MINIREVIEW

Role of Pili in *Haemophilus influenzae* Adherence and Colonization

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Haemophilus influenzae was first recognized as an important pathogen in 1892, when it was isolated from throat swab specimens and mistakenly identified as the causative agent of influenza (45). Humans are the only natural reservoir for this bacterium, which is found, most commonly, on respiratory mucosal surfaces and, occasionally, in the female genital tract. Up to 50 to 80% of healthy individuals are colonized with nontypeable *H. influenzae*, which possesses no capsular polysaccharide and fails to react with standard typing sera; fewer than 5% are colonized with strains of serotypes a through f, which possess one of six antigenically and biochemically distinct polysaccharide capsules. For reasons that are not entirely clear, children are more likely to be colonized with *H. influenzae* than are adults (63).

In addition to asymptomatic mucosal colonization, *H. influenzae* causes a wide spectrum of clinical infections (63). Organisms possessing the type b capsule are highly virulent in nonimmune hosts (primarily young, unvaccinated children) and may cause bacteremia and invasive infections such as meningitis, epiglottitis, pneumonia, septic arthritis, and buccal cellulitis. Nonencapsulated *H. influenzae* strains as well as those possessing the non-b capsules a and c through f rarely cause bacteremia or invasive infections in healthy hosts. A unique exception is the Brazilian purpuric fever strain of nontypeable *H. influenzae* biogroup aegyptius, which apparently does not possess a polysaccharide capsule and yet has caused outbreaks of bacteremia, sepsis, and vascular collapse and death in young children, primarily in Brazil (44). Nonencapsulated organisms, however, commonly cause localized respiratory tract infections such as otitis media, sinusitis, bronchitis, and pneumonia (41, 63).

Although asymptomatic in most individuals, *H. influenzae* colonization of the respiratory mucosa is the first step in a series of events that may lead to infection (1, 53). In respiratory organ culture tissues infected with *H. influenzae*, the bacterium is closely associated with mucus, damaged epithelium, and nonciliated cells (12, 13, 30). Furthermore, these organisms invade both cultured epithelial cells (31, 56) and mononuclear, macrophage-like cells within human adenoid tissue (16); survival of the bacterium within cells lining the respiratory mucosa may play a role in prolonged *H. influenzae* colonization of the respiratory tract. It is not clear whether *H. influenzae* nasopharyngeal colonization in humans results in immunity and, thus, protection against subsequent infection by the colonizing or-

ganism. In infant rats, however, *H. influenzae* type b nasopharyngeal colonization without bacteremia failed to protect animals against challenge, whereas colonized animals that were also bacteremic were protected (19).

COLONIZATION AND ADHERENCE FACTORS OF *H. INFLUENZAE*

H. influenzae, like other bacteria, has evolved specific adherence mechanisms that facilitate its continued close association with epithelial surfaces, thus preventing removal of the organism by natural mechanical and cleansing forces and sustaining successful colonization of the human respiratory tract. In the first report of *Haemophilus* adherence to eukaryotic cells, nontypeable *H. influenzae* biogroup aegyptius was noted to agglutinate human erythrocytes (10). More recently, both nontypeable and type b organisms have been shown to adhere to other human cells as well (18, 19, 35, 51, 56, 58).

By electron microscopy (EM), *H. influenzae* isolates that hemagglutinate and adhere to buccal epithelial cells (6, 17, 47, 61) exhibit long, filamentous organelles that extend from the bacterial surface and are not present on hemagglutination-negative, nonadherent bacteria (Fig. 1). These organelles have variably been termed pili or fimbriae. Because of their functional and molecular homology to Pap pili of *Escherichia coli*, we prefer the term pili in describing these surface structures (33). In addition to buccal epithelial cells, pili also mediate adherence to bronchial epithelial cells (25) but not to human foreskin fibroblasts (51), HeLa cells (of human cervical carcinoma origin), A549 cells (of human alveolar epithelial carcinoma origin), human nasal epithelial cells, human tracheal fibroblasts (25), or Chang cells (of human conjunctival origin) (58). These findings suggest that only certain cell types possess receptors for pili. Interestingly, nonpiliated *H. influenzae* variants adhere more efficiently to HEp-2 cells (of human laryngeal carcinoma origin) than do piliated isolates (25, 51), suggesting that *H. influenzae* also possesses nonpilus adhesins whose expression may be negatively coregulated with pili.

In addition to the hemagglutinating pili described above, other types of adherence organelles that do not mediate hemagglutination have been described for *H. influenzae*. Biotype IV *H. influenzae*, which is generally isolated from newborns or the female genital tract, possesses peritrichous pili that do not hemagglutinate but appear to mediate adherence to HeLa cells (50). Bakaletz et al. (4, 52) described surface fimbriae from otitis media isolates that mediate adherence to chinchilla tracheal cells and human oropharyngeal cells but do not mediate binding to human erythrocytes. Furthermore, by EM, these fimbrial structures are thinner than pili. Molecular analysis of fimbriin, the major subunit of chinchilla tracheal cell-adherent

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FIG. 1. Transmission EM of nonpiliated (A) and piliated (B) *H. influenzae*. The pili are seen as long, fine fibers extending from the bacterial surface. (Reprinted from reference 17.)

fimbriae, demonstrated strong homology with outer membrane protein A of *E. coli* (52). Brinton et al. (6) described hemagglutinating pili (referred to as LKP [long, thick, hemagglutination-positive] pili) as well as three other nonhemagglutinating pilus types, which were classified according to their morphological appearance. These other types have not been well characterized, and their relationship to the structures from biotype IV strains or otitis media isolates is unclear.

In addition to adhesins associated with pili or fimbriae, *H. influenzae* also possesses nonpilus adhesins, including the high-molecular-weight adhesins HMW1 and HMW2 (5, 59), the high-molecular-weight adhesin expressed by the *hia* gene (54, 55), and an immunoglobulin A protease-like protein (59), all of which mediate adherence to Chang conjunctival cells. Furthermore, nontypeable *H. influenzae* has been shown to adhere to human tracheobronchial mucins (34); this adherence may be mediated by *H. influenzae* outer membrane proteins P2 and P5 (49).

STRUCTURAL CHARACTERIZATION OF PILI

As seen by EM, *H. influenzae* pili are 4.7 to 18.0 nm in diameter and 209 to 453 nm in length and possess a hollow core (61). By high-magnification, quick-freeze, deep-etch EM, they appear as relatively thick flexible rods with a short, thin fibrillum at the tip, resembling *E. coli* pap pili (60). In *H. influenzae* pili, however, the rods have regularly spaced horizontal striations with cross-over repeats, suggesting that they are two-stranded, helical structures. They are distributed in a

peritrichous manner on the *H. influenzae* surface (47), and 95% of cells examined from piliated strains demonstrate pili (38). Stull et al. (61) described an average of 16 pili per bacterial cell, although EM studies by other investigators have shown the bacteria to be more heavily piliated (6, 17).

The *H. influenzae* pilus rods are composed of polymerized pilins, which are subunit proteins that show slight strain-to-strain variability in migration by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (17). As determined by amino acid sequence analysis, pilins of strain Eagan consist of 196 amino acids with a molecular mass of 21,152 (3). This kind of molecular organization is consistent with the structures of adherence pili of other organisms (33) in which subunit proteins with sizes of 20 to 30 kDa polymerize into long, filamentous, adhesive proteins.

Although hemagglutinating pili were initially identified, characterized, and purified from type b strains, similar structures are also present on nontypeable *H. influenzae* isolates (17).

FUNCTIONAL CHARACTERIZATION OF *H. INFLUENZAE* PILI

Like the expression of pilus adhesins of other organisms (33), the expression of pili by *H. influenzae* is regulated by phase variation. The rates of transition from the piliated phase to the nonpiliated phase and vice versa are 3×10^{-4} and 7×10^{-4} phase shifts/bacterium/generation, respectively (12). Most clinical isolates, either type b or nontypeable, do not express pili when they are obtained after several subcultures from the diagnostic microbiology laboratory (2, 18, 38, 46); an exception is *H. influenzae* biotype aegyptius strains (10, 48). Piliated-phase variants can, however, be obtained from most nonpiliated isolates by selective hemadsorption, taking advantage of the ability of pili to agglutinate erythrocytes (9, 37).

By light microscopy to assay eukaryotic cell adherence, piliated, adherent *H. influenzae* organisms are shown to be randomly distributed over the surface of buccal epithelial cells (18). By kinetic analysis (43), buccal epithelial cell adherence of both piliated and nonpiliated *H. influenzae* increases over time, with maximum adherence occurring between 24 and 36 h of incubation. After 30 h of incubation, epithelial cells incubated with nonpiliated *H. influenzae* are coated with piliated variants, suggesting that pilus production is induced during incubation or that incubation with epithelial cells selects for piliated variants.

van Alphen et al. (67) have shown that piliated *H. influenzae* binds to erythrocytes possessing the AnWj (previously known as the Anton antigen) phenotype. This antigen is present on the surfaces of most human erythrocytes, although the erythrocytes of newborn infants lack AnWj. The observation that neither the erythrocytes nor the buccal epithelial cells from three AnWj-negative siblings bound piliated *H. influenzae* led van Alphen et al. to conclude that both epithelial cell and erythrocyte receptor molecules possess similar binding sites for *H. influenzae* pili (65, 66). Additional studies have shown that sialic acid-containing lactosylceramides, the gangliosides GM1, GM2, GM3, and GD1a, inhibit pilus-mediated *H. influenzae* binding to both buccal epithelial cells and erythrocytes (64), consistent with the finding in other bacteria that pili bind to glycoconjugated receptors present on eukaryotic cell surface glycoproteins and glycolipids (33).

Although the role of pili in mediating adherence of *H. influenzae* to respiratory epithelial cells is clear, their role in the establishment or maintenance of nasopharyngeal colonization is more difficult to ascertain. Anderson et al. (1) observed that

a piliated *H. influenzae* strain which showed increased adherence to rat buccal epithelial cells was more effective than its nonpiliated variant in colonizing rats following intranasal inoculation. In addition, Weber et al. (72) showed that a piliated type b isolate colonized yearling Rhesus monkeys more effectively than a pilus-negative insertion mutant; the organisms recovered from the colonized monkeys, however, were nonpiliated, highlighting the complex biology of nasopharyngeal colonization, which undoubtedly involves a complicated interplay of multiple host and bacterial factors.

Although the role of pili in colonization remains unclear, their role in bacterial invasion and intervascular spread is even more cryptic. Clearly, the most important bacterial factor in the pathogenesis of bacteremic *H. influenzae* disease is the type b capsule (73, 75), and use of the type b capsular vaccine has resulted in a dramatic reduction in the incidence of invasive type b infections (7). As for the role of pili, data from Gutierrez et al. (26) suggest that infant rats inoculated with piliated *H. influenzae* b, either intranasally, intravenously, or intraperitoneally, had decreased levels of bacteremia compared to rats inoculated with nonpiliated variants. In addition, piliated *H. influenzae* organisms bind (and are killed by) antibodies directed against nonpilus surface antigens more effectively than do nonpiliated organisms (24). Furthermore, piliated *H. influenzae* stimulates enhanced opsonization-dependent phagocytosis by neutrophils as determined by chemiluminescence (62). Taken together, these data support the concept that piliated *H. influenzae* is cleared from the circulation more readily than are nonpiliated organisms. Thus, the most important roles of pili in the pathogenic pathway of *H. influenzae* infection appear to be their mediation of bacterial adherence to mucosal surfaces and their facilitation of respiratory tract colonization.

IMMUNOLOGIC CHARACTERIZATION OF *H. INFLUENZAE* PILI

H. influenzae pili are immunogenic (20), and their antigenic nature is complex because of their tertiary or quaternary structure. Polyclonal antibodies directed against native pili on intact bacteria bind to homologous piliated bacteria but do not bind to homologous denatured pilin, the major structural subunit of pili (37). Similarly, polyclonal antisera directed against gel-purified, denatured pilins bind to homologous pilins but do not bind to native pili on whole bacterial cells (37). Upon examination of pilus-antibody binding to a variety of piliated strains, 18 of 21 type b isolates reacted with antisera against type b strain M43 pili and 11 of 21 isolates reacted with antisera against type b strain Eagan pili, demonstrating immunologic heterogeneity of surface pili (23). Furthermore, antibodies directed at epitopes on denatured pilins from strain M43 recognized the denatured pilins of all strains tested, demonstrating a high level of conservation of epitopes on denatured pilins. These data confirm the highly conformational nature of epitopes on polymerized pili.

Pilus and pilin epitopes of nontypeable organisms show a pattern of conservation similar to that of type b organisms. Antisera against intact type b pili did not bind to any of the 12 piliated nontypeable *H. influenzae* strains tested, whereas antisera against denatured type b pilins did bind to denatured pilins of all 12 nontypeable strains (17). Thus, linear epitopes on pilins are highly conserved among both type b and nontypeable piliated strains, but epitopes on polymerized pili are conformational and vary significantly from strain to strain.

Protein hydrophilicity profiles of pilins from *H. influenzae* Eagan and M43 show several hydrophilic regions that correspond to areas of conserved amino acids. Antisera against

synthetic peptides representing these conserved and hydrophilic regions bind only weakly to native pili of the homologous strain but bind well to denatured pilins, again demonstrating the complexity of *H. influenzae* pili and the highly conformational nature of their epitopic domains. The targets of these antipeptide antibodies appear to be available for antibody binding on denatured pilins but unavailable on native pili, either because these epitopes are lost on polymerization of pilins into pili or because the epitopes are hidden deeply within the configuration of assembled pili (15, 20).

Antibodies against *H. influenzae* pili interfere with buccal cell adherence of the homologous piliated strain and show either reduced or no interference of strains expressing immunologically heterologous pili (37). Furthermore these antibodies are bactericidal for the homologous strain but are not bactericidal for strains expressing heterologous pili. Even though *H. influenzae* pili from different strains show immunologic heterogeneity, and thus, presumably exhibit different amino acid sequences, they appear to possess common regions that bind to eukaryotic cells, as they all bind to the erythrocyte antigen AnWj (21).

Only limited information exists concerning the antibody response to pili in humans following *H. influenzae* infection. Pichichero et al. (47) described presumably antipilin antibodies in the serum of a child colonized with piliated *H. influenzae* that were not present in a serum sample obtained prior to detection of the colonization. In addition, Erwin et al. (11) detected antipilin antibodies by Western blot (immunoblot) analysis in serum samples from 5 of 16 healthy children and from 5 of 10 children convalescing from invasive *H. influenzae* type b disease; for 4 of these latter 5 children, however, antibodies were detected in their serum samples during the acute phase of infection as well. Interpretation of these human antibody findings is difficult in light of the strong evidence by Western blotting that rabbit antibodies against either native pili or purified pili do not bind pilins (23, 37).

MOLECULAR CHARACTERIZATION OF *H. INFLUENZAE* PILI

The gene (*hifA*) that encodes the major pilus structural subunit (pilin) has been cloned and sequenced from four *H. influenzae* type b isolates (8, 15, 22, 36, 68) and from six nontypeable isolates, including two Brazilian purpuric fever strains (8, 57, 74). Each of these pilins possesses a leader sequence consisting of between 17 and 20 amino acids followed by a mature protein of between 191 and 196 amino acids. They also contain two cysteines in similar positions, as well as tyrosine and glycine residues located 2 and 14 amino acids, respectively, from the C terminus, which is the signature pattern of *E. coli* pilus structural proteins (27). Furthermore, the *H. influenzae* pilins are significantly homologous, with 63 to 81% amino acid identity (Table 1). In addition, they show sequence homology to pilins of other organisms, including *E. coli* F17, type 1c, and Pap pili as well as pili of *Klebsiella pneumoniae*, *Bordetella pertussis*, and *Serratia marcescens* (22, 68). One copy of the pilin gene is present in the chromosome of most *H. influenzae* strains studied, although strain Rd, whose entire chromosome has been sequenced, does not have a pilin gene (14), and the Brazilian purpuric fever isolates possess two complete copies (48, 59). Thus, in *H. influenzae* biotype aegyptius strains, a shift to the nonpiliated phase requires phase variation of both genes, which explains the observation that clinical isolates of these strains are more likely than other strains to express pili.

Downstream of *hifA* are two open reading frames with strong nucleotide sequence homology to the *E. coli* purine

TABLE 1. Description of *H. influenzae* pilus gene cluster

Gene	Size (bp)	Mutant phenotype			Predicted function	Predicted size (kDa) of protein
		Pilin	Pilus	HA ^a		
<i>hifA</i>	648	—	—	—	Structural	23.3
<i>hifB</i>	711	—	—	—	Chaperone	26.3
<i>hifC</i>	2,514	+	—	—	Usher	92.7
<i>hifD</i>	645	+	±	—	Structural	20.6
<i>hifE</i>	1,305	+	±	—	Structural/adhesin	45.5

^a HA, hemagglutination.

biosynthesis genes *purK* and *purE* (Fig. 2). Upstream but transcribed in the opposite direction from *hifA* are four additional open reading frames, termed *hifB* to *hifE*. Immediately downstream of *hifE* is an open reading frame whose nucleotide sequence is homologous to *pepN*, an *E. coli* gene encoding an aminopeptidase (39). These five genes, *hifA* to *hifE*, comprise the *H. influenzae* pilus gene cluster (39, 69). Recombinant *E. coli* transformed with plasmids containing this gene cluster expresses both pilin and assembled pili and demonstrates hemagglutination and buccal epithelial cell adherence (32, 68), suggesting that this cluster is sufficient for pilus assembly and function (Fig. 3).

hifB encodes a 214-amino-acid protein with strong nucleotide sequence homology to the immunoglobulin-like chaperone proteins of other organisms, including PapD of *E. coli* Pap pili (28). As with the other pilus chaperone proteins, HifB binds to HifA and to HifD, forming complexes that protect HifA and HifD from proteolytic digestion as they traverse the periplasmic space (60). *H. influenzae* possessing mutations in *hifB* no longer expresses pilin (71), does not demonstrate surface pili (39, 69), and lacks hemagglutinating activity (71). Although *hifA* is transcribed in these *hifB* mutants (71), in the absence of the chaperone, the *hifA* gene product, pilin, is most likely degraded in the periplasm (60).

hifC encodes a large, 837-amino-acid protein that has significant nucleotide sequence homology to members of the pilus assembly platform (usher) family of proteins, including PapC and FimD of *E. coli*, MrkC of *K. pneumoniae*, and FhaA of *B. pertussis* (71). *H. influenzae* with mutations in *hifC* lacks pili and hemagglutinating activity but expresses the major pilus subunit, pilin (39, 69), supporting the functional role of HifC as an assembly platform (usher) protein.

hifD encodes a 216-amino-acid lipoprotein-like protein (69) that shows nucleotide sequence homology, especially in the C-terminal region, to several bacterial pilin genes, including *hifA* (39, 69). The protein encoded by this gene is incorporated into the *H. influenzae* pilus structure and is located at its tip (40, 60). In-frame deletions in *hifD* result in *H. influenzae* mutants that express pilin but are unable to assemble pilins into pili or to agglutinate erythrocytes (39). The function of HifD remains unknown, although, similar to minor pilus com-

FIG. 2. *H. influenzae* pilus gene cluster. The five genes, the directions of transcription (indicated by the horizontal arrows), and the flanking open reading frames (*purK*, *purE*, and *pepN* homologs) are shown. See Table 1 for descriptions of the genes.

FIG. 3. Model of the role of proteins HifA to HifE in assembly of *H. influenzae* pili. HifA, the structural subunit, HifD, and HifE (by analogy to *E. coli* [28]) bind to HifB (the chaperone) in the periplasm, protecting them from degradation. These proteins are assembled at the bacterial surface through interactions with HifC (usher), such that HifA polymerizes into a pilus stalk and HifD and HifE (the pilus adhesin) localize at the pilus tip.

ponents of other bacteria (27), it may play a role in activating or terminating polymerization of pilins into pili.

hifE, which begins 27 bp downstream of *hifD*, encodes a protein of 435 amino acids, of which the first 29 are a putative leader sequence (39). This protein also shows significant nucleotide sequence homology with HifA and HifD, particularly in the C-terminal region (39, 69). The similarity of these proteins in this region may serve to maintain the correct subunit-subunit interactions between pilin subunit proteins and the chaperone proteins that carry them through the periplasmic space (29). The amino acid homology of HifE with HifA and the presence of two cysteines, as well as the tyrosine and glycine residues located 2 and 14 amino acids, respectively, from the C terminus, suggest that HifE is assembled in a fashion similar to that of HifA and HifD (33, 60). As seen in

TABLE 2. Comparison of amino acid sequences of *H. influenzae* pilus proteins to those of strain Eagan^a

Strain	Capsule type	% Homology to Eagan protein				
		HifA ^b	HifB	HifC	HifD	HifE
MA3/AO2	b	80				
770235	b	80	98	98	100	100
86-1249	Nontypeable	81				
86-0295	Nontypeable	63	95	98	86	51
81-0384	Nontypeable	67				
M37	Nontypeable	70				
F3031	Nontypeable	77	96	97	93	50

^a The accession numbers for the strain Eagan protein sequences are M64334 (HifA), X66606 (HifB), U02932 (HifC), and U13254 (HifD/E).

^b The accession numbers for the HifA protein sequence of the different *H. influenzae* strains (indicated in parentheses) are X52419 (M43/AO2), Z33502 (770235), U19761 (86-1249), U19730 (86-0295), U19795 (81-0384), and L08606 and U54780 (F3031). For the HifA protein sequence of strain M37, see reference 8.

Table 2, HifE shows strong homology (100%) between the two type b strains but only about 50% homology between the type b strains and the two nontypeable strains. This observation may reflect the genetic differences that generally exist between type b and nontypeable strains (42). HifE is located at the tips of *H. influenzae* pili (40), and mutations in the *hifE* gene result in mutants with intact pilin expression (39) but reduced pilus expression (39, 69) and negligible hemagglutination and buccal cell adherence. The epithelial cell binding site of *H. influenzae* pili appears to be located on HifE, as HifE antiserum completely blocks pilus-mediated hemagglutination. Antiserum against pili, on the other hand, only partially blocks pilus-mediated hemagglutination, and antiserum against HifD fails to block pilus-mediated hemagglutination (40).

In addition to the presence of the pilus genes in type a, b, and f strains and in nontypeable strains, they are also present in nonpiliated variants of piliated strains (15, 22, 36), consistent with the observation that pilus expression is phase variable. The promoter regions of the two divergently transcribed pilus genes, *hifA* (encoding the major pilus subunit) and *hifB* (encoding the pilus chaperone), overlap and are found in the stretch of DNA between these two genes that contains multiple TA repeats (70). Variation in the number of the TA repeats alters the spacing between the -35 and -10 sequence, suggesting a mechanism by which phase variation in the expression of pili may occur.

In summary, the hemagglutinating pili of *H. influenzae* are important organelles that facilitate colonization of the respiratory tissues by allowing the bacteria to adhere to human epithelial cells. These pili are complex proteins containing major pilus subunit proteins encoded by *hifA* and two minor tip proteins encoded by *hifD* and *hifE*. In addition, two other genes in the pilus gene cluster, *hifB* (a chaperone-like protein) and *hifC* (an assembly platform-like protein), are required for *H. influenzae* pilus biogenesis.

Because of their importance in mediating adherence to human mucosal surfaces, pili appear to be attractive candidates for vaccines against non-type-b *H. influenzae*, against which the presently available *H. influenzae* capsular vaccines offer no protection. Further study of the immunogenicity and strain-to-strain antigenic diversity of the adhesive region of pili will clarify their potential value as vaccines.

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