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Fine Tangled Pili Expressed by *Haemophilus ducreyi* Are a Novel Class of Pili

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***Haemophilus ducreyi* synthesizes fine, tangled pili composed predominantly of a protein whose apparent molecular weight is 24,000 (24K). A hybridoma, 2D8, produced a monoclonal antibody (MAb) that bound to a 24K protein in *H. ducreyi* strains isolated from diverse geographic locations. A λgt11 *H. ducreyi* library was screened with MAb 2D8. A 3.5-kb chromosomal insert from one reactive plaque was amplified and ligated into the pCRII vector. The recombinant plasmid, designated pHD24, expressed a 24K protein in *Escherichia coli* INVαF′ that bound MAb 2D8. The coding sequence of the 24K gene was localized by exonuclease III digestion. The insert contained a 570-bp open reading frame, designated *ftpA* (fine, tangled pili). Translation of *ftpA* predicted a polypeptide with a molecular weight of 21.1K. The predicted N-terminal amino acid sequence of the polypeptide encoded by *ftpA* was identical to the N-terminal amino acid sequence of purified pilin and lacked a cleavable signal sequence. Primer extension analysis of *ftpA* confirmed the lack of a leader peptide. The predicted amino acid sequence lacked homology to known pilin sequences but shared homology with the sequences of *E. coli* Dps and *Treponema pallidum* antigen TpF1 or 4D, proteins which associate to form ordered rings. An isogenic pilin mutant, *H. ducreyi* 35000*ftpA*::mTn*3*(Cm), was constructed by shuttle mutagenesis and did not contain pili when examined by electron microscopy. We conclude that *H. ducreyi* synthesizes fine, tangled pili that are composed of a unique major subunit, which may be exported by a signal sequence independent mechanism.**

Haemophilus ducreyi is the causative agent of the genital ulcer disease chancroid (46). Genital ulcer disease due to *H. ducreyi* is an independent risk factor for human immunodeficiency virus seropositivity (24, 44, 46, 47). *H. ducreyi* is a strictly human pathogen that primarily infects the skin and mucous membranes. Little is known about the mechanisms by which *H. ducreyi* causes disease (31, 46). *H. ducreyi* adheres to extracellular matrix proteins (1), several immortalized cell lines (27, 28, 37), human fibroblasts (3, 28), and human keratinocytes in vitro (12, 45). However, the identities of the surface components that mediate adherence to human tissues and the cells to which *H. ducreyi* binds in vivo are presently unknown.

Pili (fimbriae) are filamentous appendages present on the surface of most gram-negative bacteria and serve as adhesins. *H. ducreyi* synthesizes fine, tangled pili, the expression of which has been associated with binding to laminin (1, 40). Purified pili are composed predominantly of a major subunit whose apparent molecular weight is 24,000 (24K) as well as several minor higher-molecular-weight bands (18, 22, 40). Immunization with purified pili provides partial protection against experimental infection in the temperature-dependent rabbit model (18).

In order to study the role of *H. ducreyi* pili in pathogenesis, we isolated a gene, designated *ftpA* (fine, tangled pili) that encodes the 24K protein. We show that FtpA lacked homology to other pilins and was not preceded by a typical leader sequence. FtpA shared homology with *Escherichia coli* Dps and *Treponema pallidum* antigen TpF1 or 4D, proteins that polymerize to form ordered rings. An isogenic mutant, constructed by insertionally inactivating *ftpA*, did not contain pili when examined by electron microscopy, confirming that the gene product encoded by *ftpA* was the major subunit of fine, tangled pili.

(Part of this research was presented by R. J. Brentjens and S. M. Spinola at the 94th General Meeting of the American Society of Microbiology, Las Vegas, Nev., 22 to 26 May 1994.)

MATERIALS AND METHODS

Bacterial strains and culture conditions. *H. ducreyi* 35000, 85-023233, A75, CIP 542, Hd 183, Hd 188, 82-029362, 84-018676, 6644, ATCC 33921, R1, and ATCC 27722 have been described previously (41). All strains were grown on chocolate agar supplemented with 1% IsoVitaleX at 35°C in a 5% CO_2 atmosphere.

Escherichia coli INVαF′ (Invitrogen Corporation, San Diego, Calif.), Y1090r− (Promega Biotech, Madison, Wis.), HB101 (30), RDP146, and NS2114Sm (36) were grown on Luria-Bertani (LB) agar at 37°C. *E. coli* HB101(pFK10), which expresses type 3 pili of *Klebsiella pneumoniae*, was kindly supplied by Steven Clegg and Trisha Schurtz of the University of Iowa. Where appropriate, *E. coli* strains were selected on medium with appropriate antibiotics at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 40 μg/ml; tetracycline, 10 μg/ml; chloramphenicol, 30 μg/ml; and streptomycin, 100 μg/ml.

Partial purification of *H. ducreyi* pili and pilus extracts. *H. ducreyi* 35000 and 85-023233 were grown overnight to confluency on 20 agar plates (150 mm in diameter). Bacteria were harvested in 0.1 M Tris-HCl (pH 8.0) containing 0.5 M NaCl and 1% sodium dodecyl sulfate (SDS). The bacterial suspension was sonicated to disperse clumps and lysed by incubation for 30 min at 37°C. SDS-insoluble material was pelleted at 25°C for 30 min at 21,000 × *g*. The pellet was suspended in the SDS buffer by sonication with a microtip, and the procedure was repeated five times. The final pellet, termed SDS-insoluble pili, was suspended in 50 mM Tris-HCl (pH 7.2) with 0.9% NaCl.

Preparation of pilus extracts by ammonium sulfate precipitation was done exactly as described previously (40). Briefly, pili were sheared from bacteria harvested in 0.1 M Tris-HCl (pH 7.6) by blending with a Sorvall omnimixer. Whole bacteria were separated from sheared pili by centrifugation. Pili were precipitated with saturated ammonium sulfate (45%, final concentration), collected by centrifugation, and suspended in 0.1 M Tris-HCl (pH 7.6).

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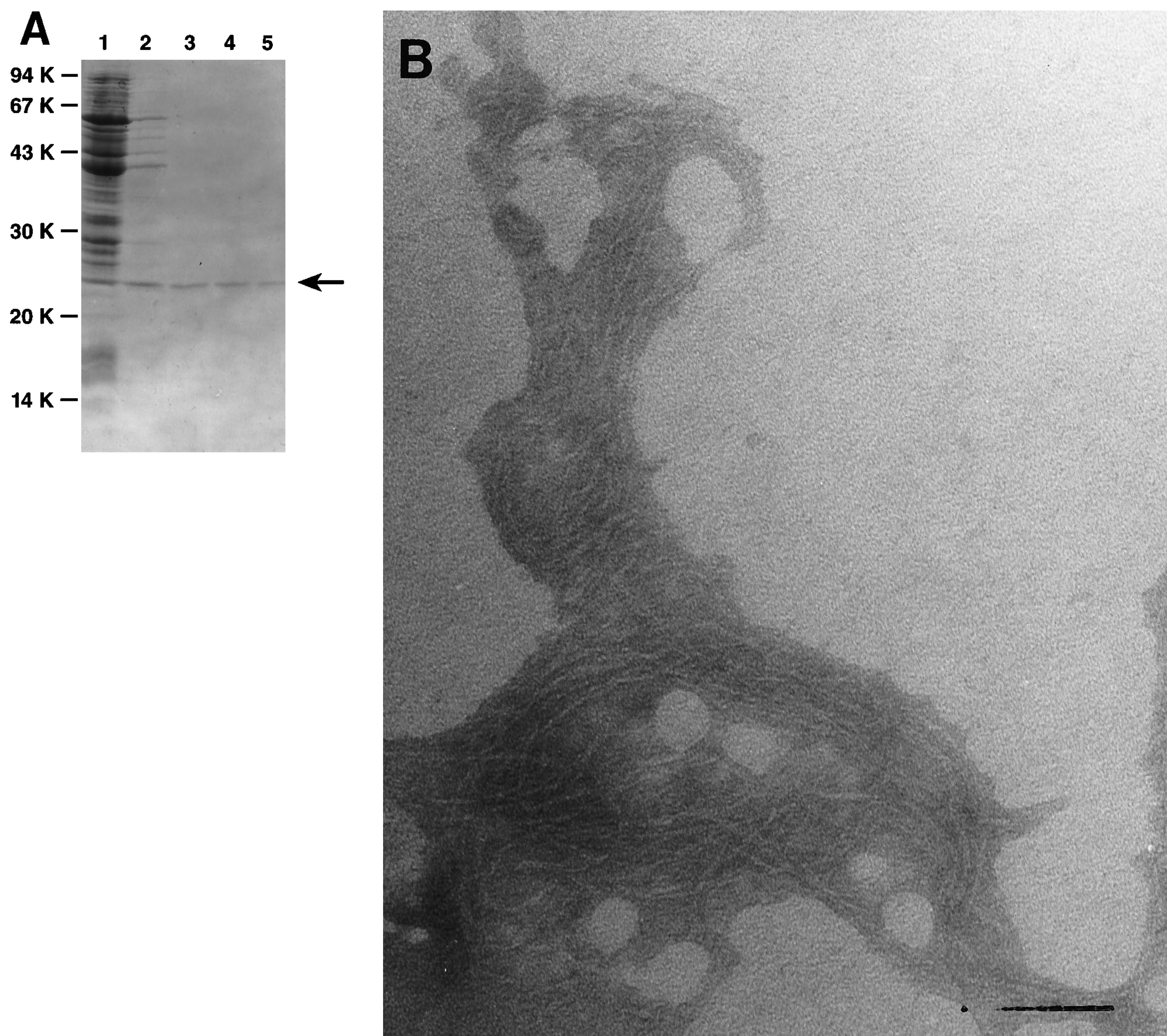

FIG. 1. (A) SDS-PAGE (15% acrylamide gel) of *H. ducreyi* after each of 5 cycles of SDS extraction (lanes 1 to 5). The arrow indicates the 24K pilin monomer. The molecular weights of the proteins are indicated on the left. Note that the higher-molecular-weight proteins present in the preparation are not evident in this photograph. (B) Electron micrograph of the final product stained with 2% ammonium acetate–2% ammonium molybdate. Bar, 0.1 μm.

Development of an anti-pilin monoclonal antibody (MAb). BALB/c mice were repetitively immunized subcutaneously with 100 μg of SDS-insoluble pili diluted in saponin. Splenic lymphocytes were fused to Sp2/0-Ag14 plasmacytoma cells as described previously (6). Tissue culture supernatants were screened for reactivity to SDS-insoluble pili in an immunodot assay as described previously (42).

Electron microscopy. SDS-insoluble pili and pilus extracts were dried on Formvar-coated copper grids (Electron Microscopy Sciences, Fort Washington, Penn.), stained with 2% ammonium molybdate–2% ammonium acetate in distilled water, and viewed with a Philips CM10 electron microscope at 60 kV.

To examine whether *H. ducreyi* cells synthesized pili, bacteria were grown for 24 h on plates, harvested, and suspended in 500 μl of phosphate-buffered saline (PBS) in Eppendorf tubes. Bacteria were dispersed by gentle agitation on a vortex mixer, and large aggregates of organisms were allowed to settle for 10 min. Samples (5 μl) obtained from the top of the suspension were deposited on glow-discharged, Formvar-coated 200-mesh nickel grids. The cells were fixed on the surface of the grids by treatment with 2% paraformaldehyde in PBS for 10 min, dried, and stained with 2% phosphotungstic acid for 1 min. The specimens were viewed with a Hitachi H-7000 transmission electron microscope at 75 kV at the University of Iowa electron microscope facility.

Immunoelectron microscopy of ultrathin sections of whole cells was performed as previously described (7). *H. ducreyi* cells were suspended in L. R. White (LRW) embedment (Ted Pella Inc., Reading, Calif.) and cut into 60- to 90-nm-thick sections with a diamond knife. The sectioned tissue was incubated with MAb 2D8 for 16 to 18 h. The sections were washed and reacted with a 20-nm-diameter colloidal gold bead–goat anti-murine immunoglobulin G conjugate (Auroprobe; Amersham) for 1 hour. Sections were counterstained with uranyl acetate. The specimens were viewed with a Hitachi H-7000 transmission electron microscope at 75 kV.

SDS-PAGE, Western blot, and colony blots. Whole bacteria, SDS-insoluble pili, and pilus extracts were solubilized in sample buffer at 100°C unless otherwise indicated and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) in 15% acrylamide gels by the method of Laemmli (26). Gels were stained with Coomassie brilliant blue or silver or transferred to nitrocellulose or Immobilon polyvinylidene difluoride membranes in a Semi-Phor blotter (Hoeffer Scientific Instruments, San Francisco, Calif.) (40). Colony blot assays were performed exactly as described previously (41). Western blots (immunoblots) and colony blots were probed with tissue culture supernatants and protein A-peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) as described previously (39).

N-terminal amino acid sequence. The N-terminal amino acid sequences of the proteins transferred to Immobilon were determined by Edman degradation at the Protein Microsequencing Facility of the University of California, Los Angeles, or at the University Peptide Sequence Facility of the State University of New York at Buffalo.

Preparation of DNA-protein complexes. Prior to the DNA-protein complexing experiments, pili were treated by the method that removes contaminating DNA from Dps (5). Briefly, SDS-insoluble pili were suspended in 50 mM Tris buffer (pH 8.0) with 2 M NaCl and 0.1 mM EDTA to dissociate DNA-protein complexes. To separate DNA from protein, the sample was applied to a Sepharose 6B column (20 by 1 cm) equilibrated in the same buffer. Fractions containing the 24K pilin subunit were pooled, dialyzed against a 50 mM Tris buffer (pH 8.0) with 50 mM NaCl and 0.1 mM EDTA, and concentrated in a Centricon 10 microconcentrator (Amicon, Beverly, Mass.).

DNA-protein complexing was performed by modification of a method described previously by Almiron et al. (5). One microgram of Dps protein (kindly provided by Roberto Kolter) or SDS-insoluble pili was added to 50 ng of *H. ducreyi* chromosomal DNA, pHD24, or λ*Hin*dIII DNA standards; incubated at room temperature for 30 min; and electrophoresed in an 0.8% agarose gel. DNA that had not been incubated with protein served as a negative control.

DNA manipulation. *H. ducreyi* chromosomal DNA was prepared as described by Maniatis et al. (30). A λgt11 library was constructed by ligating 2- to 4-kb *Rsa*I-digested chromosomal fragments of *H. ducreyi* 85-023233 to *Eco*RI adaptors (Promega Biotech). Fragments were ligated with λgt11 arms, packaged with commercially available reagents (Packagene Lambda DNA Packaging System; Promega Biotech), and amplified in *E. coli* Y1090r−.

The library was screened with MAb 2D8, and reactive clones were plaque purified three times. The insert was recovered from recombinant phage by PCR with λgt11 forward and reverse primers (Promega Biotech). Amplification reactions were performed with *Taq* polymerase in a Hybaid thermal reactor (National Labnet Company, Woodbridge, N.J.) with reagents supplied by the GeneAmp PCR kit (Perkin-Elmer, Norwalk, Conn.). Amplified DNA was ligated into the pCRII (Invitrogen) and transformed into *E. coli* INVαF′ competent cells.

Southern blots. *H. ducreyi* DNA was digested to completion with the appropriate restriction enzyme and electrophoresed on 0.8% agarose gels. Southern blots were probed with either the cloned *H. ducreyi* insert, a probe amplified by PCR with the PILIN 1 and PILIN R3 synthetic oligonucleotides (see Fig. 4A), or the chloramphenicol acetyltransferase (*cat*) cassette purified from pACYC184 by *Hha*II digestion under high-stringency conditions as described previously (39). Probes were labeled by random priming as described in the NEBlot Phototope Kit (New England Biolabs, Beverly, Mass.). Blots were developed by using the Phototope Detection Kit (New England Biolabs).

DNA sequencing and primer extension. To localize the gene encoding the 24K pilin protein, a nested set of deletions was made in the pHD24 insert with exonuclease III (Erase-a-Base; Promega Biotech) (2). Digested plasmids were transformed into *E. coli* INVαF′ competent cells, and transformants were screened by colony blot for loss of reactivity to MAb 2D8. DNA was sequenced by the dideoxy-chain termination method (35) with synthetic oligonucleotide primers (Biochemistry Biotechnology Facility at the Indiana University School of Medicine, Indianapolis) and the Sequenase version 2.0 kit (United States Biochemical, Cleveland, Ohio). Both strands of two clones derived from two independent PCR reactions were sequenced.

mRNA for primer extension was isolated from *H. ducreyi* by the method of Baker and Yanofsky (9). Primer extension was done with [γ-^{32}P]ATP-labeled oligonucleotide PREX-1 (see Fig. 4A) by using reagents and instructions provided for in the avian myeloblastosis virus reverse transcriptase primer extension system (Promega Biotech) at 42°C.

Sequencing and primer extension reactions were resolved on 8% acrylamide gels with 8 M urea. Dried gels were autoradiographed overnight.

Insertional inactivation of *ftpA*. Transposon mutagenesis of *ftpA* was done in *E. coli* by the shuttle mutagenesis system of Seifert et al. (36). Briefly, the pHD24 insert was subcloned into pHSS8 and transformed into *E. coli* RDP146(pTCA) by the method of Chung et al. (14). One MAb 2D8-reactive transformant was mated with RDP146[pOX38::mTn*3*(Cm)]. Cointegrates were resolved by subsequent conjugation with *E. coli* NS2114Sm. Transconjugants were screened for loss of pilin expression with MAb 2D8.

Strain 85-023233 was the source of the *H. ducreyi* insert in pHD24 but was resistant to chloramphenicol. Strain 35000 was used to construct an isogenic mutant because it was sensitive to chloramphenicol and transformable by electroporation (23). Plasmid DNA from one *E. coli* transconjugant that no longer expressed the MAb 2D8 epitope was transformed into *H. ducreyi* 35000 by electroporation as described by Hansen et al. (23), by using the Cel-Porator Electroporation System (Gibco-BRL, Gaithersburg, Md.). Transformants were plated on chocolate agar supplemented with 1 μg of chloramphenicol per ml and screened for loss of MAb 2D8 reactivity in a Western blot.

Laminin binding assays. The ability of *H. ducreyi* and *E. coli* strains to bind to laminin-coated glass slides was examined by the method of Westerlund et al. (48). Glass slides (Nunc Incorporated, Napersville, Ill.) were coated with a solution of laminin (60 μg/ml) (Sigma Chemical, St. Louis, Mo.) or bovine serum albumin (BSA; 50 μg/ml), washed, and blocked with PBS containing 2% BSA (wt/vol). The slides were seeded with suspensions containing 2.5 × 10^{7} CFU/ml, incubated at room temperature for 45 or 90 min, washed, and gram strained. The mean number of adherent bacteria present in five high-power fields was determined. All experiments were done in duplicate.

Nucleotide sequence accession number. The sequence of *H. ducreyi ftpA* has been submitted to GenBank under accession no. U18769.

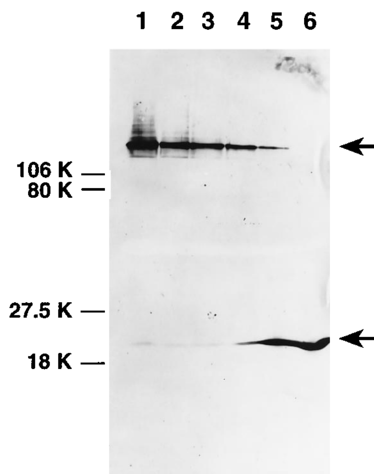

FIG. 2. Western blot of pilus extracts probed with MAb 2D8. The samples were incubated for 10 min at 25°C (lane 1), 56°C (lane 2), 62°C (lane 3), 72°C (lane 4), 85°C (lane 5), and 100°C (lane 6) prior to solubilization in sample buffer at room temperature. The upper and lower arrows indicate binding of the MAb to a 150K multimer and the 24K pilin monomer, respectively. The molecular weights of the proteins are indicated on the left.

RESULTS

Pilus purification and development of a MAb to the major pilin subunit. We previously purified *H. ducreyi* fine, tangled pili by differential centrifugation employing crystallization of pili at pH 5.0 and solubilization of pili at pH 10.5 (40). This preparation contains a predominant 24K protein and several higher-molecular-weight proteins (40). We also observed that SDS-insoluble material prepared from *H. ducreyi* consists predominantly of a protein with a similar apparent molecular weight and peptidoglycan (41). To test whether the SDS-insoluble material contained pili, whole cells were solubilized in a buffer containing 1% SDS, and insoluble material was collected. After 5 cycles of extraction, the SDS-insoluble fraction consisted of a predominant 24K protein and several minor higher-molecular-weight proteins (Fig. 1A). By electron microscopy, the preparation contained fine, tangled pili (Fig. 1B). The 24K proteins obtained by the acid-base purification technique and the SDS-insolubility technique were isolated by preparative SDS-PAGE and transferred to an Immobilon membrane. Identical N-terminal amino acid sequences (Met-Arg-Ser-Lys-Thr-Ile-Thr-Phe-Pro-Val-Leu-Lys-Leu-Thr-Gly-Glu) were obtained from both preparations. This sequence is also identical to that recently reported by Frisk et al. (22).

Splenocytes from a BALB/c mouse immunized with SDS-purified pili were fused to Sp2/0 myeloma cells. A hybridoma, 2D8, produced an immunoglobulin G 2b MAb that bound to the 24K protein in Western blot.

Effect of temperature on pilin aggregation. Pilus extracts were prepared by ammonium sulfate precipitation of mechanically sheared pili. Samples were heated in 0.1 M Tris-HCl (pH 7.6) for 10 min at temperatures ranging from 25 to 100°C, solubilized in sample buffer at room temperature, and probed with MAb 2D8 in a Western blot (Fig. 2). A 150K band that bound 2D8 was present in the preparation at room temperature. The 150K band dissociated to a 24K subunit at 72°C, and dissociation was complete at 100°C. The data suggested that the 24K subunit formed a hexameric structure that was dissociated with heat and is consistent with a previous characterization of the protein (22).

Cloning and sequencing of gene encoding 24K protein. A

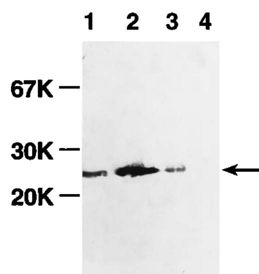

FIG. 3. Western blot of *H. ducreyi* whole cells (lane 1), SDS-insoluble pili (lane 2), *E. coli* INVαF′ (pHD24) (lane 3), and *E. coli* INVαF′ (pCRII) (lane 4) probed with MAb 2D8. The arrow indicates binding of the MAb 2D8 to the 24K protein. The molecular weights are indicated on the left.

chromosomal library was constructed from *H. ducreyi* 85-023233 in the phage λgt11. Three of 3,000 plaques screened bound MAb 2D8. A 3.5-kb insert from one reactive phage was amplified by PCR, ligated into pCRII, and designated pHD24. *E. coli* INVαF′ transformed with pHD24 expressed a 24K protein that bound 2D8 (Fig. 3). *E. coli* HB101 transformed with pHD24 also expressed the 24K protein but did not express fine, tangled pili when examined by electron microscopy (data not shown).

Restriction mapping of pHD24 and Southern blot analysis confirmed that the cloned insert originated from *H. ducreyi*. The pHD24 insert contained three *Sau*3A sites. In Southern blots, the insert bound to four similar *Sau*3A fragments of the insert and *H. ducreyi* chromosomal DNA and did not hybridize to *E. coli* DNA (data not shown).

A nested set of deletions was made in the pHD24 insert by exonuclease III digestion. *E. coli* transformants were screened for loss of reactivity to MAb 2D8. The gene encoding a MAb 2D8-reactive protein was localized to an approximately 1 kb fragment. The 1-kb fragment contained a 570-bp open reading frame (ORF) designated *ftpA*; identical sequences were obtained from subclones of two independent PCR reactions (Fig. 4). Translation of the ORF predicts a polypeptide with a molecular weight of 21.1K. The predicted N-terminal sequence of FtpA was identical to the N-terminal amino acid sequence of the purified protein. Thus, FtpA was not preceded by a typical cleavable signal sequence.

Primer extension analysis of *ftpA*. To confirm that the lack of a leader sequence was not due to cloning artifact, the transcriptional start of *ftpA* was determined by primer extension analysis using the synthetic oligonucleotide PREX-1 and mRNA isolated from *H. ducreyi*. PREX-1 is complementary to the coding strand of *ftpA* from 62 to 84 bp downstream of the ATG start (Fig. 4). The transcriptional start was 38 bp upstream of the ATG codon (Fig. 5), 27 bp upstream from a typical Shine-Dalgarno sequence, and just downstream of a TATA box (Fig. 4). These results confirmed that the lack of a predicted signal sequence was not due to cloning artifacts.

Construction of an isogenic *ftpA* mutant. The *ftpA* ORF was insertionally inactivated in *E. coli* by shuttle mutagenesis. Sequence analysis of one plasmid, pHD24*ftpA*::mTn*3*(Cm), showed that mTn*3*(Cm) had inserted 234 bp downstream from

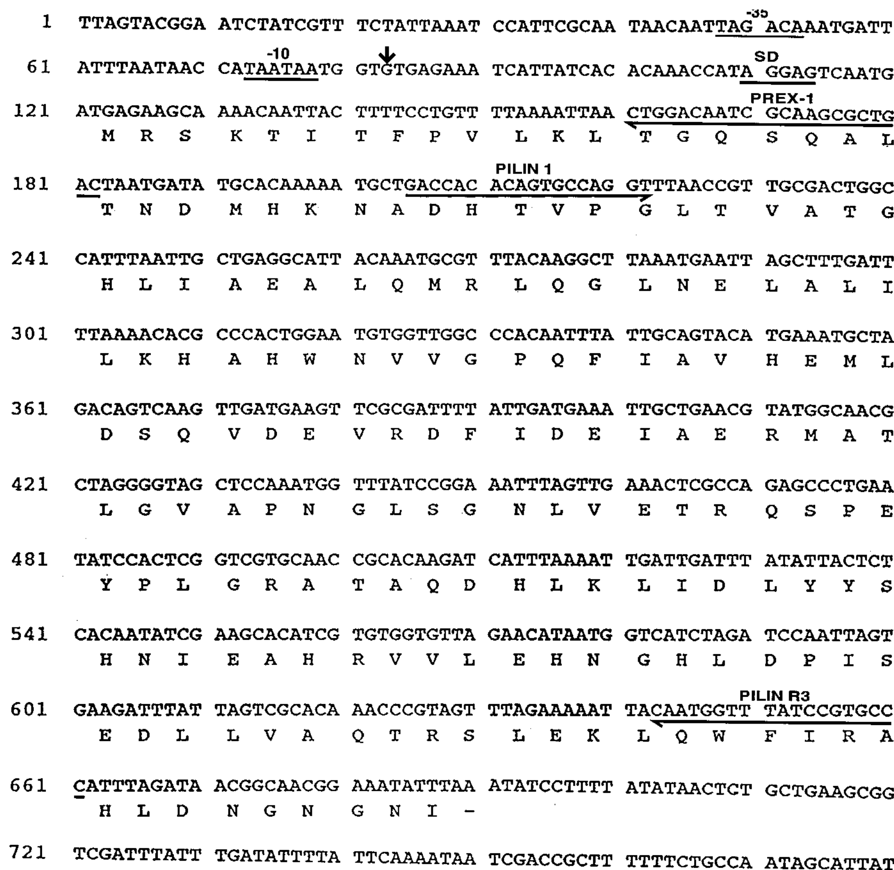

FIG. 4. Nucleotide sequence of *H. ducreyi ftpA* and predicted amino acid sequence of the pilin protein. Putative −35, −10 and Shine-Dalgarno (SD) regions and sequences corresponding to PILIN 1, PILIN R3, and PREX 1 primers are underlined. The arrow denotes the transcriptional start site determined by primer extension analysis.

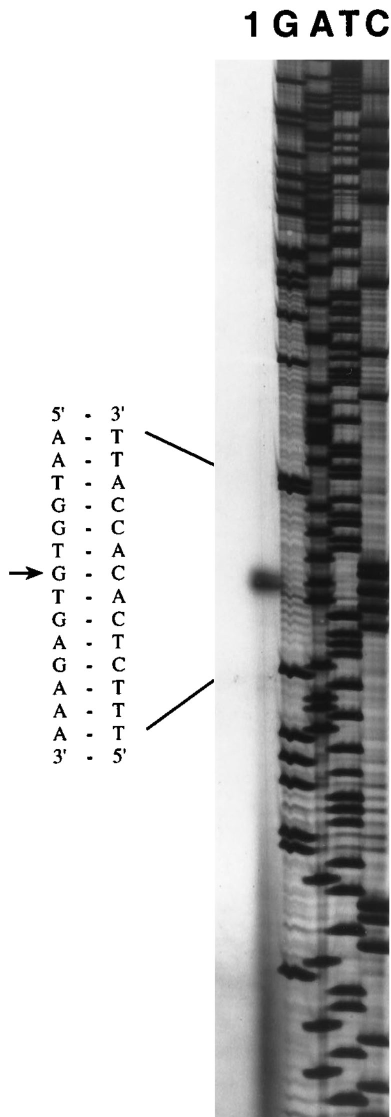

FIG. 5. Autoradiograph of a primer extension reaction mixture (lane 1) electrophoresed next to a sequencing reaction mixture with pHD24. For both reaction, primer PREX-1 was used. To the left is the sequence of pHD24 that contains the transcriptional start site, designated by the arrow.

the start of *ftpA*. Plasmid pHD24*ftpA*::mTn*3*(Cm) was transformed into *H. ducreyi* 35000 by electroporation. One transformant, *H. ducreyi* 35000*ftpA*::mTn*3*(Cm), no longer bound MAb 2D8 in a Western blot (Fig. 6).

Southern blots of *H. ducreyi* 35000 and 35000*ftpA*::mTn*3* (Cm) DNA were probed with a 450-bp fragment of *ftpA*. The probe hybridized to a 6.5-kb *Ava*I fragment in strain 35000 and to an 8.1-kb *Ava*I fragment of 35000*ftpA*::mTn*3*(Cm) (Fig. 6). *Ava*I-digested DNA from the mutant and parental strains were also probed with the *cat* cassette of pACYC184. The *cat* probe did not hybridize 35000 DNA but did hybridize to an 8.1-kb *Ava*I fragment of 35000*ftpA*::mTn*3*(Cm), confirming that allele exchange had occurred in *ftpA*.

To confirm that the *ftpA* gene product was the major subunit of fine, tangled pili, whole cells of strains 35000 and 35000*ftpA*:: mTn*3*(Cm) were examined by electron microscopy. Many piliated cells of strain 35000 were easily found, while no piliated organisms were seen in samples prepared from the mutant (Fig. 7). The outer membrane protein and lipooligosaccharide patterns of strains 35000 and 35000*ftpA*::mTn*3*(Cm) were identical (data not shown). *H. ducreyi* 35000*ftpA*::mTn*3*(Cm) colonies could be moved intact across agar, indicating that the mutation had no effect on the organism's ability to clump. There were no differences in the growth rates of the mutant and parent strains in broth. Gram strains showed that the mutant and parent formed chains when grown in broth. These data suggested that bacterial phenotypes other than the synthesis of pili were not affected by the insertional inactivation of *ftpA*. However, we cannot exclude the possibility that the mTn*3*(Cm) had polar effects on genes downstream of the *ftpA* locus.

Immunoelectron microscopy. MAb 2D8 did not bind to *H. ducreyi* in colony blot assays and did not bind to native pili in immunoelectron microscopy (data not shown). The data suggested that the epitope recognized by MAb 2D8 was present on SDS-treated material but not accessible on native structures. To exclude the possibility that the 2D8-reactive protein was abundant in the cytosol, thin sections of whole cells of strains 35000 and 35000*ftpA*::mTn*3*(Cm) were probed with 2D8. The MAb exhibited minimal binding (zero to four gold balls per cell) to strain 35000 and no reactivity to the isogenic mutant (Fig. 7), suggesting that it did not bind to intracellular structures.

Laminin-binding assays. Expression of pili by *H. ducreyi* is associated with binding to laminin (1). To test whether expression of FtpA mediated binding to laminin, we compared the abilities of 35000 and 35000*ftpA*::mTn*3*(Cm) cells to bind to laminin-coated glass slides. *E. coli* HB101(pFK10), which ex-

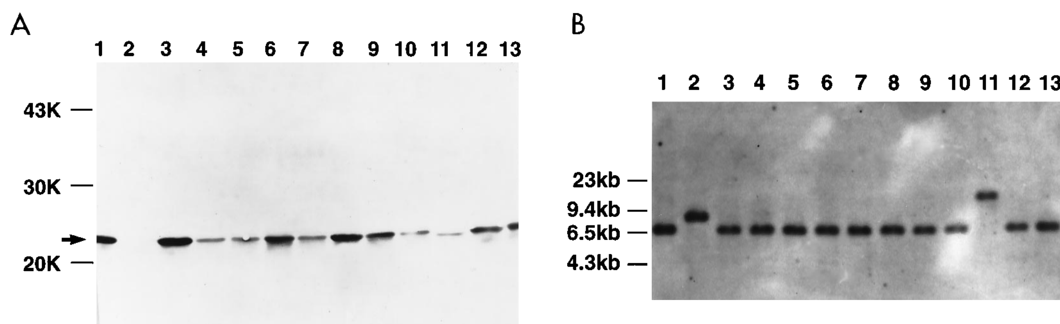

FIG. 6. (A) Western blot of whole-cell lysates of *H. ducreyi* probed with MAb 2D8 and (B) Southern blot of *Ava*I-digested chromosomal DNA probed with a fragment consisting of *ftpA* coding sequence. Lanes: 1 to 13, *H. ducreyi* strains 35000, 35000*ftpA*::mTn*3*(Cm), 85-023233, CIP A75, CIP 542, Hd183, Hd188, 82-029362, 84-018676, 6644, ATCC 33921, R1 and ATCC 27722, respectively. Molecular weights and fragment lengths are indicated on the left of panels A and B, respectively. The arrow indicates binding of MAb to the 24K pilin monomer.

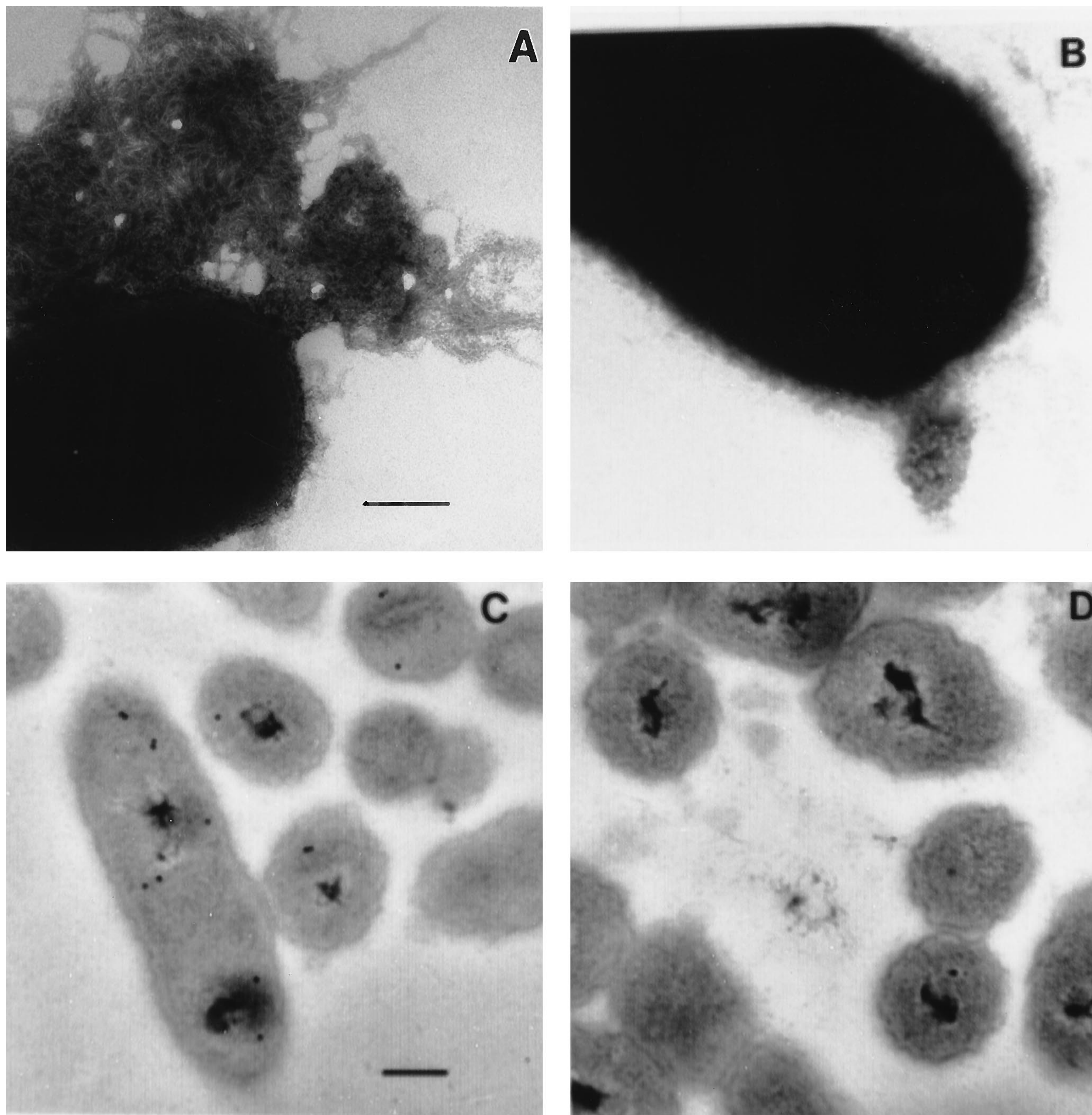

FIG. 7. Electron micrographs of strains 35000 (A and C) and 35000*ftpA*::mTn*3*(Cm) (B and D). (A and B) cells stained with phosphotungstic acid. (C and D) Ultrathin sections probed with Mab 2D8 and stained with uranyl acetate. Bars, 0.2 μm.

presses type 3 pili of *K. pneumoniae* (4) and binds to laminin (43), and *E. coli* HB101, which does not bind to laminin, served as controls in these assays. Neither of the *H. ducreyi* strains bound to the BSA-coated slides (data not shown). *E. coli* HB101 did not bind to the laminin-coated slides, while *E. coli* HB101(pFK10), 35000, and 35000*ftpA*::mTn*3*(Cm) bound equally well to laminin (data not shown). Thus, FtpA did not mediate binding of *H. ducreyi* to laminin.

Conservation of *ftpA* in *H. ducreyi*. In a Western blot, MAb 2D8 bound to a 24K protein in 12 *H. ducreyi* isolates with diverse geographic origins (Fig. 6). In a Southern blot, a 450-bp probe consisting of *ftpA* coding sequences bound 6.5-kb *Ava*I fragments in 11 of 12 strains and a 12.8-kb fragment of a Kenyan isolate (Fig. 6). Thus, there was little heterogeneity in the restriction fragments containing *ftpA*, and expression of the 2D8 epitope is conserved among *H. ducreyi* strains.

Homologies of FtpA. Search of GenEMBL, SWISSPROT, and PIR-Protein databases showed no homologies of FtpA to other pilin proteins but did show homology to *E. coli* Dps (32% identity and 72% similarity) (5) and *T. pallidum* TpF1 (also known as 4D) (21% identity and 55% similarity) (Fig. 8) over the entire sequence (32). Neither Dps nor 4D is currently

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TpF1 (4D)           MNMCTDGKKYHSTATSAAVGASAPGVPDARAIAAICEQLRQ
FtpA        MRSKTITFPVLKLTGQSQALTNDMHKNADHTVPGLTVATGHLIAEALQM
Dps                       MSTAKLVKSKATNLLYTRNDVSDSEKKATVELLNR

TpF1 (4D)   HVADLGVLYIKLHNYHWAIYGIEFKQVHELLEEYYVSVTEAFDTIAERL
FtpA        RLQGLNELALILKHAHWNVVGPQFIAVHEMLDSQVDEVRDFIDEIAERM
Dps         QVIQFIDLSLITKQAHWNMRGANFIAVHEMLDGFRTALIDHLDTMAERA

TpF1 (4D)   LQLGAQAPASMAEYLALSGIAEETEKEITIVSALARVK-RDFEYLSTRFSQ
FtpA        ATLGV-APNGLSG--NLVETRQSPEYPLGRATAQDHLK-LIDLYYSHNIEA
Dps         VQLGG-VALGTTQ--VINSKTPLKSYPLDIHNVQDHLKELADRYAIVANDV

TpF1 (4D)   TQVLAAESG--DAVTDGIITDILRTLGKAIWMLGATLKA
FtpA        HRVVLEHNGHLDPISEDLLVAQTRSLEKLQWFIRAHLDNGNGNI
Dps         RKAIGEAK---DDDTADILTAASRDLDKFLWFIESNIE
```

FIG. 8. Sequence alignment of *H. ducreyi* FtpA with *T. pallidum* TpF1 (4D) and *E. coli* Dps. Identical residues are indicated by single lines; similar residues are indicated by two dots. The positions of the amino acids of the bacterioferritins that participate in metal ion binding are designated by the arrows; only amino acids 81 (D) and 100 (E) of FtpA are identical or conservatively substituted.

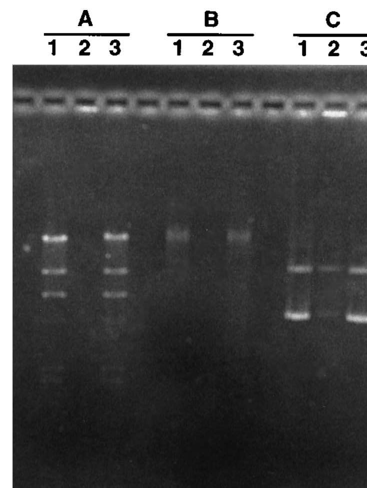

FIG. 9. Agarose gel electrophoresis of DNA (lane 1), DNA complexed with Dps (lane 2), and DNA complexed with FtpA (lane 3). (A) λ*Hin*dIII DNA standards; (B) *H. ducreyi* chromosomal DNA; (C) pHD24.

thought to be exported through the outer membrane (5, 11). Both proteins are composed of monomers that multimerize to form ordered ring structures that are 9 to 10 nm in diameter (5, 34). The 4D antigen and Dps share homology with known or putative prokaryotic bacterioferritins, which also aggregate into ordered rings or paracrystalline arrays (19). Although the function of 4D is unknown, it is thought to be a protoplasmic cylinder-associated protein. The 4D antigen contains a motif of 7 conserved amino acids dispersed along its sequence that are found in bacterioferritins and participate in metal ion binding (10). Dps contains only 4 of these 7 conserved amino acids, is produced only in stationary phase, forms hexagonal complexes that copurify with DNA, and protects *E. coli* from oxidative injury, perhaps by complexing with DNA. In vitro, Dps binds nonspecifically to DNA so that DNA-Dps complexes will not enter an agarose gel (5).

We compared the properties of FtpA to those of the bacterioferritins and Dps. FtpA contained only 1 conserved and 1 conservatively substituted amino acid of the metal ion-binding motif (Fig. 8). By Western blot analysis, FtpA was made by *H. ducreyi* in all phases of growth (data not shown). FtpA and Dps were tested for their abilities to form DNA-protein complexes (5). Unlike Dps, FtpA had no effect on the entry of DNA into an agarose gel (Fig. 9). Thus, FtpA shares homology and some physical characteristics with the bacterioferritins and Dps, but there are differences among these proteins.

DISCUSSION

To facilitate the study of *H. ducreyi* pili, we developed a rapid method of pilus purification based on SDS insolubility and a MAb that bound to the major pilin subunit. Using the MAb as a probe, we isolated the gene encoding the 24K pilin protein, *ftpA*, from an *H. ducreyi* λgt11 library. The translated amino acid sequence of FtpA matched the N-terminal sequence of the 24K protein purified by two different methods. An isogenic *ftpA* mutant, *H. ducreyi* 35000*ftpA*::mTn*3*(Cm), no longer expressed the 24K protein and did not contain pili when examined by electron microscopy. Thus, *ftpA* encoded the major pilin protein of fine, tangled pili.

H. ducreyi pili were partially purified on the basis of their insolubility in 1% SDS. Like other pilin proteins, the major pilin subunit dissociated from pili by heating the preparation (13, 22, 38). Similarly, the thin aggregative fimbriae of *Salmonella enteriditis* and curli of *E. coli* are purified on the basis of their insolubility in SDS (15, 16). However, thin aggregative fimbriae and curli dissociate into monomers only after treatment with formic acid (8, 15). Formic acid treatment of fine, tangled pili was not required to dissociate *H. ducreyi* pili into the major pilin subunit and did not reveal the presence of other proteins from the preparation (data not shown). Thus, *H. ducreyi* pili had physical characteristics similar to those of other pili.

Translation of the *ftpA* ORF predicts a polypeptide with an N-terminal sequence that was identical to that the purified pilin protein and lacked a cleavable signal sequence. We confirmed that *ftpA* lacked sequences encoding a cleavable leader peptide by primer extension analysis of mRNA isolated from *H. ducreyi*. The data suggested that the FtpA is exported by a signal sequence-independent mechanism. To our knowledge, all described pilin proteins have cleavable signal sequences. Most pilins are believed to be processed by LepB signal peptidase (signal peptidase I) of the general secretory pathway (33). Unique export mechanisms have been reported for type IV pilins, but these mechanisms are also dependent on cleavable signal sequences (25, 29). However, proteins that lack cleavable signal sequences may be exported. Examples include hemolysins, colicins, cytotoxins, and metalloproteases (17, 21, 29). Although these proteins are exported by pathways that have not been implicated in pilin export, our data suggest that a signal sequence-independent pathway exists for export of *H. ducreyi* pilin.

The *ftpA* gene predicted a 189-amino-acid polypeptide that contains 46% hydrophobic nonpolar residues and is similar to other pilins as reported previously (40). We had previously reported that the 24K protein purified by acid crystallization, base solubilization, and preparative SDS-PAGE contained two cysteine residues (40). However, the predicted amino acid sequence of FtpA lacked cysteine residues. Our previous results may have been due to possible contamination of the preparation by a comigrating protein or experimental error. Although pilin proteins often contain cysteine residues that form disulfide bonds and have a role in secondary structure, several pilins that lack cysteine residues have also been reported (13, 16).

MAb 2D8, which was raised by immunization with SDS-insoluble pili, did not bind to *H. ducreyi* colonies or pili when examined by immunoelectron microscopy and bound minimally to the cytosol of sectioned whole cells. An anti-pilus polyclonal serum raised to SDS-insoluble pili bound to purified pili but did not bind to native pilus structures on whole cells (data not shown). However, polyclonal serum raised to the 24K protein purified by acid crystallization, base solubilization, preparative SDS-PAGE, and electroelution binds to pilus structures on whole cells (22). This data suggests antibodies raised to SDS-treated material bind to epitopes that are not accessible on native pili.

Sequence analysis of *ftpA* determined that the pilin protein shared homology with Dps and 4D, which are similar to known or putative prokaryotic bacterioferritins (19). Similar to FtpA, Dps, 4D, and bacterioferritins form multimers (19) that dissociate with heat (5, 20). The number of monomers required to form the ordered ring structure of 4D is unknown (20). The ordered ring formed by Dps may consist of 12 subunits arranged in two hexameric rings (5). The 150K multimer formed by FtpA may be composed of six subunits. Although FtpA, Dps, 4D and the bacterioferritins share some common features, there are differences among these proteins. FtpA and Dps lack most of the conserved amino acids that participate in bacterioferritin metal ion binding. Dps and 4D form multimers in the shape of 9- to 10-nm-diameter ordered rings, while FtpA forms thin, tangled filaments that are 3 nm in diameter. Dps is expressed by *E. coli* only in stationary phase, while FtpA was expressed in all phases of growth. Dps forms complexes with DNA that are unable to enter an agarose gel (5), while the FtpA had no effect on DNA migration in agarose gels. Dps is soluble in 1% SDS (25a) while the FtpA is insoluble in 1% SDS. *H. ducreyi* pili were initially purified by mechanical dissociation, precipitation at pH 5.0, and solubilization at pH 10.5 (40). In contrast, the 4D antigen forms multimers at pH 9.5 and dissociates at pH 4.0 (20). Thus, FtpA has homology to proteins that form multimers but has several distinct characteristics.

We conclude that *H. ducreyi* expresses a unique class of pili. Future studies will include analysis of the pilin export mechanism and determination of pilin receptor binding activity.

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REFERENCES

1. **Abeck, D., A. P. Johnson, and H. Mensing.** 1992. Binding of *Haemophilus ducreyi* to extracellular matrix proteins. Microb. Pathog. **13:**81–84.
2. **Abu Kwaik, Y., R. E. McLaughlin, M. A. Apicella, and S. M. Spinola.** 1991. Analysis of *Haemophilus influenzae* type b lipooligosaccharide-synthesis genes that assemble or expose a 2-keto-3-deoxyoctulosonic acid epitope. Mol. Microbiol. **5:**2475–2480.
3. **Alfa, M. J., P. Degagne, and T. Hollyer.** 1993. *Haemophilus ducreyi* adheres to but does not invade cultured human foreskin cells. Infect. Immun. **61:** 1735–1742.
4. **Allen, B. L., G. F. Gerlach, and S. Clegg.** 1991. Nucleotide sequence and functions of *mrk* determinants necessary for expression of type 3 fimbriae in *Klebsiella pneumoniae*. J. Bacteriol. **173:**916–920.
5. **Almiron, M., A. J. Link, D. Furlong, and R. Kolter.** 1992. A novel DNA-binding protein with regulatory and protective roles in starved *Escherichia coli*. Genes Dev. **6:**2646–2654.
6. **Apicella, M. A., K. C. Dudas, A. Campagnari, P. Rice, J. M. Mylotte, and T. F. Murphy.** 1985. Antigenic heterogeneity of lipid A of *Haemophilus influenzae*. Infect. Immun. **50:**9–14.
7. **Apicella, M. A., R. E. Mandrell, M. Shero, M. E. Wilson, J. M. Griffiss, G. F. Brooks, C. Lammel, J. F. Breen, and P. A. Rice.** 1990. Modification by sialic acid of *Neisseria gonorrhoeae* lipooligosaccharide epitope expression in human urethral exudates: an immunoelectron microscopic analysis. J. Infect. Dis. **162:**506–512.
8. **Arnqvist, A., A. Olsen, J. Pfeifer, D. G. Russell, and S. Normark.** 1992. The Crl protein activates cryptic genes for curli formation and fibronectin binding in *Escherichia coli* HB101. Mol. Microbiol. **6:**2443–2452.
9. **Baker, R. F., and C. Yanofsky.** 1968. The periodicity of RNA polymerase initiations: a new regulatory feature of transcription. Proc. Natl. Acad. Sci. USA **60:**313–320.
10. **Blanco, D. R., K. Reimann, J. Skare, C. I. Champion, D. Foley, M. M. Exner, R. E. W. Hancock, J. N. Miller, and M. A. Lovett.** 1994. Isolation of the outer membranes from *Treponema pallidum* and *Treponema vincentii*. J. Bacteriol. **176:**6088–6099.
11. **Blanco, D. R., E. M. Walker, D. A. Haake, C. I. Champion, J. N. Miller, and M. A. Lovett.** 1990. Complement activation limits the rate of in vitro treponemicidal activity and correlates with antibody-mediated aggregation of *Treponema pallidum* rare outer membrane protein. J. Immunol. **144:**1914–1921.
12. **Brentjens, R. J., S. M. Spinola, and A. A. Campagnari.** 1994. *Haemophilus ducreyi* adheres to human keratinocytes. Microb. Pathog. **16:**243–247.
13. **Buhler, T., H. Hoschutzky, and K. Jann.** 1991. Analysis of colonization factor antigen I, an adhesin of enterotoxigenic *Escherichia coli* 078:H11: fimbrial morphology and location of the receptor-binding site. Infect. Immun. **59:** 3876–3882.
14. **Chung, C. T., S. L. Niemela, and R. H. Miller.** 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. USA **86:**2172–2175.
15. **Collinson, S. K., L. Emody, K. H. Muller, T. J. Trust, and W. W. Kay.** 1991. Purification and characterization of thin, aggregative fimbriae from *Salmonella enteritidis*. J. Bacteriol. **173:**4773–4781.
16. **Collinson, S. K., L. Emody, T. J. Trust, and W. W. Kay.** 1992. Thin aggregative fimbriae from diarrheagenic *Escherichia coli*. J. Bacteriol. **174:**4490–4495.
17. **Delepelaire, P., and C. Wandersman.** 1990. Protein secretion in gram-negative bacteria: the extracellular metalloprotease B from *Erwinia chrysanthemi* contains a C-terminal secretion signal analogous to that of *Escherichia coli* hemolysin. J. Biol. Chem. **265:**17118–17125.
18. **Desjardins, M., L. G. Filion, S. Robertson, and D. W. Cameron.** 1995. Inducible immunity with a pilus preparation booster vaccination in an animal model of *Haemophilus ducreyi* infection and disease. Infect. Immun. **63:** 2012–2020.
19. **Evans, D. J., Jr., D. G. Evans, H. C. Lampert, and H. Nakano.** 1995. Identification of four new prokaryotic bacterioferritins, from *Helicobacter pylori*, *Anabaena variabilis*, *Bacillus subtilis*, and *Treponema pallidum*, by analysis of gene sequences. Gene **153:**123–127.
20. **Fehniger, T. E., J. D. Radolf, and M. A. Lovett.** 1986. Properties of an ordered ring structure formed by recombinant *Treponema pallidum* surface antigen 4D. J. Bacteriol. **165:**732–739.
21. **Felmlee, T., S. Pellett, E. Y. Lee, and R. A. Welch.** 1985. *Escherichia coli* hemolysin is released extracellularly without celevage of a signal peptide. J. Bacteriol. **163:**88–93.
22. **Frisk, A., E. L. Roggen, and T. Lagergard.** 1995. Localisation and immunological properties of a 24-kDa surface protein of *Haemophilus ducreyi*. J. Med. Microbiol. **43:**192–200.
23. **Hansen, E. J., J. L. Latimer, S. E. Thomas, M. Helminen, W. L. Albritton, and J. D. Radolf.** 1992. Use of electroporation to construct isogenic mutants of *Haemophilus ducreyi*. J. Bacteriol. **174:**5442–5449.
24. **Hayes, R. J., K. F. Schulz, and F. A. Plummer.** 1995. The cofactor effect of genital ulcers on the per-exposure risk of HIV transmission in sub-Saharan Africa. J. Trop. Med. Hyg. **98:**1–8.
25. **Kaufman, M. R., J. M. Seyer, and R. K. Taylor.** 1991. Processing of TCP pilin by TcpJ typifies a common step intrinsic to a newly recognized pathway of extracellular protein secretion by gram-negative bacteria. Genes Dev. **5:**1834–1846.

25a. **Kolter, Robert.** Personal communication.

26. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
27. **Lagergard, T., M. Purven, and A. Frisk.** 1993. Evidence of *Haemophilus ducreyi* adherence to and cytotoxin destruction of human epithelial cells. Microb. Pathog. **14:**417–431.

28. **Lammel, C. J., N. P. Dekker, J. Palefsky, and G. F. Brooks.** 1993. In vitro model of *Haemophilus ducreyi* adherence to and entry into eukaryotic cells of genital origin. J. Infect. Dis. **167:**642–650.
29. **Lory, S.** 1992. Determinants of extracellular protein secretion in gram-negative bacteria. J. Bacteriol. **174:**3423–3428.
30. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Morse, S. A.** 1989. Chancroid and *Haemophilus ducreyi*. Clin. Microbiol. Rev. **2:**137–157.
32. **Noordhoek, G. T., P. W. M. Hermans, A. N. Paul, L. M. Schouls, J. J. VanDerSluis, and J. D. A. VanEmbden.** 1989. *Treponema pallidum* subspecies pallidum (Nichols) and *Treponema pallidum* subspecies pertenue (CDC 2575) differ in at least one nucleotide: comparison of two homologous antigens. Microb. Pathog. **6:**29–42.
33. **Pugsley, A. P.** 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol. Rev. **57:**50–108.
34. **Radolf, J. D., L. A. Borenstein, J. Y. Kim, T. E. Fehniger, and M. A. Lovett.** 1987. Role of disulfide bonds in the oligomeric structure and protease resistance of recombinant and native *Treponema pallidum* surface antigen 4D. J. Bacteriol. **169:**1365–1371.
35. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
36. **Seifert, H. S., M. So, and F. Heffron.** 1986. Shuttle mutagenesis: a method of introducing transposons into transformable organisms, p. 123–134. *In* J. K. Setlow and A. Hollander (ed.), Genetic engineering, principles and methods. Plenum Press, N.Y.
37. **Shah, L., H. A. Davies, and R. A. Wall.** 1992. Association of *Haemophilus ducreyi* with cell-culture lines. J. Med. Microbiol. **37:**268–272.
38. **Sojar, H. T., J. Y. Lee, G. S. Bedi, M. I. Cho, and R. J. Genco.** 1991. Purification, characterization and immunolocalization of fimbrial protein from *Porphyromonas* (*Bacteroides*) *gingivalis*. Biochem. Biophys. Res. Commun. **175:**713–719.
39. **Spinola, S. M., Y. Abu Kwaik, A. J. Lesse, A. A. Campagnari, and M. A. Apicella.** 1990. Cloning and expression in *Escherichia coli* of a *Haemophilus influenzae* type b lipooligosaccharide synthesis gene(s) that encodes a 2-keto-3-deoxyoctulosonic acid epitope. Infect. Immun. **58:**1558–1564.
40. **Spinola, S. M., A. Castellazzo, M. Shero, and M. A. Apicella.** 1991. Characterization of pili expressed by *Haemophilus ducreyi*. Microb. Pathog. **9:**417–426.
41. **Spinola, S. M., G. E. Griffiths, J. A. Bogdan, and M. A. Menegus.** 1992. Characterization of an 18,000-molecular-weight outer membrane protein of *Haemophilus ducreyi* that contains a conserved surface-exposed epitope. Infect. Immun. **60:**385–391.
42. **Spinola, S. M., G. E. Griffiths, K. L. Shanks, and M. S. Blake.** 1993. The major outer membrane protein of *Haemophilus ducreyi* is a member of the OmpA family of proteins. Infect. Immun. **61:**1346–1351.
43. **Tarkkanen, A. M., B. L. Allen, B. Westerlund, H. Holthofer, P. Kuusela, L. Risteli, S. Clegg, and T. K. Korhonen.** 1990. Type V collagen as the target for type-3 fimbriae, enterobacterial adherence organelles. Mol. Microbiol. **4:**1353–1361.
44. **Telzak, E. E., M. A. Chiasson, P. J. Bevier, R. L. Stoneburner, K. G. Castro, and H. W. Jaffe.** 1993. HIV-1 seroconversion in patients with and without genital ulcer disease. Ann. Intern. Med. **119:**1181–1186.
45. **Totten, P. A., J. C. Lara, D. V. Norn, and W. E. Stamm.** 1994. *Haemophilus ducreyi* attaches to and invades human epithelial cells in vitro. Infect. Immun. **62:**5632–5640.
46. **Trees, D. L., and S. A. Morse.** 1995. Chancroid and *Haemophilus ducreyi*: an update. Clin. Microbiol. Rev. **8:**357–375.
47. **Wasserheit, J. N.** 1992. Epidemiological synergy. Interrelationships between human immunodeficiency virus infection and other sexually transmitted diseases. Sex. Transm. Dis. **19:**61–77.
48. **Westerlund, B., P. Kuusela, J. Risteli, L. Risteli, T. Vartio, H. Rauvala, R. Virkola, and T. K. Korhonen.** 1989. The 075X adhesin of uropathogenic *Escherichia coli* is a type IV collagen-binding protein. Mol. Microbiol. **3:**329–337.