Molecular Cloning, Expression, and Sequence of the Pilin Gene from Nontypeable *Haemophilus influenzae* M37

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Nontypeable *Haemophilus influenzae* M37 adheres to human buccal epithelial cells and exhibits mannose-resistant hemagglutination of human erythrocytes. An isogenic variant of this strain which was deficient in hemagglutination was isolated. A protein with an apparent molecular weight of 22,000 was present in the sodium dodecyl sulfate-polyacrylamide gel profile of sarcosyl-insoluble proteins from the hemagglutination-proficient strain but was absent from the profile of the isogenic hemagglutination-deficient variant. A monoclonal antibody which reacts with the hemagglutination-proficient isolate but not with the hemagglutination-deficient isolate has been characterized. This monoclonal antibody was employed in an affinity column for purification of the protein as well as to screen a genomic library for recombinant clones expressing the gene. Several clones which contained overlapping genomic fragments were identified by reaction with the monoclonal antibody. The gene for the 22-kDa protein was subcloned and sequenced. The gene which the type b pilin from *H. influenzae* type b strain MinA was also cloned and sequenced. The DNA sequence of the strain MinA gene was identical to that reported previously for two other type b strains. The DNA sequence of the strain M37 gene is 77% identical to that of the type b pilin gene, and the derived amino acid sequence is 68% identical to that of the type b pilin.

Nontypeable *Haemophilus influenzae* is commonly found in the nasopharynx of children and is responsible for approximately 20% of acute bacterial middle ear infections in children (8, 45). This organism also is thought to play a role in exacerbation of chronic bronchitis and to cause pneumonia in the elderly population (33). It is estimated that in the developing world, more than 4 million children under the age of 5 die of lower respiratory infection each year (21).

Although most cases of lower respiratory infection are thought to be caused by viral agents, the majority of the severe disease and death is thought to be due to bacterial pathogens (5, 27, 32). In four published studies of children with pneumonia in which lung puncture or blood specimens were cultured, nontypeable *H. influenzae*, *H. influenzae* type b, and *Streptococcus pneumoniae* were the predominant pathogens identified (4, 38, 46, 47).

Pili in both type b and nontypeable *H. influenzae* have been described. Type b organisms isolated from the blood or cerebrospinal fluid of patients are hemagglutination deficient and do not express pili (13, 35). Strains expressing a hemagglutination-associated pili as well as strains not expressing this pilus have been isolated from the human nasopharynx (10, 24, 35). Variants of type b strains expressing pili can also be isolated by enrichment for cells which adhere to human erythrocytes (13, 35). The cloning and sequencing of the gene for the type b pilus from two strains have been reported recently (11, 20, 44).

Pili in nontypeable isolates have also been observed and characterized. Several morphologically distinct structures have been described (1, 2, 6). One of these, the LKP pilus, appears to be similar to the type b pilus. The cloning of the LKP pilin operon from a nontypeable strain has been reported (17). Although these pili are thought to be important colonization factors and antibody directed against the LKP pilus is protective in an experimental model of nontypeable *Haemophilus otitis* media (18), studies to date have been hampered by the lack of isogenic strains which stably express or do not express pili. Further, no data regarding the structure of these pili, the molecular basis for phase variation, or the biogenesis of these pili are available. As a first step toward addressing these questions, we report the cloning, expression in *Escherichia coli*, and primary sequence of a pilin gene from the nontypeable *H. influenzae* strain M37.

MATERIALS AND METHODS

Strains. The M37 strain of *H. influenzae*, a nasopharyngeal isolate, was a gift from J. Gilsdorf (10). Supernatants from overnight cultures of M37 were tested for reactivity with typing sera for capsular serotypes a through f by counter-current immunoelectrophoresis (16). No positive reactions were observed. Southern blot analysis of chromosomal DNA from strain M37 revealed one weakly hybridizing chromosomal EcoRI fragment when probed with plasmid pUO38, a plasmid which contains sequences common to all encapsulated strains of *H. influenzae* and their nonencapsulated derivatives (19). Thus, M37 is not an encapsulated strain, nor does it appear to be a capsule-deficient mutant of an encapsulated strain.

A hemagglutination-deficient variant of strain M37 was isolated by enriching for M37 cells which did not bind to human erythrocytes (42). Individual clones were then screened for hemagglutinating activity. A clone with no detectable hemagglutinating activity was saved for further analysis; this clone was designated M37-1. When grown in broth without shaking, M37-1 cells settle to the bottom of the tube, whereas M37 cells grow as a turbid suspension. In order to select a variant which grew like strain M37, we transferred 100 μl from the top of an overnight growth culture of M37-1 into fresh broth and allowed the culture to grow overnight. After several transfers, the overnight cul-

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tures were evenly turbid. A loop of culture was streaked on chocolate agar, and clones were tested individually for hemagglutinating activity. A hemagglutination-proficient derivative designated M37-2 was saved for further analysis.

H. influenzae type b strain MinnA was provided by J. Gilsdorf and has been described elsewhere (30). E. coli K-12 strains y2819 (7) and LE392 were gifts from Roy Curtiss III. E. coli BL21(DE3)/plysS was a gift from F. William Studier. Strain BL21(DE3) contains a single copy of the T7 RNA polymerase gene under the control of the lac regulatory system (41). Plasmid plysS contains the T7 lysozyme gene. T7 lysozyme binds to the T7 RNA polymerase in vitro, and plysS stabilizes many toxic T7 expression constructs, presumably by binding and inactivating the low quantities of T7 RNA polymerase produced by BL21(DE3) in the absence of IPTG (isopropyl-β-D-thiogalactopyranoside) (26). E. coli JM101 was obtained from New England Biolabs. Plasmid pcos2EMBL (36) was obtained from Roy Curtiss III, and plasmid pT7-7 was a gift from Stan Tabor (43). Plasmid was a gift from E. Richard Moxon (19).

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The H. influenzae strains were grown on chocolate agar or in supplemented brain heart infusion broth as described previously (3). The E. coli strains were grown on L agar plates or L broth supplemented with 50 μg of ampicillin per ml, 35 μg of kanamycin per ml, and/or 25 μg of chloramphenicol per ml as appropriate.

Agglutination. Hemagglutination assays of H. influenzae and E. coli strains were performed as described previously (35). Briefly, plate-grown cells were suspended in phosphate-buffered saline (PBS) (pH 7.4) at an A500 of 1.0. Fifty-microliter aliquots of twofold bacterial dilutions were placed in U-bottom microtiter wells (Dynatech Laboratories, Inc., Chantilly, Va.). An equal volume of a 0.6% suspension of human type O erythrocytes in PBS (in some experiments 2% mannose was present in the PBS) was added, and the two volumes were mixed and then incubated without shaking at room temperature. Hemagglutination was determined visually after 1 h. The highest bacterial dilution with visible agglutination was reported as the titer.

Direct bacterial agglutination was determined by mixing plate-grown bacterial cells suspended in PBS with dilutions of antiserum or PBS. Agglutination was observed with an indirect light source.

Monoclonal antibodies. Mice were immunized with live M37 cells, and splenic lymphocytes were isolated and fused to cells of the P3X63Ag8.6.5.3 murine cell line by the Washington University Hybridoma Center using standard procedures. Hybridoma supernatants were screened by dot blotting for antibodies that recognized strain M37 but not strain M37-1, the isogenic hemagglutination-deficient variant. Wells containing hybridomas of interest were cloned by limiting dilution. An immunoglobulin G (IgG) clone and two IgM clones were isolated from a single fusion.

One of the IgM antibodies, designated 3H12, had strong agglutinating activity for strain M37 but no activity against strain M37-1. This antibody was employed for affinity purification of the M37 pilin and immunologic screening of recombinant libraries. Ascitic fluid was induced by intraperitoneal immunization of the cell line into pristane-stimulated mice. Crude ascitic fluid was centrifuged at 12,000 × g for 10 min. The supernatant was retained, and ammonium sulfate was added up to a final concentration of 50%. After overnight incubation at 4°C, the suspension was centrifuged at 4,300 × g for 10 min. The pellet was suspended in PBS, and approximately 20 mg of protein in 1.2 ml was fractionated on a Sepharose CL-6B column (2 by 60 cm) at room temperature. The fractions were monitored by A280 and an enzyme-linked immunosorbent assay (ELISA). Column fractions which were reactive with M37 cell extracts by ELISA were pooled, concentrated with a Centricon-10 microconcentrating unit (Amicon, Danvers, Mass.), and analyzed for immunoglobulin purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Immunosassays. A colony blot immunoassay was performed by transfer of colonies to nitrocellulose (BA85; Schleicher & Schuell, Inc., Keene, N.H.). For analysis of recombinant clones, colonies were transferred to nitrocellulose and lysed with chloroform vapor (15). The remaining protein binding sites were blocked with 2% gelatin in Tris-buffered saline (TBS) (0.02 M Tris-HCl, 0.5 M NaCl [pH 7.5]). Twentyfold dilutions of tissue culture supernatants or dilutions of purified monoclonal antibodies were used as primary antibody; the secondary antibody was goat antimouse (IgG + IgM) alkaline phosphatase conjugate diluted 1,000-fold with 1% gelatin–TBS-0.4% Tween 20 (Tago Inc., Burlingame, Calif.). Blots were developed with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indoly phosphate as described elsewhere (30). A dot blot immunoassay was performed similarly, except that sonicates or partially purified proteins were applied directly to nitrocellulose and the cholroform step was omitted.

ELISA determinations were made with EIA microtitration plates (Linbro, McLean, Va.). The wells were coated overnight at 37°C with a sarcosyl-insoluble fraction (3) from M37 cells suspended at 1 μg/ml in borate-buffered saline (8.76 g of NaCl per liter, 6.18 g of boric acid per liter, 9.52 g of sodium borate per liter [pH 8.2]). Unbound protein was removed by aspiration, and the wells were washed three times with 0.05% Tween 20–PBS. Monoclonal antibody (3H12) or fractions from the Sepharose column were diluted in 0.5% bovine serum albumin–PBS and incubated with bound antigen at 37°C for 1 h. The monoclonal antibody solution was removed, the wells were washed, and secondary antibody diluted 1,000-fold in 0.05% Tween 20–PBS was added. Incubation was continued for 1 h at 37°C. The secondary antibody was subsequently removed, the wells were washed, and the bound antigen-antibody complexes were developed with p-nitrophenyl phosphate (1 mg/ml) dissolved in 10% diethanolamine. After 30 to 90 min of incubation at room temperature, the A405 was determined.

Purification and N-terminal analysis of M37 pilin. M37 cells from eight confluent 13.5-cm-diameter chocolate agar plates were scraped into PBS (pH 7.4) and heated at 65°C for 60 min. Cells were removed by centrifugation at 12,000 × g for 10 min. The supernatant from heat-treated M37 cells was mixed with an IgM (3H12)-Sepharose 4B affinity matrix (volume, 0.4 ml) previously equilibrated with 10 mM KPO4 buffer (pH 8.0), and the mixture was rocked overnight at 4°C. The mixture was poured into a 10-ml plastic column and washed with 5 ml of 10 mM KPO4 buffer (pH 8.0). Bound proteins were eluted with 1 ml of 100 mM triethylamine (pH 11.5) and quickly neutralized with 0.05 ml of 1 M KPO4 buffer (pH 6.8). Proteins with apparent molecular weights of 22,000 and 24,000 were eluted under these conditions.

The triethylamine eluate was fractionated on a modified Laemmli SDS–11% polyacrylamide gel, electrotransferred to a polyvinylidene difluoride (PVDF) membrane (25), and stained with Coomassie brilliant blue R. The 22- and 24-kDa proteins were cut from the PVDF membrane, and their N-terminal sequences were determined by the Protein Chemistry Laboratory at Washington University. Sequence
Molecular cloning. Genomic DNA from strain M37 was isolated as described elsewhere (37). Sau3A partial digests of M37 chromosomal DNA were fractionated by preparative agarose electrophoresis; DNA fragments >20 kb long were isolated and ligated into BamHI-digested pCO2EMBL. The ligation mixture was packaged in vitro with Gigapack (Stratagene) and transduced into E. coli x2819. Kanamycin-resistant clones were screened for reactivity with monoclonal antibody 3H12 by colony blot analysis. Plasmid DNA was isolated from eight immobilized clones and analyzed by restriction analysis on 0.7% agarose gels.

The sequence of the pilin gene of H. influenzae type b strain 770235* b° was reported by van Ham et al. (44). We prepared oligonucleotide primers 5' and 3' to the reported type b pilin gene such that sequences between nucleotides 20 and 831 (numbering of van Ham et al.) would be amplified. A fragment of approximately 800 bp was amplified from the DNA of immunoreactive cosmid clones as well as from genomic DNA of strains M37 and MinnA when these primers were used in a polymerase chain reaction (PCR) (GeneAmp; Perkin Elmer Cetus, Norwalk, Conn.). The sequence of the 5' oligonucleotide employed for amplification is 5'ACGATTCTCGATTATAGGTCTTAG. The sequence of the complementary 3' oligonucleotide is 5'AGCTGATCTTGAGGGTTAGGCC. The 5' oligonucleotide contains an EcoRI site and the complementary 3' oligonucleotide contains a BamHI site to facilitate subcloning.

The cosmid clone designated pRSM774 was chosen for further analysis. The amplified 800-bp fragment from two independent PCRs employing plasmid DNA from pRSM774 as a template was cloned into the replicative form of M13mp18 and M13mp19 as described elsewhere (28). The ligation mixtures were transformed into E. coli JM101, and the DNA sequence was determined in both directions with Sequenase (U.S. Biochemicals) as described elsewhere (28).

Similarly, the pilin gene from H. influenzae type b strain MinnA was cloned from genomic DNA and sequenced. Sequence comparisons were performed with the University of Wisconsin Genetics Computer Group GAP program, which employs the Needleman and Wunsch algorithm (34).

In order to express the M37 pilin gene in E. coli, the EcoRI-to-BamHI fragment containing the pilin gene was cloned from the replicative form of one of the M13 clones into the bacteriophage T7 expression plasmid pT7-7. The ligation mixture was transformed into E. coli JM101, and the recombinants were screened by restriction analysis of plasmid minipreparations. One plasmid containing the M37 pilin gene was designated pRSM865. To obtain high-level expression of the M37 pilin, pRSM865 was transformed into E. coli BL21(DE3)pLysS. Expression of the M37 pilin gene product under control of the 380 promoter was achieved by induction of T7 RNA polymerase synthesis by the addition of IPTG (41).

Restriction and ligation conditions were those suggested by the manufacturers. Other methods used were described previously (23, 37).

Southern blotting. Genomic DNA from M37 and M37-1 and DNA from cosmid clones reactive with monoclonal antibody 3H12 were digested with PstI, separated on 0.7% agarose gels, and transferred to PhotoGene nylon membranes according to the manufacturer's protocol (PhotoGene Nucleic Acid Detection System; Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, Md.). A biotinylated DNA probe containing the pilin gene was prepared. Plasmid DNA from pRSM865 was digested with EcoRI and BamHI, and the fragment containing the pilin gene was isolated. Nick translation of the pilin gene with biotin-ATP was performed according to instructions provided by the manufacturer (BioNick Labeling System; Bethesda Research Laboratories Life Technologies). The biotinylated probe was hybridized to blotted DNA overnight at 42°C and washed at 65°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate; 1× SDS for 1.5 h, and the blot was developed according to the manufacturer's instructions (PhotoGene DNA Hybridization Detection; Bethesda Research Laboratories Life Technologies).

Analytical methods. Preparation and analysis of sonic extracts, envelope fractions, and sarcosyl-insoluble fractions were performed as described previously (31). SDS-PAGE was performed on 11% polyacrylamide gels as described by Lugtenberg and coworkers (22). Protein concentrations were determined by the bichinchoninic acid method (39) according to the manufacturer's instructions (Pierce Chemical Co., Rockford, Ill.).

RESULTS AND DISCUSSION

The M37 strain described by Gilsdorf and Ferrieri (10) was chosen for study, as it was hemagglutination positive and was adherent to buccal epithelial cells. The hemagglutination titer of M37 cells was 1/32. After enrichment for cells which did not bind human erythrocytes, clones were tested for hemagglutination activity. A clone designated M37-7 had no detectable hemagglutination activity. M37-2 was a spontaneous variant of M37-1 which had regained hemagglutination activity. Sarcosyl-insoluble preparations of all three strains were analyzed by SDS-PAGE. A protein with an apparent molecular weight of 22,000 was observed in preparations from strains M37 and M37-2 but was not observed in preparations from M37-1 (Fig. 1). This protein was presumed to be the pilin.

Purification of the pilin from H. influenzae A02 by shearing, differential pH of extraction buffers, and ammonium sulfate precipitation has been described elsewhere (14). In contrast, we observed that the hemagglutination titer of M37 was only slightly affected by prolonged shearing. Stull et al. (42) reported that the hemagglutination capacity of three strains of H. influenzae type b was lost by heating cells to 60°C, and heat shock has been employed to strip colonization factor antigens (40) from the E. coli cell surface. The hemagglutinating activity of the M37 cells was lost by heating and was accompanied by the release of a number of proteins into the supernatant.

An IgM monoclonal antibody designated 3H12 was characterized. Monoclonal antibody 3H12 was reactive in dot blots with M37 cells but not with M37-1 cells, MinnA cells, or cells of a hemagglutination-proficient variant of MinnA. Similarly, M37 cells were strongly agglutinated by 3H12, whereas the other strains were not agglutinated by this antibody. Two additional monoclonal antibodies that demonstrated the same specificity for M37 and M37-1 cells were isolated and characterized; these antibodies were not strongly agglutinating. None of the antibodies were reactive with proteins from strain M37 in Western immunoblots.

An antigen reactive with monoclonal antibody 3H12 was released into the heat shock supernatant. Proteins with apparent molecular weights of 22,000 and 24,000 were then purified from the heat shock supernatant by affinity chromatography over a monoclonal antibody 3H12 coupled to Seph-
arose. The two proteins were separated by SDS-PAGE and electrotransferred to PVDF paper. The bands were cut from the PVDF paper and subjected to automated Edman degradation. The first amino acid from both proteins was indeterminate. Amino acids 2 through 17 were identified for the 24-kDa protein. The sequence was NPQVSAETSGKVT FGG. Amino acids 2 through 8 were determined for the 22-kDa protein and were identical to those found in the 24-kDa protein. Thus, it is likely that the 22-kDa protein is a proteolytic fragment of the 24-kDa protein, although it is possible that the one of the proteins is derived from the other by posttranslational modification. The N-terminal sequence has homology to the type b pilin as well as homology to other pilins, as has been previously reported (11).

A genomic library of H. influenzae M37 was prepared in the cosmid vector pCOS2EMBL and propagated in E. coli K-12 strain &chi;2819. Eight clones which were recognized by monoclonal antibody 3H12 were further characterized. Seven of these clones were not identical; however, restriction analysis indicated that they contained overlapping genomic fragments. Since the N-terminal sequences of the 22-kDa protein and the type b pilin were similar, we reasoned that we might be able to subclone the M37 gene from the cosmids by using the PCR. Primers homologous to the 5′ upstream and 3′ downstream region of the H. influenzae type b pilin gene (44) were generated. A DNA fragment of approximately 800 bp was obtained from the PCR employing genomic DNA from the type b strain MinnA, M37 genomic DNA, M37-1 genomic DNA, and cosmid DNA from several immunoreactive clones.

The M37 pilin gene derived from two independent PCR clones was cloned into M13mp18 and M13mp19, and both clones were sequenced. The sequences were identical, indicating that no PCR errors had occurred. The sequence of the M37 gene is shown in Fig. 2. The chemically determined residues from the affinity-purified 24-kDa protein were identical to residues 2 to 17 of the derived sequence. The protein has an 18-amino-acid leader peptide which is typical of the leader peptides identified for other Haemophilus surface proteins as well as the leader peptides of other prokaryotic secreted proteins (48). The molecular weight of the mature protein, as determined from the derived amino sequence, is 21,077.

The sequence of the type b gene from strain MinnA was also determined. It is identical to that reported by van Ham et al. (44) as well as to the sequence determined by Gilford et al. for the gene from strain M43 (A02) (11). The sequence of the gene from strain A02 determined by Langermann and Wright (20) differs by a single nucleotide, resulting in a single
amino acid change. The nucleic acid and derived amino acid sequences of the type b MinNA and M37 pilins were compared. The nucleic acid sequences coding for the mature protein were 77% identical. The derived amino acid sequences were 68% identical and 77% similar. Similarity was as defined by Dayhoff and normalized by Gribskov and Burgess (12). The conserved cysteines and the penultimate tyrosine observed in the type b pilin and other pilins are conserved in the M37 pilin (residues 24, 64, and 193, respectively). One interesting feature of the sequence comparison is the region surrounding the signal peptide cleavage site. In contrast to the 18-amino-acid leader peptide in the M37 pilin, the type b pilin has a 20-amino-acid leader peptide. The sequence immediately surrounding the cleavage site differs between the two pilins, while the sequences surrounding this stretch of residues are nearly identical. It will be of interest to determine whether the two pilins can be assembled into a common pilus and whether the putative chaperones will recognize the pilin from the heterologous strain.

Total genomic DNA from M37 and M37-1 cells and plasmid DNA from the immunoreactive cosmid clones were digested with PstI and probed with the M37 pilin gene. A 4.2-kb PstI restriction fragment hybridized strongly to the pilin probe (Fig. 3). Two less intensely hybridizing bands of about 9.4 and 4.4 kb were also observed. The pilin probe hybridized to a single 4.2-kb PstI fragment in pRSM781 and the other immunoreactive clones (data not shown). To rule out the possibility that the 9.4- and 4.4-kb hybridizing fragments observed in the chromosomal digests were due to hybridization with plasmid pT7-7 sequences rather than pilin sequences, we probed PstI-digested chromosomal DNA from strains M37, M37-1, and MinNA with nick-translated pT7-7. The plasmid-related sequences (b-lactamase gene) from strain MinNA were readily visualized; no hybridization was observed with the M37 or M37-1 DNA. Thus, M37 DNA contains a single strongly hybridizing fragment and two weakly hybridizing fragments. Previous studies have proposed that type b H. influenzae has a single pilin gene; the significance of the weaker hybridization signals in the M37 DNA remains to be established. In type b strains, no chromosomal DNA rearrangements have been correlated with the expression or lack of expression of the type b pilus. Similarly, we observed no changes in the size of the PstI fragment containing the pilin gene in strains M37 and M37-1.

The M37 pilin gene was digested with BamHI and EcoRI from the replicative form of M13mp18 and cloned into the bacteriophage T7 expression vector pT7-7. A plasmid designated pRSM865 had the correct restriction map. A partial restriction map of pRSM865 is shown in Fig. 4. In this T7 expression system, T7 RNA polymerase synthesis is induced by addition of IPTG to the medium; transcription of genes cloned downstream of T7 610 promoter ensues. A 22-kDa protein was observed in extracts of cells after induction of BL21(DE3)/pLysS/RSM865 cultures with IPTG (Fig. 5). An increase in a protein with an apparent molecular mass of 24 kDa is also observed, although the interpretation of the gel is complicated by a protein with the same apparent molecular mass in cells which do not harbor the plasmid. Sonicates of IPTG-induced BL21(DE3)/pLysS/pRSM865 cells were reactive with monoclonal antibody 3H12. Extracts prepared from uninduced cells or from BL21(DE3)/pLysS cells did not contain the 22-kDa protein and were not reactive with monoclonal antibody 3H12. These data indicate that monoclonal antibody 3H12 reacts with an epitope present on the pilin protein and not with an epitope present on a protein whose expression is coregulated with expression of the pilin gene. The data further indicate that the recombinant pilin folds in E. coli such that the 3H12 epitope is expressed. This folding occurs in the absence of the other gene products of the Haemophilus pilin operon.

Gilsdorf and coworkers (9) recently reported that the hemagglutination-associated pilus from 11 nontypeable H. influenzae isolates was reactive with an antisera prepared against denatured type b pilin or against a synthetic peptide corresponding to residues 5 through 17 of the type b pilin, indicating that the nontypeable and type b pilin share common epitopes. Gilsdorf and coworkers also reported that antiserum which recognized conformational epitopes on type b pilin were reactive with only 1 of the 11 piliated nontypeable strains. Our results with strain M37 are consistent with these observations. The M37 pilin and type b pilin are highly conserved; however, the conformational epitope recognized by 3H12 is not present in MinNA pilin.

Van Ham and coworkers (44) and Brinton and coworkers (17) report that the Haemophilus pilin is assembled and that recombinant clones express a hemagglutination-positive...
Fimbriation of *Haemophilus* species isolated from the respiratory tract of adults. J. Infect. Dis. 150:40–43.


