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# Nucleotide Sequences of Genes Coding for Fimbrial Proteins in a Cryptic Genospecies of *Haemophilus* spp. Isolated from Neonatal and Genital Tract Infections

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**Nineteen isolates belonging to a cryptic genospecies of *Haemophilus* (referred to here as genital strains) isolated from genital tract infections (6 strains) and from neonatal infections (13 strains) were studied for fimbrial genes. Sixteen strains exhibit peritrichous fimbriae observed by electron microscopy. By PCR with primers corresponding to the extreme ends of the *Haemophilus influenzae* type b (Hib) *hifA* and *hifD* genes and Southern blotting, a *hifA*-like gene (named *ghfA*) and a *hifD*-like gene (named *ghfD*) were identified in 6 of the 19 strains. Five of these six strains were from the genital tracts of adults, and one was from a neonate. For each gene, the nucleotide sequence was identical for the six strains. A *hifE*-like gene (named *ghfE*) was amplified from only one of the 19 genital strains of *Haemophilus*, but the *ghfE* probe gave a signal in Southern hybridization with the five other strains positive for *ghfA* and *ghfD*. Therefore, these strains may carry a *ghfE*-like gene. The Hib fimbrial gene cluster is located between the *purE* and *pepN* genes as previously described. For the 13 genital *Haemophilus* strains that lack fimbrial genes, this region corresponds to a noncoding sequence. Another major fimbrial gene designated the fimbrin gene was previously identified in a nontypeable *H. influenzae* strain. A fimbrin-like gene was identified for all of our 19 genital strains. This gene is similar to the *ompP5* gene of many *Haemophilus* strains. Therefore, other, unidentified genes may explain the piliation observed in electron microscopy on genital *Haemophilus* strains which do not possess LKP-like fimbrial genes. Fimbrial genes were significantly associated with strains isolated from the genital tract. They may confer on the strain the ability to survive in the genital tract.**

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*Haemophilus influenzae* strains are gram-negative rods which colonize human respiratory and genital mucosa. Although they are commensal bacteria, they can cause serious respiratory tract infections, meningitis, and genital and neonatal infections. Adhesion to epithelial cells is the first step in host colonization by many bacteria (5, 9). Adhesion can be mediated by nonfimbrial and fimbrial structures. Most nontypeable strains of *Haemophilus* (95%) adhere to human epithelial cells either by high-molecular-weight surface proteins (84%) or by Hia protein (16%) (4, 34, 35). Short, thin surface fibrils involved in *H. influenzae* type b (Hib) adherence to human epithelial cells were recently identified (31, 32). Both Hib and nontypeable *H. influenzae* (NTHi) strains commonly express fimbriae, which are polymeric structures composed of a major structural protein associated with several other minor proteins. The fimbrial proteins of a large number of gram-negative bacteria have been purified, and their sequences have been determined. The major fimbrial proteins from different species are often very similar (14, 15, 36, 38). Fimbria-mediated adhesion to cells has also been reported for *H. influenzae* strains that colonize the nasopharynx and that are responsible for various infections, including meningitis, chronic bronchitis, otitis media, and Brazilian purpuric fever (3, 12, 21, 29, 37).

The genes coding for the major fimbrial subunit (*hifA* genes) in several Hib strains and in some NTHi strains responsible for various diseases have been characterized. The deduced amino acid sequences of these proteins are about 80% similar, and

the amino acid sequences are particularly well conserved at the N- and C-terminal ends (2, 7, 10, 15, 33, 38, 42). The genes from some Hib strains coding for minor proteins that constitute fimbriae (*hifD* and *hifE* genes) have been characterized (18, 39). These two genes map downstream from the *hifA* gene. The complete fimbrial gene cluster in Hib has been identified and lies between the *purE* and *pepN* genes (11, 39). The adhesive component in *H. influenzae* fimbriae has not been clearly defined; it could be the major subunit (HifA) or HifE, a minor subunit located at the tips of fimbriae (17, 40).

Another fimbrial gene in a strain of NTHi has been characterized. It encodes a fimbrin protein, an adhesin homologous to OmpA proteins of many gram-negative bacteria (30).

In the last 20 years, urogenital, mother-infant, and neonatal infections caused by *Haemophilus* strains have been reported with increasing frequency (1, 16, 23, 41). The members of a group of *Haemophilus* strains isolated from neonatal and genital tract infections (referred to here as genital strains) and commonly identified as *H. influenzae* biotype IV have several unusual features (19, 24, 26). Genetic analysis of these strains demonstrates that they constitute a cryptic genospecies which forms a monophyletic unit with *H. influenzae* and *Haemophilus haemolyticus*, most closely related to *H. haemolyticus* (25, 27). These isolates may have a specific tropism for the genital tract. They were not detected among a large number of *H. influenzae* isolates recovered from invasive, respiratory, and other infections in children and adults (1, 20). Most express peritrichous fimbriae, adhere better to HeLa cells than to HEp-2 cells and do not cause agglutination of human erythrocytes expressing the AnWj antigen (28), contrasting with numerous fimbriated *H. influenzae* strains responsible for respiratory infections and meningitis (3, 12, 21, 37). Therefore, if these fimbriae are

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TABLE 1. Identification, origin, piliation, and presence of fimbrial genes for *Haemophilus* strains

| Strain | Identification[a] | Biotype (capsular type) | Origin | Anatomic site | Presence of[c]: | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Fimbriae | *ghfA* | *ghfD* | *ghfE* |
| Control strain 770235 | *H. influenzae* | I (b) | The Netherlands | Cerebrospinal fluid | + | + | + | + |
| **Collection strains** | | | | | | | | |
| CIP 52152 | *H. influenzae* | I (b) | Unknown | Unknown | 0 | 0 | 0 | 0 |
| CIP 102514 | *H. influenzae* | II (NT[b]) | Unknown | Unknown | + | 0 | 0 | 0 |
| CIP 102284 | *H. influenzae* | III (NT) | France | Sputum | ND | 0 | 0 | 0 |
| CIP 5424 | *H. influenzae* | IV (NT) | Unknown | Unknown | 0 | 0 | 0 | 0 |
| CIP 5483 | *H. influenzae* | IV (d) | Unknown | Unknown | 0 | 0 | 0 | 0 |
| CIP 5494 | *H. influenzae* | IV (b) | United States | Cerebrospinal fluid | + | + | + | + |
| CIP 5484 | *H. influenzae* | IV (e) | Unknown | Unknown | 0 | 0 | 0 | 0 |
| CIP 52154 | *H. influenzae* | IV (d) | Unknown | Unknown | 0 | 0 | 0 | 0 |
| CIP 52155 | *H. influenzae* | IV (e) | Unknown | Unknown | 0 | 0 | 0 | 0 |
| CIP 103290 | *H. haemolyticus* | | Unknown | Unknown | + | 0 | 0 | 0 |
| CIP 102348 | *H. haemolyticus* | | France | Sputum | 0 | 0 | 0 | 0 |
| CIP 52129 | *H. influenzae* biogroup Aegyptius | | United States | Conjunctiva | 0 | + | 0 | + |
| **Clinical isolates** | | | | | | | | |
| 189 | Genital *Haemophilus* | | United States | Amniotic fluid | +[d] | 0 | 0 | 0 |
| 421 | Genital *Haemophilus* | | United States | Blood of neonate | 0 | 0 | 0 | 0 |
| 422 | Genital *Haemophilus* | | United States | Blood of neonate | 0 | 0 | 0 | 0 |
| 427 | Genital *Haemophilus* | | United States | Amniotic fluid | 0 | 0 | 0 | 0 |
| 799 | Genital *Haemophilus* | | United States | Blood of neonate | + | 0 | 0 | 0 |
| 847 | Genital *Haemophilus* | | United States | Scrotal abcess | + | 0 | 0 | 0 |
| 911 | Genital *Haemophilus* | | United States | Cerebrospinal fluid of neonate | + | 0 | 0 | 0 |
| 1595 | Genital *Haemophilus* | | United States | Blood of neonate | + | 0 | 0 | 0 |
| 1610 | Genital *Haemophilus* | | United States | Blood of neonate | + | 0 | 0 | 0 |
| 3N | Genital *Haemophilus* | | France | Gastric fluid of neonate | +[d] | 0 | 0 | 0 |
| 10N | Genital *Haemophilus* | | France | Gastric fluid of neonate | + | 0 | 0 | 0 |
| 12N | Genital *Haemophilus* | | France | Gastric fluid of neonate | + | 0 | 0 | 0 |
| 15N | Genital *Haemophilus* | | France | Gastric fluid of neonate | + | + | + | 0 |
| 16N | Genital *Haemophilus* | | France | Gastric fluid of neonate | + | 0 | 0 | 0 |
| 10U | Genital *Haemophilus* | | France | Urethra (male) | + | + | + | 0 |
| 11PS | Genital *Haemophilus* | | France | Urethra (male) | + | + | + | 0 |
| 26E | Genital *Haemophilus* | | France | Uterus | + | + | + | + |
| PIZ | Genital *Haemophilus* | | France | Endocervix | + | + | + | 0 |
| 2406 | Genital *Haemophilus* | | France | Vagina | + | + | + | 0 |

[a] Genital *Haemophilus* strains were phenotypically identified as *H. influenzae* biotype IV, except strain 2406, which was phenotypically identified as *H. parainfluenzae*.
[b] NT, nontypeable.
[c] +, present; 0, absent; ND, not determined. The presence of fimbriae was determined by electron microscopy; the presence of fimbrial genes (*ghfA*, *ghfD*, and *ghfE*) was assessed by PCR and confirmed by Southern blotting.
[d] This strain expressed fimbriae only after enrichment.

involved in the colonization of the genital tract, they may differ from those produced by strains isolated from the respiratory tract.

Our objective was to explore a group of 19 strains genetically assigned to the cryptic genital *Haemophilus* genospecies (27) for the presence of fimbrial genes. In addition, this population was investigated for the presence of the gene coding for fimbrin. When present, these genes were sequenced, and the sequences were compared to those of previously described genes coding for proteins that are components of the fimbriae of *H. influenzae*. If no such gene was detected, the absence of these genes was confirmed by Southern blotting experiments and the nucleotide sequence between the *purE* and *pepN* genes was analyzed. For each isolate, the expression and the morphology of fimbriae were studied by electron microscopy.

## MATERIALS AND METHODS

**Bacterial strains.** Thirty-one *Haemophilus* strains were studied, including 1 *H. influenzae* biotype I strain, 1 *H. influenzae* biotype II strain, 1 *H. influenzae* biotype III strain, 6 *H. influenzae* biotype IV sensu stricto strains, 2 *H. haemo-*

*lyticus* strains, 1 *H. influenzae* biogroup Aegyptius strain, and 19 strains previously assigned to the cryptic genital *Haemophilus* genospecies on the basis of DNA-DNA hybridization and small-subunit ribosomal DNA sequencing (25, 27). Genital strains of *Haemophilus* from the United States were kindly provided by J. M. Musser (Department of Pathology, Baylor College of Medicine, Houston, Tex.). The anatomic and geographic origins of these strains are reported in Table 1. Strains were stored at $-80°C$ in Schaedler-vitamin $K_3$ broth (bioMérieux, Marcy l'Etoile, France) with 10% glycerol. A heavily fimbriated acapsular variant of *H. influenzae* (strain 770235), kindly provided by L. van Alphen (Department of Medical Microbiology, University of Amsterdam, Amsterdam, The Netherlands), was used as a control. This strain harbors long, thick, hemagglutination-positive (LKP) pili. The nucleotide sequences of the genes coding for proteins constituting the fimbriae of this strain have been reported by van Ham et al. (39).

**Electron microscopy.** The presence and appearance of fimbriae were examined by electron microscopy before and after selection of piliated *Haemophilus* strains as previously described (28). Bacteria grown to stationary phase were washed three times in saline buffer, settled onto 400-mesh copper grids coated with carbon film, negatively stained with a 1.5% uranyl acetate solution in distilled water as previously described (28), and then examined with a JEOL 1010 electron microscope at 80 kV.

**Genomic DNA extraction.** Each strain was subcultured on four chocolate agar plates (15 by 15 cm) for 18 to 24 h at 37°C in 8% $CO_2$. The cultures were checked visually for purity, harvested in 15 ml of buffer (40 mM Tris, 2 mM EDTA, pH 8), and lysed by adding 150 µl of a 25% (wt/vol) aqueous solution of sodium dodecyl sulfate and 22.5 µl of 2% self-digested pronase (Sigma, St. Louis, Mo.).

TABLE 2. Nucleotide sequences of PCR primers used to amplify a
*hifA*-like gene from genital *Haemophilus* strains

| Primer[a] | Nucleotide sequence |
|---|---|
| N1[b] ............................5′ | CTCTTTATGGAGCAATTTATTATG 3′ |
| N34[b] ..........................5′ | GGTAAGGTTGTTGAGAATACTTGT 3′ |
| N51............................5′ | AGTGTAGTATTAAATGATGTGGTTAAA 3′ |
| N69............................5′ | GCAATGCCAACGCCATTTACGATT 3′ |
| C186...........................5′ | GTAATATTGGGCGATAAAGTGGAG 3′ |
| C198...........................5′ | GAGAACATGAAATGGTCGTCAACG 3′ |
| C207[b] .........................5′ | TTATTCGTAAGCAATTTGGAAATTTACTGA 3′ |
| Crypt N[b] ...................5′ | ACTGATTGTGCTGTAGCAAATGGA 3′ |
| Crypt C[b] ...................5′ | AGTTTTACCATTTAATTCAGTTGC 3′ |

[a] Primers N1, N34, N51, and N69 were identical to the leading strand; primers
C186, C198, C207, and Crypt C were identical to the lagging strand.
[b] This primer gave an amplification product.

TABLE 3. Nucleotide sequences of PCR primers used to amplify
a fimbrin-like gene and genes coding for minor fimbrial
proteins from genital *Haemophilus* strains

| Primer[a] | Nucleotide sequence | Position[b] |
|---|---|---|
| fim5 | 5′ GGACATCAAAATGAAAAAAACTGC 3′ | 396 |
| fim3 | 5′ TTATTTAGTACCGTTTACTGCGAT 3′ | 1485 |
| hifD5 | 5′ CAAAAAACACCCAAAAAATTAACC 3′ | 5467 |
| hifD3 | 5′ AGTTATAACTGCACTTGAAAAGTT 3′ | 6112 |
| hifE5 | 5′ ATGAAAACTTTAACAACATACGCA 3′ | 6138 |
| hifE3 | 5′ ATTGATATGACATTGTGAAAGTGG 3′ | 7443 |
| purE | 5′ GACCCCATCACAACGGCAATTTGT 3′ | 447 |
| pepN | 5′ CTGTGACCGTAAAATCTGGTTGTT 3′ | 7017 |

[a] Primers fim5, hifD5, hifE5, and purE were identical to the leading strand;
primers fim3, hifD3, hifE3, and pepN were identical to the lagging strand.
[b] For primers fim5 and fim3, the numbers correspond to the position of the
first 5′ base of the oligonucleotide by the numbering of Sirakova et al. (30); for
all other primers, the numbers correspond to the position of the first 5′ base of
the oligonucleotide by the numbering of van Ham et al. (39).

The mixture was incubated overnight at 37°C, and DNA was extracted and
purified as previously described (6), dialyzed on 0.025-μm-pore-size filters (Millipore, St Quentin en Yvelines, France), and diluted in water to a concentration
of 10 μg/ml.

**PCR assay.** One hundred nanograms of DNA was used for PCR. Eleven
primers were used to amplify genes coding for a major fimbrial subunit (Eurogentec, Seraing, Belgium) (Tables 2 and 3). Primers N1, N34, N51, N69, C189,
C198, and C207 were designed to correspond to conserved sequences in the *hifA*
genes of Hib 770235, AO2, and Eagan, NTHi M37 and 1128, and *H. influenzae*
biogroup Aegyptius, described previously (Fig. 1 and Table 2) (2, 8, 10, 39, 42).
Primers were named as follows: the letters N and C indicate the localization of
the primer sequence in the 5′ and 3′ ends of the gene, respectively, and the
number following the letter corresponds to the position of the first or the last
translated codon, respectively (Fig. 1). The Crypt N and Crypt C primers were
designed to correspond to two regions that are different in the major fimbrial
protein genes of genital *Haemophilus* strains and of *H. influenzae* (Fig. 1 and
Table 2). Primers fim5 and fim3 were deduced from a gene coding for the
fimbrial subunit (fimbrin) of the NTHi 1128 strain sequenced by Sirakova et al.
(30) (Table 3).

Two sets of primers were used to amplify the genes coding for minor proteins.
Each set of primers was defined at the extreme ends of the *hifD* and *hifE* genes
of Hib strain 770235 (39) (Table 3).

In addition, two primers (purE and pepN) (Table 3) correspond to the ends of
the *purE* and *pepN* genes, respectively. These two genes were previously demonstrated to flank the Hib fimbrial gene cluster (11, 39). We used these primers
to verify that a fimbrial cluster was not present in *Haemophilus* genital strains

which did not possess previously described major or minor fimbrial protein
genes.

The PCR mixture (20 μl) contained primers (0.5 μM each), genomic DNA
(100 ng), deoxynucleoside triphosphates (100 μM each) (Boehringer, Mannheim, Germany), *Taq* polymerase (1.5 U) (Appligène, Illkirch, France), MgCl₂
(1.5 mM), 10 mM Tris HCl (pH 8.3), and 50 mM KCl. The PCR consisted of a
first long denaturation step (5 min at 95°C); 25 cycles each of 1 min of denaturation at 95°C, 2 min of annealing at 50°C, and 2 min of elongation at 72°C; and
then a 10-min final elongation step (Cetus 480; Perkin-Elmer Cetus, Norwalk,
Conn.).

PCR products were analyzed by agarose gel electrophoresis for 1 h at a
constant voltage (110 V). Gels contained 1% agarose (Eurogentec) in TBE
buffer (pH 8.0) (89 mM Tris, 89 mM borate, 2.5 mM EDTA). A 100-bp ladder
(Pharmacia Biotech, Saclay, France) was used as the molecular size standard.
Gels were stained with ethidium bromide (1 μg/ml) (Bioprobe System, Montreuil, France) for 30 min.

**DNA sequencing.** PCR products were purified by a 15-min spin at 500 × g on
a Microcon 100 (Amicon, Beverly, Mass.) to remove unincorporated deoxynucleoside triphosphates, primers, and salts. Nucleotide sequences were determined by the dideoxy chain termination method of Sanger et al. (29a) with
Thermo Sequenase dye terminator cycle sequencing premixed version 2 (Amersham, Les Ulis, France) and PCR primers with an Abi Prism 377 sequencer
(Perkin-Elmer) according to the manufacturer's instructions.

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                         N1                                              N34             N51
Hib 770235   ctctttatggagcaatttatt MKKTLLGSLI LLAFAGNVQA ---DINTETS GKVTFFGKVV ENTCKVKTEH KNLSVVLNDV GKNSLSTKVN
Hib AO2=M43                         .......... .......... ---....... .......... .......... ..........
Hib Eagan    tct...............     .......... .......... ---AANAD.K .T........ ....Q..D.. .......... ....KD.G.
NTHi M37     ....................... .......... ....T.A-- ANPQVSA... .......... ....Q.S.GN RDM....... ...........
NTHi 1128                           AA.A...... .......... .........N RDM....... ..SHFKN.GD
H. Aegyptius ....................... .......... .......... -ATTT.A... .......... .......DN Q.M....... ......G.
genital H.   ....................... .......... .......... ---AP.AD.. .......... .......AGN ..M.....N. ..AH..NVG.  67

                 N69        Crypt N
Hib 770235   TAMPTPFTIT LQNCDPTTAN GTANKANKVG LYFYSWKNVD KENNFTLKNE Q-TTA-DYAT NVNIQLMESN GTKAISVVGK ETEDFMHTNN
Hib AO2=M43  .......... .......... .......... .......... .......... .-...-.... .......... .......... ..........
Hib Eagan    .......... ....NL.A.. SST....... ....E.A... .......... ....K T-S.SN.F.. M......... D...E.K.... ...V.K.A
NTHi M37     .........K ....NANR.T ....N..... I.....N.T. .D........ K--M.N.... K....I..AD ..NQ.E.... SVD..T.K-.
NTHi 1128    .......... .TD.A-.VGV .DT-..K... V.....E.A. ...DY....T H--MGA.K.N ......FKD. .VDP.K.... ..N..T.---
H. Aegyptius .........S .D.NNV.T- -.....S... V.....E.A. .D.DY....K ---MSN.F.. M........D ..TP.K.... T....V.KGA
genital H.   I.......... ....TD.AVANG- A.DK..K... V.....E.A. .D.AY....I HENNG-.K.S M.....FKE. ..DS.E.... ..K..I--SQ  153

                         Crypt C    C186              C198      C207
Hib 770235   NGVAL--NQT HPNNAHISGS TQLTTGTNEL PLHFIAQYYA TNK--ATAGK VQSSVDFQIA YE taa
Hib AO2=M43  .....--.... .......... .......... .......... ...--..... .......... .. ...
Hib Eagan    T.AGVALT.. ..D.D..... ....GV.GD. .........- SLGS-T.... .......... .. ...
NTHi M37     ..STN---SS AVTKD....K .T.DNTKS.Y D......... .DA--..... .....N.... .. ...
NTHi 1128    ---NSSA.S. K.TKN...A. .G..-N.S.I ....V....S .GNDV-.... .......... .. ...
H. Aegyptius ..S.V--.S. AVTSK....N .A.NSN.S-I D....S.... .GI--..... .E...N.... .. ...
genital H.   ..-QN--TI. K-.--....A .E.NGK.GDI D.Y...... .GQ--..PV. .K...N.... .. ...   207
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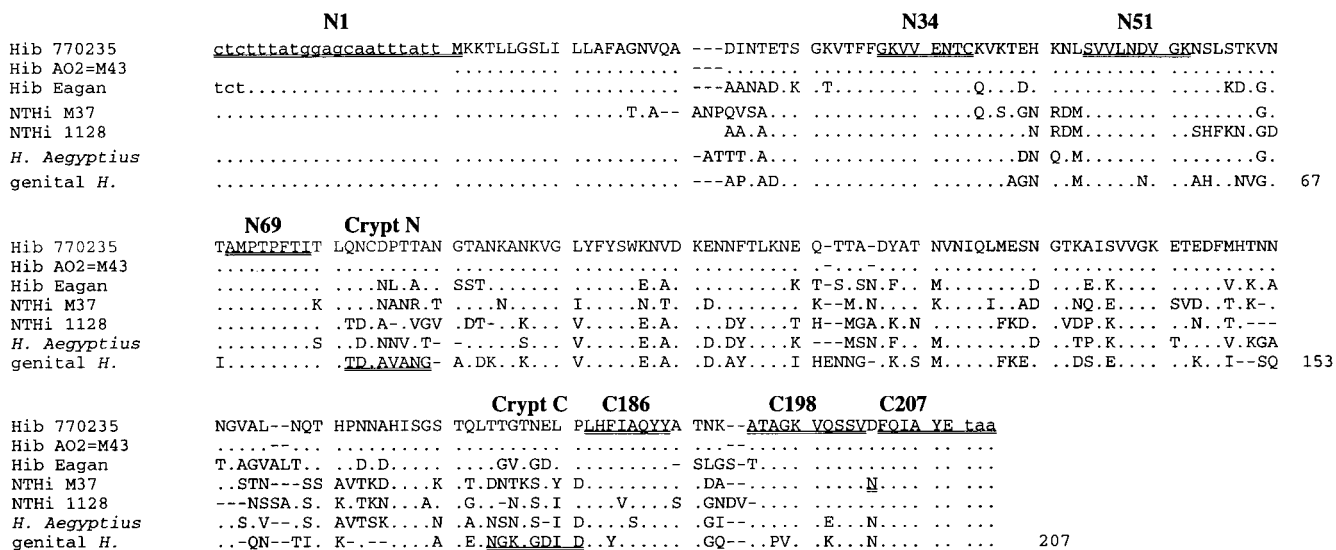
FIG. 1. Amino acid sequence deduced from major fimbrial protein gene sequence of genital *Haemophilus* strain. The sequence is compared with previously reported
HifA sequences (2, 8, 13, 38, 42). Amino acid residues chosen as the basis of primer sequences are double underlined, and the name of each primer is shown above
the line. Dots represent identity. Dashes indicate gaps introduced to optimize alignment. *H. Aegyptius*; *H. influenzae* biogroup Aegyptius; genital *H.*, genital cryptic
strains of *Haemophilus*.

**Southern blotting.** To confirm the absence of major or minor fimbrial protein genes for strains which did not give a PCR product with the fimbrial primers, probes for major and minor fimbrial protein genes labeled with digoxigenin (DIG) were synthesized by PCR with alkali-labile DIG-11–2′-deoxyuridine-5′-triphosphate (DIG-11–dUTP) (Boehringer). For this PCR assay 30 μM dTTP was replaced by 30 μM DIG-11–dUTP. One probe was prepared with the N1 and C207 primers and DNA from strain 11PS, which carries the *hifA*-like gene observed in genital strains. Another probe was constructed with the N1 and C207 primers and the Hib reference strain 770235. Two other probes were prepared with a genital strain of *Haemophilus*: the first was prepared from strain 11PS by using the hifD5 and hifD3 primers, and the second was prepared from strain 26E by using the hifE5 and hifE3 primers.

Two micrograms of DNA from each of the 31 strains of *Haemophilus* was digested with 100 U of *Eco*RI overnight. The restriction fragments were resolved on a gel containing 0.8% agarose for 20 h at a constant voltage (30 V). The DNA was transferred by capillarity with 20× SSC (trisodium citrate, 0.3 M; NaCl, 3 M; pH 7) onto a positively charged nylon membrane (Boehringer Mannheim). The DNA on the membrane was then tested for hybridization at 65°C with the various probes in a hybridization buffer (sodium dodecyl sulfate, 1%; NaCl, 1 M; Tris, 50 mM [pH 7.5], blocking reagent, 1% [wt/vol]). The hybridized probe was immunodetected with anti-DIG Fab fragments conjugated to alkaline phosphatase and visualized with the chemiluminescent substrate disodium 3-(4-methoscyspiro-{1,2-dioscetane-3,2′-(5′-chloro)tricyclo-[3 · 3 · 7 · 7^3,7^]decan)-4-yl)phenylphosphate (Boehringer). Light emission was recorded on Hyperfilm MP (Amersham).

**Nucleotide sequence accession numbers.** The sequence of the genital *Haemophilus* major fimbrial protein gene (*ghfA*) has been deposited in the EMBL sequence database under accession no. AJ000653. The accession numbers of the *hifA*-like gene sequences of Hib strain 5494 and *H. influenzae* biogroup Aegyptius strain 52129 are AJ000636 and AJ000637, respectively. The accession numbers corresponding to the genes coding for minor fimbrial proteins are AJ006783 for the *ghfD* gene of the genital strains 15N, 10U, 11PS, 26E, PIZ, and 2406 and AJ006784 for the *ghfE* gene of the genital strain 26E. The accession number of the *omp* gene of the genital strain 16N is AJ007317.

## RESULTS

**Characterization of a genital *Haemophilus* major fimbrial gene with primers corresponding to conserved sequences in *hifA* genes of Hib.** PCR assay with the primers N34 and C207 (Table 2; Fig. 1) amplified a fragment of about 550 bp from DNA of the control strain (Hib 770235). A comparable fragment was obtained from 6 of the 19 genital *Haemophilus* strains. These six strains were all from French patients; five were from the genital tracts of adult patients (strains 10U, 11PS, 26E, PIZ, and 2406), and only one was from a neonate (strain 15N) (Table 1). A fragment was also amplified from two *H. influenzae* reference strains: the Hib CIP 5494 strain, which is of biotype IV sensu stricto, and the *H. influenzae* biogroup Aegyptius CIP 52129 strain (Table 1). Except for CIP 52129, all strains for which a fragment was amplified exhibited peritrichous piliation under our culture conditions (Table 1).

To determine the 5′ end of the *hifA*-like gene, a PCR was performed with primers N1 and C207, which bracket the entire gene (Table 2; Fig. 1). An amplified fragment of about 650 bp was obtained for the same nine strains that gave an amplified fragment with primers N34 and C207: six genital *Haemophilus* strains and the control strain (Fig. 2A) and *H. influenzae* strains CIP 5494 and CIP 52129. No amplified product was obtained for the other strains. The PCR fragments obtained with primers C207 and N1 or N34 from the control strain Hib 770235 had slightly lower electrophoretic mobilities than the fragments amplified from the genital *Haemophilus* strains (Fig. 2A). No DNA amplification was obtained from any strain except the control strain Hib 770235 by using primer N51 or N69 with primer C186 or C198 (Fig. 1; Table 2).

The fragments amplified from all strains with primers N1 and C207 were sequenced. For the six cryptic strains, the nucleotide sequences were strictly identical.

The genital *Haemophilus* PCR fragment included a 621-bp open reading frame which we designate *ghfA* (for genital *Haemophilus* fimbria gene A). *ghfA* has 72% identity with the *hifA* gene of the control strain Hib 770235. It is 18 bp shorter than
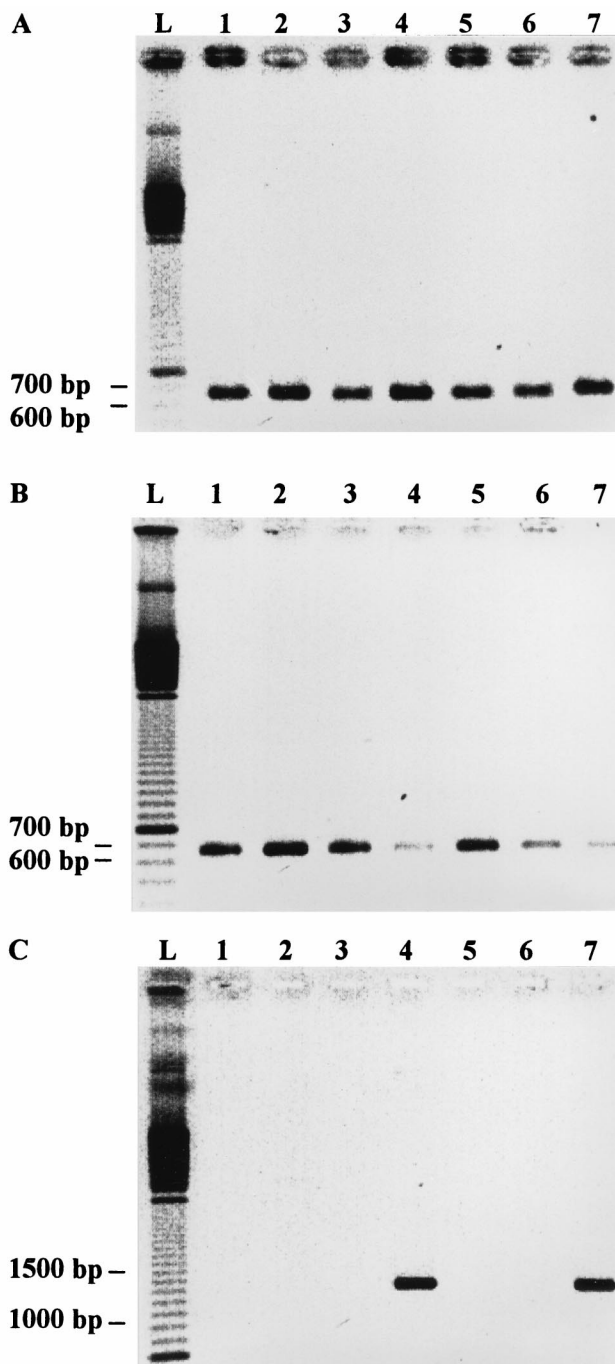
FIG. 2. PCR products obtained with fimbrial primers corresponding to the extreme ends of the three genes coding for the three proteins constituting the fimbria. (A) Primers N1 and C207 were used to amplify a *hifA*-like gene fragment. The fragment amplified from the control strain Hib 770235 (lane 7) had a slightly lower electrophoretic mobility than the fragments obtained from genital *Haemophilus* strains (lanes 1 to 6). (B) PCR products obtained with hifD5 and hifD3. (C) PCR products obtained with hifE5 and hifE3. Lanes 1 to 6, genital cryptic *Haemophilus* strains 15N, 10U, 11PS, 26E, PIZ, and 2406, respectively; lane 7, Hib control strain 770235; lane L, 100-bp ladder.

the Hib 770235 *hifA* gene, consistent with the small difference observed in electrophoretic mobility in agarose gels (Fig. 2A). It has a G+C content of 34%. It encodes a 207-amino-acid protein, named GhfA, which includes a predicted 20-amino-acid leader sequence. The leader sequence is typical of pro-
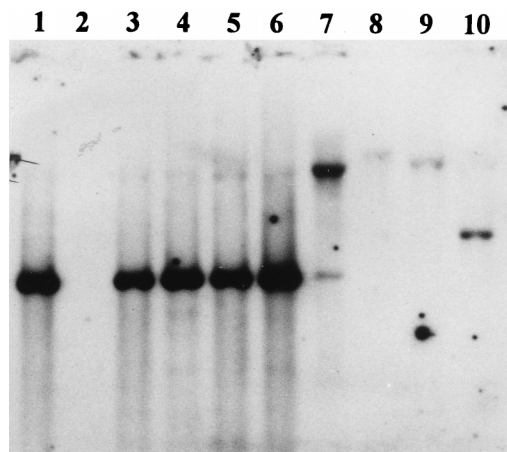
FIG. 3. Southern blots obtained with the major fimbrial protein gene of genital *Haemophilus* (*ghfA*) labeled with DIG as a probe. Hybridization was observed only with DNA from strains giving a PCR product with primers N1 and C207 (Hib strains 770235 and 5494, *H. influenzae* biogroup Aegyptius strain 52129, and genital *Haemophilus* strains 15N, 10U, 11PS, 26E, PIZ, and 2406). Lanes 1 to 7, genital cryptic *Haemophilus* strains 15N, 16N, 10U, 11PS, 26E, PIZ, and 2406, respectively; lane 8, Hib control strain 770235; lanes 9 and 10, Hib strain CIP 5494 and *H. influenzae* biogroup Aegyptius strain CIP 52129, respectively.

karyotic secreted proteins (43) and is identical to HifA leader sequences previously described for NTHi strain 1128, *H. influenzae* biogroup Aegyptius strain F3031 and Hib strains 770235, M43, and Eagan (2, 10, 13, 15, 38, 42). The remaining 561 bp of *ghfA* encodes a mature protein of 187 amino acids with a calculated molecular mass of 19,652 Da. GhfA is 63% identical and 71% similar to Hib strain 770235 HifA described by van Ham et al. (38).

The nucleotide sequence of the PCR fragment from cryptic *Haemophilus* shows 69 and 74% identity with those of two other strains we sequenced: Hib CIP 5494 and *H. influenzae* biogroup Aegyptius CIP 52129, respectively. There are 63 and 64% identity and 65 and 72% similarity between the translated sequences, respectively.

To try to identify a *hifA*-like gene in the 13 genital *Haemophilus* strains that did not give an amplified fragment, additional primers, Crypt N and Crypt C (Fig. 1; Table 2), were synthesized to correspond to regions of low sequence similarity between *H. influenzae* and genital *Haemophilus* strains. An amplification product was obtained only with the same six cryptic strains which gave amplified fragments with N34 (or N1) and C207 and with the *H. influenzae* biogroup Aegyptius strain. No amplification product was obtained for the Hib control strain (770235) or for the Hib biotype IV sensu stricto strain (CIP 5494).

To determine more accurately how many of the genital strains of *Haemophilus* contain a *hifA*-like gene, Southern hybridizations were performed with two probes. The first was the *ghfA* gene. Hybridization was obtained only for the nine strains giving a PCR fragment with the N1 and C207 primers (Fig. 3). The hybridization band intensities for Hib strain 770235, Hib strain CIP 5494, and *H. influenzae* biogroup Aegyptius strain CIP 52129 were lower than those for the six genital *Haemophilus* strains. This is consistent with the percentages of identity between the *ghfA* probe and the *hifA* or *hifA*-like genes: 100% for genital *Haemophilus* strains, 63% for Hib 770235, 69% for Hib CIP 5494, and 74% for *H. influenzae* biogroup Aegyptius CIP 52129. The second probe prepared from the Hib control strain 770235 was a *hifA* probe. A similar result was obtained, but the hybridization signal was more intense for Hib 770235

than for the other strains, also in accordance with the percentages of identity between the *hifA* probe and the *ghfA* or *hifA*-like genes. The size of the restriction fragment of the genital strain 2406 hybridizing with the probes was different from those for the other genital strains (Fig. 3). This strain exhibits the phenotypic characteristics of *Haemophilus parainfluenzae*, whereas the other genital *Haemophilus* strains are phenotypically identified as *H. influenzae* biotype IV. Nevertheless, previous DNA-DNA hybridization experiments indicated that this strain belongs to the genital *Haemophilus* genospecies (100.0% relative binding with the genital *Haemophilus* reference strain) and not to *H. parainfluenzae* (14.3% relative binding with the type strain of *H. parainfluenzae*) (22).

**Identification of genes coding for minor fimbrial proteins for genital *Haemophilus*.** Two sets of primers were used to amplify the genes coding for the two minor fimbrial proteins. The hifD5 and hifD3 primers (Table 3), corresponding to the extreme ends of the *hifD* gene of the Hib strain 770235, were used to amplify the *hifD*-like gene, and hifE5 and hifE3 (Table 3), corresponding to the extreme ends of the *hifE* gene of the control strain, were used to amplify the *hifE*-like gene.

With the hifD5 and hifD3 primers, a PCR fragment of about 650 bp was obtained for eight strains: the Hib control strain 770235, the six genital strains having the *ghfA* gene (Fig. 2B), and the *H. influenzae* biotype IV sensu stricto strain CIP 5494 (Table 1). Only the *H. influenzae* biogroup Aegyptius strain was positive for a *hifA*-like gene and negative for a *hifD*-like gene. The PCR fragments of the six genital strains of *Haemophilus* were sequenced. The sequences were strictly identical for the six strains. The sequence included a 648-bp open reading frame, which we designate *ghfD* (for genital *Haemophilus* fimbria gene D), that encodes a 218-amino acidprotein (GhfD). The amino acid sequence of GhfD was compared with the HifD sequence of Hib strain 770235; it was 79% identical and 82% similar.

With the hifE5 and hifE3 primers, a PCR fragment of 1,308 bp was obtained only for the genital strain 26E; it was named *ghfE* (Fig. 2C). The derived amino acid sequence was 53% identical and 59% similar to the Hib 770235 HifE sequence. No amplification was obtained for the genital strains 15N, 10U, 11PS, PIZ, and 2406, which possess *ghfA* and *ghfD* genes, but hybridization with the *ghfE* probe was observed for these strains.

For the other strains of genital *Haemophilus*, no amplification was obtained with the hifD5-hifD3 and hifE5-hifE3 primers. The absence of these genes was confirmed by Southern blotting.

**Analysis of the region between the *purE* and *pepN* genes for *ghfA*-negative strains.** By using primers derived from the conserved flanking regions (*purE* and *pepN* genes), a PCR fragment of about 450 bp was obtained for all *ghfA*-negative genital strains of *Haemophilus*, except for two (genital strains 1595 and 3N) for which the PCR fragment was of about 250 bp. A 250-bp fragment was also amplified for the two strains of *H. haemolyticus* (CIP 102348 and CIP 103290).

The 450-bp fragment from the genital strain 16N was sequenced. It had 95% identity with a noncoding nucleotide sequence between the *purE* and *pepN* genes previously described for the fimbria-negative strain *H. influenzae* Rd. This fragment included a 126-bp sequence which shows some identities with a part of the *purK* genes of several *H. influenzae* strains (determined with FASTA software). The 250-bp PCR fragments from strain 1595 and one of the two strains of *H. haemolyticus* were sequenced. The sequences were identical and correspond to the noncoding sequence of *H. influenzae* Rd.

**Characterization of a gene in our genital *Haemophilus* collection possibly involved in adherence and previously described as a fimbrial gene in an NTHi strain.** The primers fim5 and fim3 (Table 3) were deduced from the sequence of the gene coding for the fimbrin previously studied by Sirakova et al. (30). A fragment of about 1.1 kb was amplified for all genital strains of *Haemophilus*, for the 10 strains of *H. influenzae* sensu stricto, for the strain of *H. influenzae* biogroup Aegyptius, and for a strain of *H. haemolyticus*. Only one strain of *H. haemolyticus* (strain 102348) was negative. This PCR fragment was sequenced for the genital strain 16N. The sequence of the fimbrial gene in an NTHi strain (strain 1128) described by Sirakova et al. (30) is 84% identical to the sequence of the PCR fragment from 16N. A search for homology by using the FASTA program indicated that the fimbrin-like proteins encoded by these genes were similar to several *H. influenzae* outer membrane P5 proteins.

**Electron microscopic observation of the fimbriae.** Fourteen of the 19 genital *Haemophilus* strains showed abundant peritrichous piliation (Table 1). There was no notable difference between the appearances of the fimbriae on strains having LKP-like fimbrial genes and those on the genital strains lacking these genes. The appearances of fimbriae before or after several contacts with human erythrocytes were the same. Nevertheless, two of the five nonpiliated strains (189 and 3N) exhibited fimbriae after this enrichment. No evident difference was observed between the electron microscopic appearances of these fimbriae and those of the 14 other strains.

## DISCUSSION

The aim of this study was to identify and sequence genes coding for proteins participating in fimbria biogenesis in a particular group of *Haemophilus* strains that are specifically associated with genital, maternofetal, and neonatal infections.

As previous studies demonstrate about 80% similarity between the primary sequences of the major fimbrial subunits in all Hib and NTHi strains, including *H. influenzae* biogroup Aegyptius (2, 7, 10, 42), we used primers corresponding to conserved sequences at the 3′ and 5′ ends of previously described *hifA* genes. A *hifA*-like gene (named *ghfA*) was found in only 6 of the 19 genital *Haemophilus* strains studied. These PCR results were confirmed by Southern blotting with the *hifA* and *ghfA* genes as probes. This gene had 71 to 80% identity with the known *hifA* genes of various *Haemophilus* strains, percentages of identity which are consistent with those observed between *H. influenzae* genes encoding the major fimbrial subunit (2, 7, 10, 42). No amplification was obtained with the two *H. haemolyticus* strains, although this genospecies is the most closely related to genital *Haemophilus* genospecies strains (25, 27). *ghfA* codes for a protein (GhfA) whose sequence has 63% identity and 71% similarity to the translated amino acid sequence of the Hib 770235 *hifA* gene (38). It is 3 to 9 amino acids shorter than known HifA proteins in *Haemophilus* strains and includes two cysteines, 40 residues apart, and a penultimate tyrosine, a characteristic of *H. influenzae* HifA proteins (2, 8, 10, 13, 38, 42). Differences between the amino acid sequences were not localized to any one region but were distributed throughout the protein. In addition, the FASTA software (GenBank) found an identity of 62 to 68% between GhfA and major fimbrial subunit precursors of several *H. influenzae* strains. Therefore, GhfA appears to be a member of a family of major fimbrial proteins which includes almost all HifA proteins characterized to date from Hib, NTHi, and *H. influenzae* biogroup Aegyptius strains. There are also identities of 35% with the *Escherichia coli* F17 fimbrial

protein precursor and 36% with the *Klebsiella pneumoniae* fimbrial subunit type 3 precursor.

Geluk et al. suggest that the complete fimbria gene cluster is either always present or entirely absent (11). Southern blotting suggest the presence of *ghfE* (a *hifE*-like gene) in the six genital *Haemophilus* strains which possess *ghfA* and *ghfD*. Nevertheless, at least one of the extreme ends (hifE5 or hifE3 sequences) of the gene in five of these six strains differs, because no amplification was obtained with these primers.

For 13 of the 19 genital strains, no PCR product was obtained with any of the primers used. This result was confirmed by Southern blotting with the three fimbrial gene probes and by sequencing of the products amplified with primers defined on the two genes surrounding the fimbrial cluster (*purE* and *pepN*). These data confirm the absence of LKP-like fimbrial genes from most (68%) of the genital *Haemophilus* strains. However, 10 of these 13 genital *Haemophilus* strains were piliated (Table 1). Therefore, other genes coding for proteins that constitute these fimbriae must be located elsewhere on the chromosome.

Because genital *Haemophilus* strains are commonly phenotypically identified as *H. influenzae* biotype IV, we also sequenced the PCR fragment of an *H. influenzae* biotype IV sensu stricto strain (Hib CIP 5494) for comparison. The nucleotide sequence is 69% identical to that of *ghfA*. The translated sequences are 63% identical and 65% similar. These percentages correspond to the identities and similarities observed between GhfA and *H. influenzae* HifA proteins as assessed by using FASTA software.

van Ham et al. demonstrated that HifA mediated *H. influenzae*-specific adherence (40). They found that the hydrophilicity pattern of HifA was different from that of HifD, which is a minor subunit of fimbriae and is closely related to HifA but which is not required for adherence. They suggested, therefore, that hydrophilic domains of HifA are responsible for binding to a specific eukaryotic receptor. Recently, McCrea et al. (17) suggested that the adhesion is mediated by the HifE protein localized at the tips of fimbriae rather than by the HifA major subunit, because hemagglutination by their Hib Eagan strain was inhibited by the presence of antibodies directed against HifE. Cryptic genital *Haemophilus* strains do not agglutinate erythrocytes (28). Nevertheless, the three hydrophilic domains previously observed in HifA were also identified in GhfA from the cryptic *Haemophilus* strains. This suggests that the three hydrophilic domains of HifA may be involved in an interaction other than binding to a specific eukaryotic receptor and that the adhesive site may not be localized on the HifA protein. These observations are in part in agreement with a previous study (2) in which an LKP *hifA*-like gene was amplified by PCR from a strain responsible for otitis that did not agglutinate erythrocytes. In this strain, pili were morphologically different from LKP pili, and an additional fimbrial subunit gene (fimbrin gene) having a low sequence similarity with the LKP gene has since been identified by Sirakova et al. (30). By using primers derived from this fimbrin gene, an *omp* gene which has 84% identity with the fimbrin gene described by Sirakova et al. and about 85% identity with the *ompP5* genes of many *H. influenzae* strains was amplified for all our genital strains. Thus, the product of this gene could be an adhesin commonly observed for Hib, NTHi, and genital strains of *Haemophilus*, but it seems doubtful that it is the major protein constituting fimbriae. Therefore, other genes, as yet unidentified, may explain the piliation observed by electron microscopy on genital *Haemophilus* strains which do not carry LKP-like fimbrial genes.

Genital *Haemophilus* strains that possess the *ghfA* and *ghfD* genes were isolated more often from mucosal cells of adults (5 of the 6 strains) than from neonates (only 1 of the 13 strains). In addition, *ghfA* and *ghfD* were present only in French strains. Previous genetic studies give no evidence for differences between French and U.S. strains (25, 27). Therefore, the apparent relationship between the geographic origin of strains and the presence of *ghf* genes may reflect the fact that most strains isolated from urogenital epithelial cells were from French adults, whereas most of the strains isolated in the United States were from neonates. Thus, these fimbrial genes may confer on the strain the ability to adhere to urogenital epithelial cells and to persist in the ecological and physiological conditions of the adult genital tract. In contrast, *ghfA* and *ghfD* do not appear to be essential for colonizing neonates at birth. The study of a larger number of strains may confirm this observation.

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