iron. Then, the bacteria were harvested, negatively stained by 1% phosphotungstic acid, and the pili examined by electronmicroscopy. (*A*) Bacteria grown in TSB at 22°C expressed both straight and flexible pili. Straight pili appear as rigid, rod-like structures, whereas flexible pili are curvilinear (×20,000). (*B*) Bacteria grown on TSA at 37°C expressed predominantly straight pili (×20,000). (*C*) Bacteria grown on TSA at 22°C expressed more of the flexible pili than straight pili (×20,000). (*D*) Bacteria grown at 37°C on TSA containing a final concentration of 200 μM deferoxamine mesylate expressed a high level of flexible pili (×20,000). S, straight pili; F, flexible pili.

then incubated with synthetic peptide antiserum at a dilution of 1:400 in PBS-BSA for 3 h at room temperature with gentle shaking, or overnight at 4°C; after which the blot was washed three times for 10 min each in 50 ml PBS containing 0.5% (vol/vol) Tween 20 (PBS-Tween). 5 μCi of ^{125}I protein A was added to the blot in PBS-BSA followed by gentle shaking for 2 h at room temperature. After incubation, the blot was washed three times in PBS-Tween (30 min each) followed by three washes in PBS (10 min each) at room temperature. The blot was dried and the bound antibody was assessed by autoradiography.

Hemagglutination Studies. Immediately before use, human type O, guinea pig, sheep, horse, and chicken RBC (sources mentioned in Materials and Methods) were washed three times in PBS and collected by centrifugation (3,000 *g* for 15 min).

For slide hemagglutination, a 3% (vol/vol) RBC suspension was prepared in PBS. Bacteria were harvested from agar plates and suspended in PBS to a concentration of 10^{11} bacteria per ml. The bacterial suspension (20 μl) was mixed with the RBC suspension (20 μl) on a glass slide, and the slide was rocked gently at room temperature. Hemagglutination was determined to be negative if it was not apparent within 5 min. RBC alone without bacteria were used as a negative control. Inhibition of hemagglutination was attempted by the addition of 1% (wt/vol) D-mannose, D-galactose, or D-fucose to the bacterial suspension.

A microtiter plate hemagglutination assay was also performed using serial twofold dilutions of purified pili in PBS-BSA. To each well, 50 μl of a 0.5% (vol/vol) suspension of RBC in PBS-BSA was added. Hemagglutination was performed in the presence of 0.1 M magnesium chloride. Inhibition of hemagglutination by D-mannose, D-fucose, and D-galactose was also evaluated by adding the particular sugar to each well at a final concentration of 1% (wt/vol). The hemagglutination assay was performed at both 4°C and at room temperature.

Results

Expression of Pili by A. hydrophila Strain AH26. Strain AH26 was specifically chosen for this study because electronmicroscopic evaluation revealed that it was heavily piliated. Upon closer inspection, two morphologically distinct pilus types were noted on bacteria grown in TSB at room temperature for 16 h (Fig. 1 *A*). The rod-like filaments depicted in Fig. 1 are referred to as "straight pili"; the curvilinear filaments are referred to as "flexible pili." Both pilus types have a diameter of 7–9 nm, and, when grown at room temperature in TSB, they are simultaneously produced in about the same proportion (20–50 pilus filaments of each type) by each bacterium. Straight pili are attached to the bacterial envelope more firmly than flexible pili. Even upon gentle stirring of the bacterial suspension, most of the flexible pili become detached from the bacterial surface, as seen in Fig. 1 *A*.

Growth-dependent Expression of the Flexible and Straight Pilus Types. The relative proportion of each pilus type could be modulated by propagation of strain AH26 under different growth conditions. These results (Table 1) were obtained by electronmicroscopic enumeration of the number of pilus filaments of each kind on each of the first 100 piliated bacteria encountered on several grids. When bacteria were grown in a liquid medium using TSB, either at room temperature or at 37°C (as shown in Fig. 1 *A*), both pilus types were present in about the same amounts. However, when bacteria were propagated at 37°C on a solid medium using TSA, straight pili were expressed almost exclusively (Fig. 1 *B*). In contrast, bacteria grown on TSA at temperatures ranging from 22 to 30°C expressed more flexible than straight pili (Fig. 1 *C*).

Table 1. *Expression of Straight and Flexible Pili Under Different Growth Conditions by A. hydrophila Strain AH26*

Growth conditions	Straight pili	Flexible pili
TSA + 37°C	4+	1+
TSA + 22–30°C	3+	4+
TSB + 37°C	3+	3+
TSB + 22–30°C	3+	3+
TSA + D_{50} + 37°C	4+	1+
TSA + D_{100} + 37°C	3+	3+
TSA + D_{200} + 37°C	3+	3+
TSA + D_{100} + 22–30°C	3+	4+
TSB + D_{100} + 37°C	3+	3+
TSB + D_{100} + 22–30°C	3+	3+

Strain AH26 was grown under the indicated conditions for 16 h and then examined by electronmicroscopy as described in Materials and Methods. Straight and flexible pilus filaments were identified by their characteristic morphology (see Fig. 1). The average number of pilus filaments of each type per bacterium was estimated by examining ~ 100 bacteria on each of several carbon-, formvar-coated grids for each growth condition. The degree of pili expression is denoted in this table as follows: 1+ = 0–5 pili/bacterium; 2+ = 6–20 pili/bacterium; 3+ = 21–50 pili/bacterium; 4+ = 51–100 pili/bacterium. D_{50} = 50μM deferoxamine mesylate; D_{100} = 100 μM deferoxamine mesylate; D_{200} = 200 μM deferoxamine mesylate.

The availability of free iron is usually low on mucous membranes and in blood due to the presence of lactoferrin and transferrin, respectively, and similar compounds exist in the blood and secretions of cold-blooded vertebrates. Therefore, the effect of iron concentration on pilus expression was examined by incorporating different concentrations of deferoxamine mesylate, an iron chelator, in the growth medium. Bacteria were grown at 37°C overnight on TSA containing concentrations of deferoxamine mesylate of 0, 50, 100, and 200 μM. Flexible pili expression was markedly enhanced at the two highest deferoxamine mesylate concentrations (100 and 200 μM) (Fig. 1 *D*). Thus, growth in a liquid medium, growth at 22 to 30°C, and the elimination of free iron all independently favored the expression of flexible pili. In contrast, straight pili were expressed constitutively at the same level under all the tested growth conditions (Table 1).

Purification and Biochemical Characterization of Flexible and Straight Pili. The purification of straight or flexible pili was accomplished by using growth conditions that favored or diminished the expression of the flexible pilus type. Thus, to purify straight pili, bacteria were grown on TSA at 37° for 24 h. Under this condition, straight pili were expressed almost exclusively. Because straight pili are tightly associated

with the bacterial surface, they were sheared from the bacteria using a blender that provided a strong mechanical force. The sheared bacteria were removed by centrifugation, and the pili in the supernatant were precipitated by the addition of 20% saturated ammonium sulfate; they were then purified to homogeneity as described in Materials and Methods.

Flexible pili were purified from bacteria grown on TSA for 24 h at 22°C. Since flexible pili are more readily detached from the bacterial surface than straight pili, they could be differentially freed from the bacterial envelope by gently stirring the bacterial suspension. After removal of the bacteria by centrifugation, the released flexible pili in the supernatant were precipitated with 0.1 M magnesium chloride.

The purity of the resulting straight and flexible pilus proteins was determined by electronmicroscopy (Fig. 2, *A* and *B*), SDS-PAGE (Fig. 3), and NH_2-terminal amino acid sequencing. Each pilus preparation appeared to be pure by electronmicroscopy, and only one major polypeptide was detected by SDS-PAGE for each of the two pilus proteins. The estimated subunit molecular weights of the straight and flexible pilus subunits were 17,000 and 4,000, respectively. These pilus protein preparations were used in all subsequent studies.

The amino acid compositions and NH_2-terminal amino acid sequences of the straight and flexible pilus proteins were determined and are shown in Table 2 and Fig. 4 *A*. The amino acid composition of the straight pilus protein was similar to the reported compositional analysis of the *E. coli* type 1 and Pap pilins (21, 22), including the presence of two cysteine residues per subunit. This similarity was confirmed by inspection of the NH_2-terminal amino acid sequence of the straight pilin through residue 54 (approximately one-third of the molecule), which revealed 28 identical residues between positions 7 and 47 (Fig. 4 *B*). Especially notable in this regard was the tentative identification of the conserved cysteine at about position 20, which in the type 1 and Pap sequences is known to participate in a disulfide bond with a second cysteine located at approximately position 61 (21, 22). How-

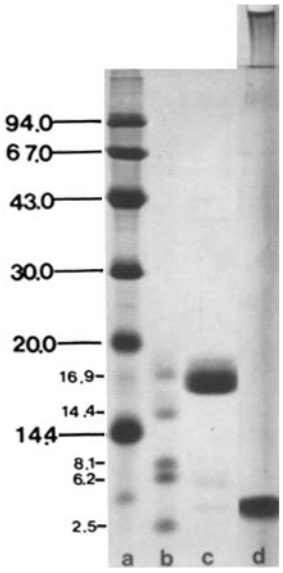

Figure 3. Apparent molecular mass of the straight and flexible pilus subunits. Purified straight and flexible pili were analyzed by SDS-PAGE as described in Materials and Methods. Lanes *a* and *b*, molecular weight standards. Lane *c*, purified straight pili showing a single pilus subunit of 17 kD. Lane *d*, purified flexible pili showing a single subunit of 4 kD; nondepolymerized flexible pilus protein is retained in the stacking gel.

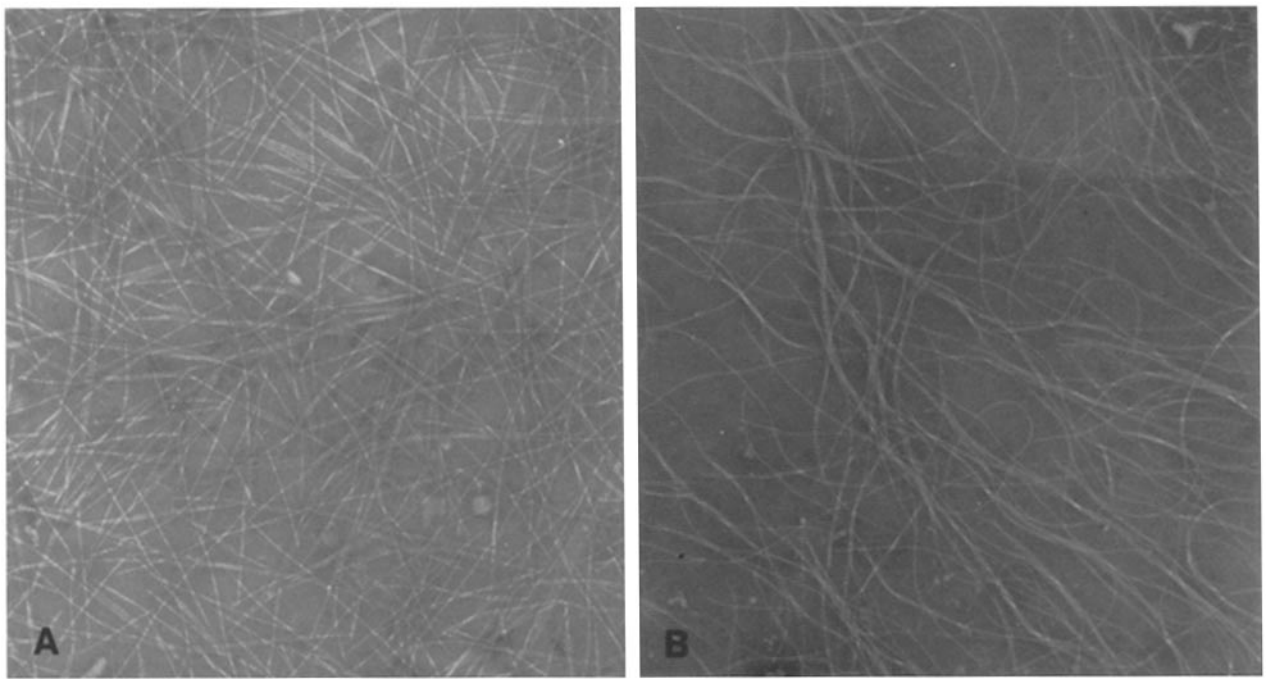

Figure 2. Ultra-structural characterization of purified straight and flexible pili. Pili were purified as described in Results, negatively stained with 1% phosphotungstic acid, and examined using electronmicroscopy. (*A*) Purified straight pili (×39,000). (*B*) Purified flexible pili (×39,000). Both pili have a diameter of 7–9 nm.

Table 2. *Amino Acid Compositions of A. hydrophila Strain AH26 Straight and Flexible Pilins*

Amino acid	Straight pilin analysis*	Flexible pilin	
		Analysis	Sequence‡
Neutral			
Aliphatic:			
Alanine	22	8	8
Glycine	23	5	5
Isoleucine	9	4	4
Leucine	13	2	2
Serine	15	2	2
Threonine	12	3	3
Valine	14	6	7
Aromatic:			
Phenylalanine	8	0	0
Tryptophan	ND	ND	0
Tyrosine	2	0	0
S containing:			
Cystein§	2	0	0
Methinine‖	2	4	4
Iminoacids:			
Proline	8	1	1
Charged			
Dicarboxylic acids:			
Aspartate¶	19	2	3
Glutamate**	14	3	3
Basic:			
Arginine	0	1	1
Histidine	0	0	0
Lysine	11	3	3
Total residues	174	44	46

Residues per subunit are shown.
* Integral number of residues based on the amino acid composition analysis described in Materials and Methods (calculation based on the subunit molecular weights of 17,000 and 4,000 of the straight and flexible pilins, respectively).
‡ Number of residues derived from the sequence shown in Fig. 4 *A*.
§ Cysteine analyzed as cysteic acid.
‖ Methionine analyzed as methionine sulfone.
¶ Total number residues of aspartic acid and asparagine.
** Total number of residues of glutamic acid and glutamine.

ever, in the case of the straight pilus subunit of *A. hydrophila*, a second cysteine was tentatively identified at position 49, indicating the probable existence of a disulfide loop composed of only 30 residues.

The complete amino acid sequence of the flexible pilus subunit was determined by Edman degradation. The entire subunit is composed of only 46 amino acids, the smallest pilin polypeptide reported thus far. Its calculated molecular mass is 4,615 daltons, in good agreement with the mass estimated from SDS-PAGE. In contrast to the straight pilin sequence, no homology is apparent with other pilin sequences or with other sequences by performing protein homology searches with the National Protein Sequence Data Library accessed through the Genetics Computer Group (University of Wisconsin). Besides its relative small size, the flexible pilin sequence is remarkable for the complete absence of cysteines and aromatic amino acids (Phe, Tyr, or Trp).

Overall, flexible pilin is a hydrophobic polypeptide, as indicated by the presence of 13 valine, leucine, and isoleucine residues, and by its elution from a C18 reverse-plasma column at an acetonitrile concentration of ~80% (data not shown). Most of the hydrophobic residues are clustered in the COOH-terminal one-half of the subunit (Fig. 5), a region that may mediate noncovalent subunit-subunit interactions. The strength of these interactions for the stability of the polymeric structure of the pilus filament was evident from our unsuccessful attempts to completely dissociate the pilus polymer using 2% SDS, 8 M urea, and 8 M guanidine. In each case, a substantial fraction of the pilus protein remained in the SDS-PAGE stacking gel (Fig. 3). Proof that this nonmigrating protein is composed solely of the 4,615-dalton pilus polypeptide comes from amino acid sequence and compositional data of the protein isolated from the high molecular weight band and from Western blotting analysis using antiserum to a synthetic pilin peptide corresponding to the first 27 amino acids of the NH_2-terminal flexible pilus sequence (Fig. 4 *A*). In addition to this large aggregate, a ladder-like pattern of immuno-reactive bands was apparent (Fig. 6), indicating the presence of smaller discrete aggregates of the flexible pilus subunit.

Localization of the Flexible Pilus Subunit. The biochemical characteristics of the 46 amino acid flexible pilus subunit provided little information about the topography of this peptide within the oligomeric structure of the pilus filament. This question was addressed through immunogold electron-microscopic examination of intact bacteria using antiserum to the 27 amino acid flexible pilus peptide described above. This antiserum bound the entire length of the flexible pilus filament (Fig. 7 *A*), indicating that the 46 amino acid subunit depicted in Fig. 4 *A* is present as part of the repeating polymeric structure that comprises the longitudinal axis of flexible pili. Moreover, Fig. 7 *A* also reveals that straight pili were not bound by this antiserum and by inference that the 46 amino acid peptide is exclusively associated with flexible pili. As a control, antiserum was also elicited to purified straight pili. This antiserum only bound straight pili (Fig. 7 *B*), providing further evidence that straight and flexible pili are not related, either biochemically or immunologically.

Functional Properties of Straight and Flexible Pili. The capacity of straight and flexible pili to agglutinate erythrocytes was assessed as an index of their ability to bind eukaryotic cell surfaces. Straight pili, purified as described above, did not agglutinate human type O or guinea pig red cells at the highest tested concentration (40 μg/ml) at 22 or 4°C or in the presence or absence of 0.1 M magnesium chloride. How-

A

 # amino acid residue

	1				5					10
S	Ala	Gly	Gly	Ala	Gly	Ser	Gly	Lys	Val	Thr
F	Glu	Gly	Gly	Ile	Ala	Glu	Ala	Ala	Gly	Lys
	11				15					20
S	Phe	Asn	Gly	Glu	Ile	Ile	Asn	Ala	Pro	(Cys)
F	Ala	Leu	Asp	Ser	Ala	Gln	Ser	Asp	Val	Thr
	21				25					30
S	Ser	Val	Ala	Pro	Glu	Ser	Val	Asp	Gln	Val
F	Ile	Thr	Ala	Pro	Lys	Val	Met	Met	Val	Val
	31				35					40
S	Val	Glu	Met	Gly	Gln	Ile	Ser	Thr	Lys	Glu
F	Ala	Thr	Val	Val	Gly	Val	Gly	Ile	Leu	Ile
	41				45					50
S	Leu	Ala	Asn	Gly	Gly	Glu	Ser	Asn	(Cys)	Lys
F	Asn	Met	Met	Arg	Lys	Ala	--COOH			
	51			54						
S	Pro	(Phe)	(Ser)	Phe						

B

# of amino acid residue

	1				5					10					15				20
Straight pilin	A	G	G	A	G	S	G	K	V	T	E	N	G	E	I	I	N	A	P (C)
	3				7					12					17				22
Pap pilin	T	I	P	Q	G	Q	G	K	V	T	E	N	G	T	V	V	D	A	P C
	2				6					11					16				21
Type 1 pilin	A	T	T	V	N	G	G	T	V	H	E	K	G	E	V	V	N	A	A C
	21				25					30					35				40
Straight pilin	S	V	A	P	E	S	V	D	Q	V	V	E	M	G	Q	I	S	T	K E
	23				27					32					37				42
Pap pilin	S	I	S	Q	K	S	A	D	Q	S	I	D	F	G	Q	L	S	K	S F
	22				26					31					36				41
Type 1 pilin	A	V	D	A	G	S	V	D	Q	T	V	Q	L	G	Q	V	R	T	A S
	41				45					50									
Straight pilin	L	A	N	G	G	E	S	N	(C)	K	P	(F)	(S)	F					
	43				47					52									
Pap pilin	L	G	A	G	G	V	S	K	P	M	D	L	D	I					
	42				46					51									
Type 1 pilin	L	A	Q	E	G	A	T	S	S	A	V	G	F	N					

Figure 4. (*A*) Amino acid sequences of the straight and flexible pilus subunits of *A. hydrophila* strain AH26. The entire primary structure of flexible pilin is shown and is composed of 46 amino acids. The NH_2-terminal 54 amino acids of straight pilin are shown (the entire subunit is estimated to be composed of 174 amino acids). Amino acids at positions 20 and 49 of the straight pilin sequence were not definitively identified; they are assigned Cys residues by analog with the *E. coli* type 1 and Pap A pilus sequences. S, straight pilin; F, flexible pilin. (*B*) Amino acid sequence homology between the straight pilin of *A. hydrophila* AH26 and the *E. coli* type 1 and Pap pilins. Identical amino acids are underlined. Type 1 pilin, the amino acid sequence of the Fim A subunit of *E. coli* (21). Pap A pilin, the amino acid sequence of the Pap A subunit of uropathogenic *E. coli* (22).

ever, the minimum hemagglutinating concentration of purified flexible pili for human type O, equine, ovine, bovine, and avian red cells was 25 μg/ml, and for guinea pig red cells, was 10 μg/ml. Hemagglutination by flexible pili occurred at 4°C, and was reduced by 50% at 22°C and required the presence of 0.1 M magnesium chloride. Hemagglutination by flexible pili was not inhibited by 1% (wt/vol) fucose, mannose, or galactose.

Structural and Functional Properties of a Synthetic Flexible Pilus Subunit. The polymeric assembly and hemagglutinating properties of *E. coli* Pap fimbriae require the participation the participation of several polypeptides in addition to the major, repeating pilus subunit, Pap A (24). To determine if the flexible pilus subunit, in the absence of other *A. hydrophila* products, could self-assemble as a polymeric filamentous array with hemagglutinating function, the entire 46 amino acid pilin polypeptide was prepared by solid-phase, synthetic chemistry. After cleavage and deprotection with trifluoroacetic acid and ether extraction, the freeze-dried polypeptide was solubilized either in 8 M urea or 30 mM octyl glucoside. Removal of

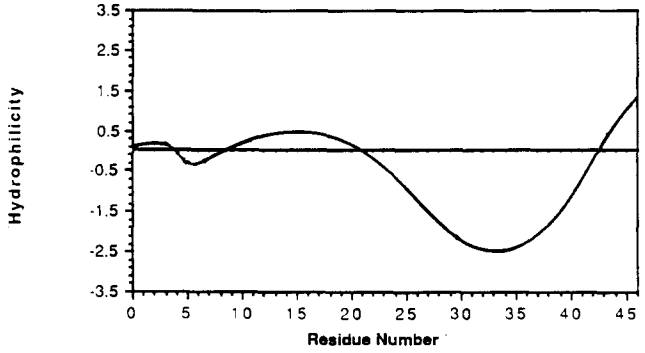

Figure 5. Hydrophilicity analysis of flexible pilin. The average hydrophilicity was calculated using the algorithm of Kyte and Doolittle (23). Positive values indicate areas of hydrophilic sequence, and negative values indicate areas of hydrophobic sequence.

Figure 6. Aggregates of the flexible pilus subunit. Western blot analysis and an antiserum to a synthetic peptide corresponding to the NH_2-terminal 27 amino acids of flexible pilin were used to identify nondepolymerized aggregates of flexible pilin (see Materials and Methods). Lane *a*, analysis of the purified flexible pilus protein. The antiserum crossreacts with the 4-kD monomeric subunit band, with nondepolymerized pilin aggregates retained in the stacking gel, and with smaller aggregates of the pilus subunit that form a ladder-like pattern indicative of a family of oligomers containing 10–20 pilins. Lane *b*, analysis of purified straight pilus protein using the same antiserum. The antiserum does not crossreact with the straight pilus subunit.

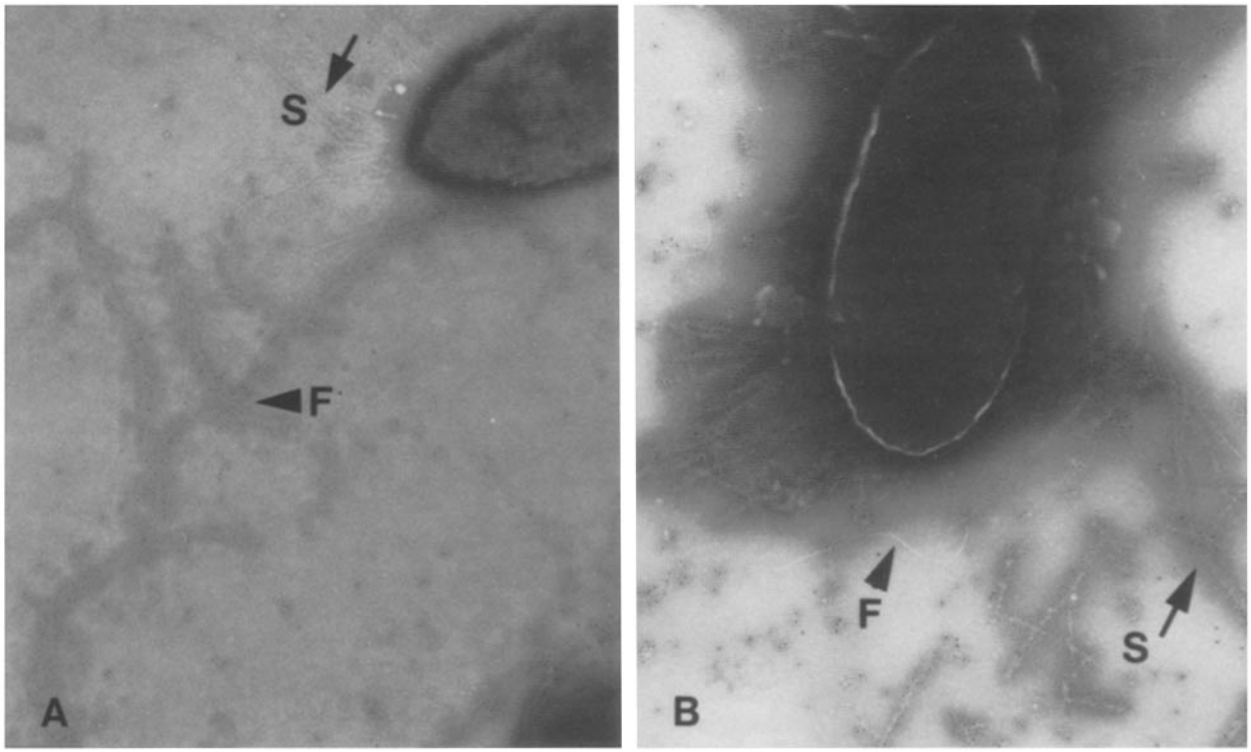

Figure 7. Location of the flexible pilin within the pilus filament. Transmission electronmicroscopy with a synthetic peptide antiserum to the 27 NH_2-terminal residues of flexible pilin was used to localize flexible pilin in negatively stained *A. hydrophila* strain AH26. The location of the bound antibody was determined using gold-conjugated goat anti-rabbit IgG (see Materials and Methods). (*A*) The antiserum (diluted 1:50) bound along the entire length of the flexible pilus filaments, but not to straight pili, expressed simultaneously by the same bacterium (×28,000). (*B*) An antiserum was also elicited to the purified straight pili. This antiserum (diluted 1:400) bound straight pili, but not flexible pili (×28,000). S, straight pili; F, flexible pili.

urea was then accomplished by step-wise dialysis against water containing successively lower concentrations of urea. Octyl glucoside was removed by dialysis against water only. The resulting preparations were examined for the presence of filamentous structures by electronmicroscopy and for hemagglutinating function with guinea pig RBC at 4 and 22°C, and the presence or absence of 0.1 M magnesium chloride. Although amorphous globular aggregates of negatively stained material were evident, the octyl glucoside–treated synthetic subunit also formed linear assembles resembling foreshortened flexible pili (Fig. 8); the diameter of these rod-like structures was 7–9 nm, approximately the same diameter as native, flexible pilus filaments. The urea-treated synthetic flexible pilus subunit also yielded curvilinear filamentous structures, but with an estimated diameter of ~5 nm (data not shown).

The hemagglutinating concentration of the octyl glucoside–treated synthetic polypeptide was 62 μg/ml, compared with 10 μg/ml for native pili. In addition to this difference, the optimal hemagglutinating temperature for the synthetic pilus preparation was 22°C instead of 4°C. The urea-treated synthetic pilus preparation, however, did not cause hemagglutination at the highest tested concentration.

Discussion

The objectives of this study were to determine the biochemical properties of the straight and flexible pili of *A. hydrophila* and how their expression might be regulated by physical and chemical signals encountered by the organism as a free-living form in water versus its role as a pathogen in warm- and cold-blooded vertebrate hosts.

The major repeating subunit of the *A. hydrophila* straight pilus filament is a 17,000–mol wt polypeptide with NH_2-terminal amino acid sequence homology with the *E. coli* type 1 and P pilins (21, 22) and with a pilin of *H. influenzae* type b (25). This structural relatedness suggests that descendents of a common pilus ancestral gene exist in at least three genera, including one species of the family *Vibrionaceae*. However, in contrast to the above-noted *E. coli* pilus types, the straight pili of *A. hydrophila* lack hemagglutinating capacity when tested with human and guinea pig erythrocytes. While this does not exclude a role for the straight pilus as an adhesin, it does separate it functionally from the mannose-sensitive and globoside-binding receptor specificities of these *E. coli* pili.

The straight pili described in this study appear to be similar morphologically to the "type S" pili first noted in electronmicroscopic studies of 46 Australian strains of *A. hydrophila* and *A. sobria* (13). Moreover, they biochemically and functionally resemble pili isolated from clinical strains of *A. hydrophila* from Japan and Thailand; these pili did not agglutinate rabbit, human, and guinea pig erythrocytes, and like the straight pili reported here, were comprised of a 17,000–mol wt subunit (14, 26). The apparent association

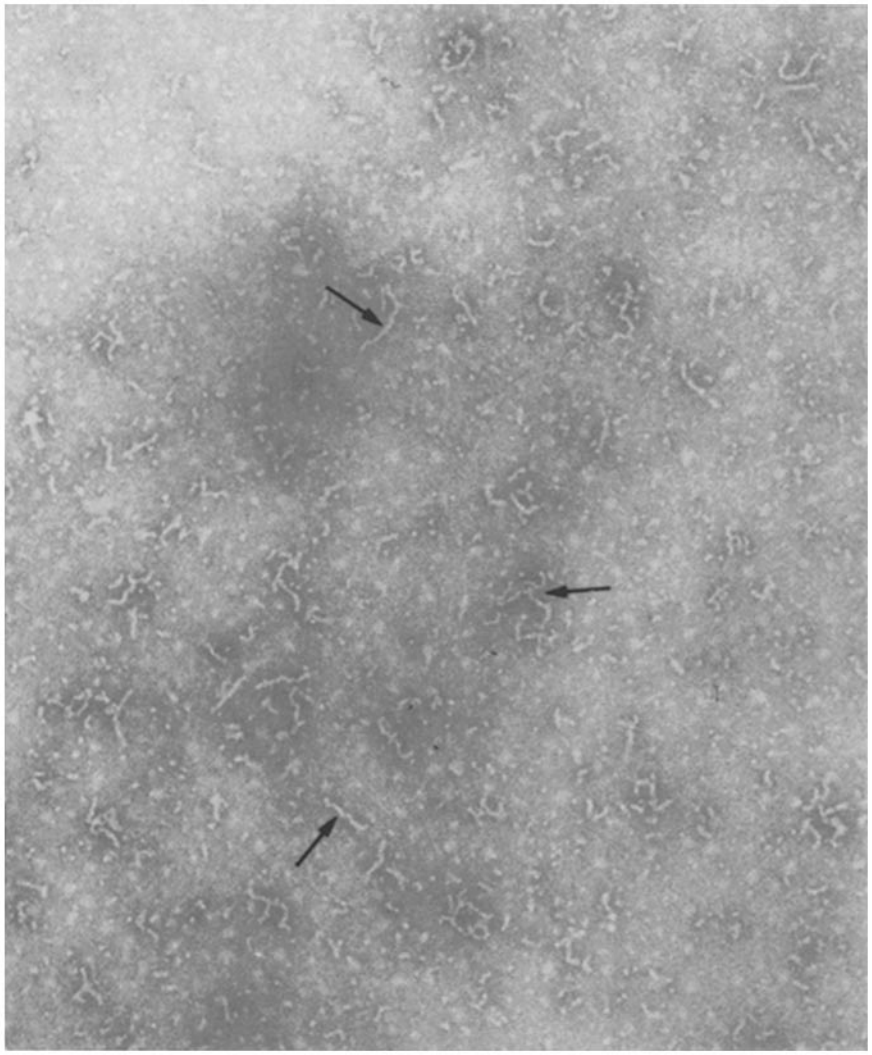

Figure 8. Self-assembly of a synthetic 46 amino acid flexible pilin analogue. The entire 46-residue pilin polypeptide was prepared by solid-phase synthesis, solubilized in 30 mM octyl glucoside, which was then removed by dialysis against water, and the resulting aggregates of the synthetic pilin were examined by transmission electronmicroscopy. Filamentous structures (7–9 nm in diameter) are evident (*arrows*) (×51,000).

of straight pili with strains from diverse geographic locations suggests that this pilus type is expressed by most clinical and environmental strains of *A. hydrophila*.

In contrast to straight pili, the flexible pili of *A. hydrophila* are composed of a biochemically unique pilin polypeptide. Flexible pilin is 4 kD, by far the smallest pilus subunit reported to date, even when compared with the 8-kD pilin of *Myxococcus xanthus* (27). Moreover, its primary structure is entirely novel. These unusual structural features led us to question whether the 46 amino acid sequence reported in Fig. 4 *A* really corresponded to the principal repeating structural subunit of the flexible pilus filament. This issue was addressed in three experiments. First, antibodies to a synthetic peptide corresponding to the first 27 amino acids of the flexible pilus sequence bound the entire longitudinal axis of the native flexible pilus filament (Fig. 7 *A*), indicating that this sequence must exist as a repeated motif throughout the pilus polymer. Second, the amino acid composition that was deduced from the primary structure of flexible pilin agreed exactly with the empirically determined composition of purified flexible pili, indicating quantitatively that the major component of these filaments is a protein with the same amino acid composition as that proposed for flexible pilin. Finally, a synthetic peptide corresponding to the entire 46 amino acid flexible pilin sequence spontaneously aggregated to form pilus-like filamentous structures, indicating that this sequence is sufficient for macromolecular assembly.

A remarkable feature of the flexible pilus is its polymeric stability. When purified flexible pili are boiled in 2% SDS and 2-ME, only about one-half of the protein migrates as a monomer when analyzed by SDS-PAGE (Fig. 3). Most of the remainder of the protein is retained in the stacking gel or migrates as discrete macromolecular aggregates (Fig. 6) estimated to be composed of 10–20 subunits. Even when exposed to 8 M urea or 8 M guanidine, or when boiled in 0.1 N HCl, the complete disaggregation of flexible pili into its constituent subunits was not observed. Because these treatments reportedly disaggregate other kinds of pilus filaments, we initially considered the possibility that the high molecular weight material might be a protein other than simple aggregates of the 46 amino acid subunit. However, direct sequencing of the protein retained in the stacking gel through

residue 29 revealed only the flexible pilin sequence depicted in Fig. 4 *A*. Moreover, no evidence of derivatized lysine residues at positions 10 and 25 was found, indicating that covalent crosslinking of the subunits had not occurred at these positions. Final proof that the polymer could be stabilized by noncovalent interactions came from SDS-PAGE analysis of the synthetic 46 amino acid flexible pilin, a polypeptide that is not covalently crosslinked. This polypeptide was also retained in the stacking gel in the same manner as the native flexible pili (data not shown).

The last 20 amino acids of the flexible pilin sequence probably mediate subunit-subunit interactions and, by inference, the stability of the polymer. These residues comprise a hydrophobic domain (Fig. 5) that according to the Kyte and Doolittle (23) algorithm of hydrophilicity is predicted to be buried in the interior of the protein or within a lipid bilayer. The former possibility is strongly supported by two kinds of biochemical data. First, attempts under a variety of conditions to digest the COOH terminus (an alanine residue) with carboxypeptidase Y were unsuccessful, even in the presence of low detergent concentrations, indicating that residues near the COOH terminus may be inaccessible. Second, the stepwise yields of amino acids released by Edman degradation fell by at least 50% after residue 26. Analysis of this phenomenon led to the recognition that the remaining sequence (a peptide corresponding to residues 27–46) was not retained in the sequencer, having been extracted into the organic phase. Only when the hydrophobicity of this region was diminished by performic acid oxidation of the methionine residues at positions 27, 28, 42, and 43 to methionine sulfone was the peptide retained in the sequencer and the sequence completed through the COOH terminus. Finally, although a synthetic peptide corresponding to the entire subunit spontaneously formed stable polymeric aggregates, a smaller peptide corresponding to residues 1–27 of the subunit appears to exist only as a monomer, indicating that the first one-half of the molecule probably lacks a polymer stabilization site.

Agglutination of human, bovine, ovine, equine, guinea pig, and avian erythrocytes by purified flexible pili could readily be demonstrated at 4°C and in the presence of 0.1 M magnesium chloride. However, the hemagglutinating power of flexible pili was reduced by ~50% when tested at 22°C, and was lost entirely when tested in buffers lacking a divalent cation. Inhibition of hemagglutination by mannose and fucose was not observed. These hemagglutinating properties could not be reconciled with the agglutinating characteristics of the whole, piliated strain from which these pili had been purified. Propagation of this strain under conditions favoring the simultaneous expression of the straight and flexible pilus types yielded bacteria that agglutinated human, guinea pig, and avian erythrocytes, but this reaction occurred at 22°C, did not require divalent cations, and was inhibited by mannose and fucose. Moreover, under growth conditions in which straight pili are expressed predominantly, the same pattern of mannose- and fucose-inhibitable hemagglutination was observed, even though purified straight pili, when tested alone, lack hemagglutinating capacity. Taken together, these results point to the existence of both pilus and nonpilus hemagglutinins of *A. hydrophila*, and suggest that their role as critical adhesins of the species will need to be addressed under conditions that more closely resemble the organism's natural habitat.

A. hydrophila exists as a free-living organism in aquatic environments; as a common pathogen of poikilotherms, especially fish; as an opportunistic pathogen of mammals; and as an increasingly recognized cause of intestinal infections in humans. Its capacity to survive in diverse environments suggests the existence of environmentally regulated genes that might specify two or more adaptive phenotypes, each corresponding to a particular habitat or host. This possibility was examined by carrying out experiments designed to identify physical and chemical variables that might influence the expression of the straight and flexible pilus types.

Straight pili are expressed under all growth conditions, whether propagated on agar or in broth, at 22 or 37°C, and in the presence or absence of available iron. In contrast, these variables caused substantial (e.g., 10-fold) differences in the expression of flexible pili. Flexible pili expression increased from an average of five filaments per bacterium to an average of 60 filaments per bacterium when the organism was propagated at 22 vs. 37°C; when the availability of free iron was limited by deferoxamine mesylate concentrations of 100 μM or more; and by growth in a liquid vs. a solid medium. Moreover, this effect on flexible pili expression was observed even if only one of the three variables favoring their expression was present (Table 1). These findings suggest that very different environmental signals might act through a common regulatory pathway. Indeed, this aspect of flexible pilus regulation by *A. hydrophila* is similar to the regulation of the enterotoxin and pilin genes of *V. cholerae* by pH, temperature, iron, and other environmental variables (28–30) where the effects of these variables all seem to be controlled through the *tox*R system of transcriptional activation. For *V. cholerae*, the organism's capacity to live as an esturine organism and as a pathogen of the human intestine appears to depend on the ability of the ToxR protein to respond to different environmental cues. Similarly, the results depicted in Table 1 indicate that the flexible pili of *A. hydrophila* are expressed both under conditions that exist in cool aquatic environments and at 37°C, providing the availability of free-iron is reduced, for example, by the presence of lactoferrin in the mucosal secretions of the small intestine. Thus, we can surmise that the parameters that positively regulate the expression of flexible pili serve to facilitate the transmission of this species from an aqueous reservoir such as contaminated water to the gastrointestinal tract of a mammalian host. Expression of flexible pili in that reservoir and during the organism's acquisition by a susceptible host would greatly favor rapid colonization of mucous membranes through the capacity of pili to mediate bacterial attachment to eukaryotic cell surfaces. Siderophore production and the elaboration of a hemolysin by *A. hydrophila* are also increased by iron limitation (31, 32), raising the possibility that the expression of three gene products (flexible pili, a high affinity iron uptake system, and a cytolytic protein) are all part of a coordinately

regulated pathogenic system. Studies are now being conducted to determine the pathogenic role of the flexible pilus in both cold-blooded vertebrates and the mammalian intestine in order to learn more about the pathogenic significance of its regulation by the physical and chemical variables that characterize these habitats.

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