INFECTION AND IMMUNITY, Nov. 1998, p. 5053–5059 Vol. 66, No. 11
0019-9567/98/$04.00+0
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MINIREVIEW

Antigenic Diversity and Gene Polymorphisms in *Haemophilus influenzae*

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Haemophilus influenzae is a gram-negative bacillus that lives symbiotically in the upper respiratory tracts of humans. With the occasional exception of the human genital tract, the human nasopharynx is the sole ecologic niche occupied by this organism; *H. influenzae* is not found in the environment and does not colonize, or infect, other animal species. Epidemiologic studies have documented that *H. influenzae* nasopharyngeal colonization rates vary between 25 and 80% in humans sampled (80), with higher rates for children than for adults.

In addition to asymptomatically colonizing humans, *H. influenzae* also causes significant human disease. *H. influenzae* strains that possess the type b capsule (Hib), composed of polyribose-ribitol phosphate (PRP), cause life-threatening, sometimes fatal, infections such as bacteremia and meningitis in nonimmune infants and children. Vaccines consisting of the PRP capsule conjugated to various protein carriers are highly immunogenic, even in very young children (81) and are protective against serious Hib infections (13). Although widespread use of these vaccines in the United States has resulted in the nearly complete disappearance of serious Hib infections in children, they do not protect against *H. influenzae* strains that possess one of the other types of capsule (a or c to f) or against those strains that possess no capsule (so-called nontypeable, as they do not react with antisera specific for capsular types a through f). Nonencapsulated (nontypeable) *H. influenzae* rarely causes life-threatening, invasive infections in otherwise healthy children or adults but is a significant cause of localized respiratory infections, such as otitis media, sinusitis, pneumonia, and bronchitis.

Nasopharyngeal colonization of *H. influenzae*, which precedes *H. influenzae* infection and disease, is a dynamic process (79). Colonization begins early in life and may persist for prolonged periods of time (21, 33, 75, 77). Epidemiologic studies, which utilize recently developed molecular typing systems that more accurately define bacterial strain differences, show rapid turnover of strains in some individuals—the duration of colonization by a single strain may be less than 1 month. In addition, chronically colonized individuals acquire variants of their original *H. influenzae* strain that differ in their surface antigens, and individuals may be cocolonized with more than one *H. influenzae* strain (33, 75, 77).

The means by which *H. influenzae* is able to colonize the human nasopharynx over an extended period of time in the face of *H. influenzae*-specific mucosal antibodies is unclear. Possible explanations for prolonged carriage include its known ability to persist intracellularly (24), the induction of a weak inflammatory response by *H. influenzae* carriage (9), and the presence of antigenic variation among colonizing *H. influenzae* strains (11). The human respiratory tract is bathed with antibody-containing (mostly immunoglobulin A [IgA]) secretions, and in *H. influenzae*-immune individuals, *H. influenzae*-specific antibodies are found in these secretions. A total of 80% of Hib-infected infants, 36% of colonized contacts of these patients, 53% of noncolonized contacts, and 30% of control children demonstrated antibodies specific for the type b capsule in their salivary secretions (29). This study assessed the point prevalence of salivary antibody against just one *H. influenzae* antigen (the type b capsule) and did not explore the relationship between antibody response and persistence of colonization, nor did it explore the prevalence of antibodies directed at other *H. influenzae* antigens. On the other hand, an increased immune response to P6, a highly conserved *H. influenzae* outer membrane protein, was associated with reduction of colonization (38). Thus, the role of antibody in preventing, or modulating, asymptomatic *H. influenzae* colonization remains incompletely understood.

In addition to chronic, asymptomatic colonization of healthy individuals, *H. influenzae* also chronically infects patients with underlying respiratory tract disease, such as cystic fibrosis or chronic obstructive pulmonary disease (33, 60). Although these patients may be infected with one *H. influenzae* strain for a prolonged period of time, subtle changes in the surface antigens of the organisms result in new epitopes that are not recognized by antibodies specific for the original infecting strain (34). Thus, variation of its surface antigens appears to be an important mechanism by which *H. influenzae* avoids attack by mucosal antibodies and survives in the only environmental site it occupies, the human nasopharynx.

DIVERSITY OF *H. INFLUENZAE* ANTIGENS

Capsule. Encapsulated *H. influenzae* strains express one of six chemically and antigenically distinct capsules, designated types a through f. Among these, the type b capsule is the most important as a virulence factor for invasive *H. influenzae* disease (61). Although *H. influenzae* organisms that appear to have lost type b capsule expression have been identified among the nasopharyngeal isolates of patients with Hib infection (40), most nontypeable strains possess unique outer membrane patterns compared to those of typeable strains and are phylogenetically distinct from typeable strains (65). Thus, nontypeable strains are, for the most part, not merely typeable strains that no longer express capsule.

Capsule expression is relatively unstable, and loss of capsule expression occurs due to loss of genetic material required for

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TABLE 1. Antigenic and amino acid diversity of *H. influenzae* antigens

Antigen	Antigenic diversity	Surface exposed	Amino acid conservation (%)	Antigen function	Reference(s)
Proteins					
HMW1, HMW2	Yes	Yes	?	Adhesin	4, 5
P1	Yes	Yes	≥86	?	20, 62
P2	Yes	Yes	≥76	Membrane porin	8, 36, 91
P4	No	Yes	?	?	31, 32
P5	Yes	Yes	No	Adhesin	18, 70, 71
P6	No	Yes	97	?	66
HifA	Yes	Yes	≥63	Pilus subunit	28
HifD	Yes	Yes	≥86	Pilus subunit	28
HifE	Yes	Yes	≥50	Pilus adhesin	28
Hia (Hsf)	?	Yes	72	Adhesin	78
IgA protease	Yes	Yes	69	IgA protease	69
D15	No	Yes	96	?	53
Lipoprotein D	No	Yes	98	Glycerophosphodiester Phosphodiesterase	1, 76
HxuA	?	Yes	≈87	Heme/hemopexin binding	16
Hemopexin binding protein	?	Yes	?	Hemopexin binding	90
Pilus	Yes	Yes	—[a]	Adherence	28
Capsule	Yes	Yes	—	Antiphagocyte	2, 61, 67
LOS	Yes	Yes	—	Membrane integrity	3, 12, 44, 82

[a] —, complex antigen that requires several gene products for expression.

its synthesis (48). Early genetic studies of *H. influenzae* demonstrated the ability of rough (nonencapsulated) mutants of encapsulated isolates to gain expression of different capsular types following transformation with DNA from encapsulated strains (2). Acquisition of expression of a new capsular serotype by an encapsulated *H. influenzae* strain has not been observed in nature, although it has been described for *Streptococcus pneumoniae* (7, 15, 39). In addition, expression of capsule by inherently nontypeable *H. influenzae* strains that have been transformed with DNA of encapsulated organisms has not been described. Although the amount of DNA that is specific to a particular capsular type is relatively small, many genes that are not capsular-type specific are required for capsule synthesis and expression, and in light of the large amount of genetic material necessary to synthesize capsular material, transfer of these capsular genes from strain to strain by transformation and homologous recombination would most likely be an extremely rare event.

LOS. The lipooligosaccharide (LOS) of *H. influenzae* differs from lipopolysaccharide (LPS) of gram-negative enteric organisms in that it possesses considerably shorter oligosaccharide side chains (25). Although *H. influenzae* LOS lacks the highly variable O-side-chain-repeating units that are characteristic of LPS, it still displays strain-to-strain structural heterogeneity (44). At least 10 serotypes of *H. influenzae* LOS have been identified (12, 82). Antigenic heterogeneity of LOS results from differences in lipid A as well as differences in its core polysaccharide or short carbohydrate side chains (3, 12). Furthermore, in some strains LOS is sialylated and, thus, similar in structure to the LOS of *Neisseria* species (54), in which the presence or absence of sialic acid modifies the antigenic specificity of the LOS (54). Also, *H. influenzae* LOS variants possess phosphocholine residues that alter their immunologic reactivity and render the organisms more susceptible to the bactericidal activity of human serum (89).

The role of anti-LOS antibodies in protection against *H. influenzae* infection is unclear. Although antibodies directed against *H. influenzae* LOS are opsonic (58) in vitro and are somewhat protective against otitis media in chinchillas (35), they do not protect against invasive disease in humans (73) or in infant rats (72). In part, this may be explained by LOS antigenic variation, as acquisition of new LOS epitopes is associated with increased resistance to complement-mediated serum killing (26, 46). Furthermore, LOS antigenic variation occurs rapidly among *H. influenzae* isolates, both in vitro (46, 47) and in vivo (59). While the presence of antibodies in the microbial environment may select for LOS antigenic variation, environmental changes unrelated to the immune response may also stimulate epitopic diversity, as broth grown *H. influenzae* shows antigenic changes in LOS similar to those of the same strain grown in vivo (59).

Proteins. A number of *H. influenzae* surface proteins have been identified and characterized (Table 1). Although the functions of some of these proteins have been defined, for many others their roles in pathogenesis or microbial physiology remain unknown. The major outer membrane proteins (P1 through P6) were first described as the predominant bands seen by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of preparations of outer membranes from Hib strains (6). Other proteins were identified as targets of antibodies in sera of convalescent patients (5, 53). Adherence proteins have been identified because of their functional capacity to promote *H. influenzae* adhesion to human epithelial cells (50, 78).

Although a few of the *H. influenzae* surface proteins, P4 (31), P6 (66), lipoprotein D (1, 76), and D15 (53), are immunologically highly conserved, many show significant immunologic heterogeneity. For example, over 30 serotypes of IgA protease (52) and at least 14 serotypes of pili (10) have been identified. The immunologic heterogeneity of *H. influenzae* proteins depends upon their structural differences, which are determined by amino acid heterogeneity.

Among *H. influenzae* proteins whose structural and immunologic characteristics have been well studied, such as IgA protease and the pilus major subunit protein HifA (14, 27), the variable amino acid sequences coincide with regions that are predicted to be hydrophilic and antigenic. Similarly, the variable

sequences of P2 and P5 are localized on the surface-exposed loops (8, 18, 91). On the other hand, regions of conserved sequences exist in areas of proteins known to be responsible for essential function, such as the membrane spanning regions of P2 and P5 (8, 18); the C termini of HifA, HifD, and HifE, which are thought to interact with the pilus chaperone (28); and both the regions surrounding the proposed active serine of IgA protease and its C-terminal domain, which forms a pore in the bacterial outer membrane to facilitate protease secretion (69). Thus, while the conserved amino acid sequences assure preservation of protein function, their variable regions allow these bacteria to present a changing face to their antibody-laden environment, which may allow them to evade the human immune response.

Various biologic functions have been ascribed to antibodies raised against *H. influenzae* surface proteins. Many (anti-P1, -P2, -P4, -P6, and -lipoprotein D) have been shown to be bactericidal (1, 31, 32, 51, 64, 91). Some (P6 and possibly P1) are protective against Hib bacteremia and/or meningitis in an infant rat model (30, 32), while anti-P5 is not (63). Antibodies directed against *H. influenzae* pili (23, 50) and its subunit proteins HifA and HifE (57) reduce, or block, *H. influenzae* adherence to human buccal epithelial cells in vitro. Although the antibodies directed against HMW1 and HMW2 adhesins have been shown to modify otitis media in an experimental chinchilla model (4), no studies of their effect on *H. influenzae* adherence have been reported.

The role of antibodies against specific *H. influenzae* proteins in facilitating protein antigenic variation and its resultant effect on *H. influenzae* mucosal colonization is difficult to directly assess in the absence of ideal animal models. Nevertheless, in a subcutaneous tissue cage model of chronic *H. influenzae* infection in rabbits (86), a P2 variant arose that was not killed by serum shown to be bactericidal for the original strain. Furthermore, in a longitudinal study of patients with chronic obstructive pulmonary disease who were chronically infected with *H. influenzae*, the isolate from one patient showed a change in both its IgA1 protease cleavage specificity and its antibody inhibition pattern (52), suggesting that the presence of mucosal antibodies directed against IgA protease selected for the emergence of the variant strain. Thus, indirect evidence suggests that protein-specific antibodies, which are stimulated by *H. influenzae* colonization or infection, select for antigenic variants that possess a survival advantage over the parent strains, thus allowing for persistent *H. influenzae* colonization and/or infection.

MOLECULAR MECHANISMS OF *H. INFLUENZAE* ANTIGENIC DIVERSITY

To facilitate evasion of the immune response generated by the human host, *H. influenzae* has evolved several molecular mechanisms (Table 2) by which to alter the antigens on its surface.

Point mutations. Antigenic diversity of proteins occurs most simply, and perhaps most commonly, by spontaneous point mutations in the genes encoding protein antigens, such that the physical topography of the molecule is altered and antibodies directed against the original antigen do not recognize the altered one. Nucleotide sequence analysis of the genes encoding several *H. influenzae* surface proteins reveals many single base changes distributed throughout the variable regions. Interestingly, in the variable regions of P5, the base pair changes were predominately nonsynonymous (nonsilent) (18). This pattern was also seen in the variable regions of P1 (62) and HifA (14). The meaning of this observation is unclear, as a selection bias

TABLE 2. Genetic mechanisms relating to antigenic variation of *H. influenzae*

Mechanism	Effect(s)	Example(s) in *H. influenzae*
Point mutation	Altered antigenicity secondary to altered protein structure	P2 and P5 variable regions
Gene amplification	Increased production of gene product	Capsule
Phase variation	Alteration of promoter structure	Pili
	Translational frame shift secondary to slipped strand mispairing	LOS genes Uncharacterized *H. influenzae* gene products
Horizontal gene transfer and homologous recombination	Importation of DNA possessing new antigenic domains	Pili IgA protease Capsule P2

that does not favor silent changes in the amino acids in the variable regions seems unlikely. The explanation may be simply a sampling problem in that the highly variable regions contain many fewer conserved amino acids in which to observe silent changes compared to the many conserved amino acids in the constant regions.

Because of the many nucleotide differences seen in the genes that encode the variable regions of *H. influenzae* proteins, ascribing altered immunoreactivity of the protein to any single point mutation is often difficult. One example of point mutations that may affect immunoreactivity, however, is in the variable regions of the *H. influenzae* gene encoding the P2 outer membrane protein. Nucleotide sequence analysis of P2 genes from *H. influenzae* organisms sequentially isolated from rabbits with experimental chronic *H. influenzae* infections revealed several base pair substitutions. Serum bactericidal antibodies against the initial strain did not kill the variant isolates that arose later in the chronic infection. These findings suggested that immune pressure by antibodies directed at the early isolates selected for later isolates containing the point mutations (86). Antibodies in these sera mapped to the tip of loop 6, a region that would be exposed to mucosal immune pressure. In this region, the variants contained nonsynonymous nucleotide substitutions that resulted in alterations in four contiguous amino acids (19).

Detailed analysis of the gene encoding *H. influenzae* outer membrane protein P5 from sequential *H. influenzae* isolates from patients with chronic bronchitis also revealed amino acid substitutions that resulted from point mutations in the variable regions. Since these variable regions are located in surface-exposed loops, amino acid substitutions in them may play a role in avoidance of immunity and in persistent *H. influenzae* colonization (18).

In HifA (the major *H. influenzae* pilus subunit), only two amino acids, which are located 17 amino acids apart, are unique to strain M43 compared to those from six other strains that do not bind antibodies against pili of strain M43 (14). The nucleotides encoding these two amino acids differ in one base each, Val (gta)75→Gly (gga) and Pro (cca)92→Leu (cta). This story is more complicated, however, because the epitopes defined by this polyclonal antiserum are not known and are unlikely to be linear amino acids (68).

Thus, point mutations are common in the nucleotide sequences of many heterogenous *H. influenzae* proteins, and

nonsynonymous mutations that result in amino acid substitutions undoubtedly play an important role in antigenic diversity. Without epitope mapping studies, however, direct association of a specific point mutation and altered antibody reactivity remains speculative.

Gene amplification. The Hib capsular genes have been studied in detail and are located in a chromosomal region characterized by two tandem 18-kilobase repeats (42). The quantity of type b capsular material expressed is proportional to the number of tandem repeats present in an isolate, with a maximum of five repeats identified (17). Nonencapsulated variants arise at relatively high frequency (0.1 to 0.3%) and are characterized by deletion of one of the tandem repeats such that the remaining copy lacks *bexA*, a gene required for polysaccharide export (41).

Phase variation. Phase variation is a method by which organisms alter the expression of surface molecules in a reversible fashion. Phase variants arise from the background population of bacteria at relatively high frequencies and may be selected during changing environmental conditions. Two *H. influenzae* surface molecules, pili and LOS, undergo phase variation, and the genetic mechanisms responsible for this variation have been, at least partially, elucidated. The factors that stimulate, or facilitate, phase variation of these antigens have not been defined, although the presence of a specific antibody may result in enhanced survival of either organisms expressing an alternative antigenic form of phase variable antigens, as in LOS, or organisms not expressing the antigen at all, as in pili (11).

H. influenzae pili are multimeric proteins that mediate epithelial cell adherence and phase vary with a P+ to P− switch frequency of 3×10^{-4} switches per bacterium per generation and a P− to P+ switch frequency of 7×10^{-4} switches per bacterium per generation (22). Expression of functional, assembled pili requires the products of five clustered genes: *hifA*, which encodes the major subunit of pili, is transcribed in the opposite direction from the other four genes in the cluster; *hifB* (a chaperone); *hifC* (an usher protein); *hifD* (a pilus subunit located at the pilus tip); and *hifE* (the pilus adhesin, also located at the pilus tip) (28). The P+↔P− phase variation is controlled by a series of TA repeats located in the intragenic region between *hifA* and *hifB*, a region that contains the promoter region for both genes. Addition or deletion of AT repeats changes the spacing of the −10 and −35 sequences, thus disrupting the transcription of *hifA* and *hifB* (84).

H. influenzae LOS is a highly variable, complex surface antigen, whose expression requires a number of genes encoding various enzymes and regulatory proteins. One genetic mechanism that contributes to LOS phase variability involves gain or loss of expression of core oligosaccharides because of slipped strand mispairing. The 5′ regions of open reading frames within *lic1*, *lic2*, and *lic3*, which are genetic loci important in synthesis of *H. influenzae* LOS, contain multiple tetrameric repeats (5′-CAAT-3′). Addition or deletion of one of the repeats moves the transcriptional start site in or out of frame with the remainder of the coding sequence, thus resulting in variable expression of the gene products (87, 88).

Analysis of the genomic sequence of *H. influenzae* Rd revealed nine novel loci with multiple tandem tetranucleotide repeats, all within putative open reading frames (43). Among the open reading frames containing the repeats were homologues of genes encoding hemoglobin-binding proteins of *Neisseria*, a glycosyltransferase of *Neisseria*, and YadA, an adhesin of *Yersinia* spp. Subsequent analysis (83) of the Rd sequence revealed five regions containing dinucleotide repeats, one region containing a trinucleotide repeat, two regions containing septanucleotide repeats, and three regions containing hexanucleotide repeats. Thus other, up-to-now-unstudied, *H. influenzae* proteins that are possibly antigenic or that regulate expression of antigens also possess the potential for phase variation by slipped strand mispairing.

Horizontal gene transfer and recombination. *H. influenzae*, like other human mucosa commensals such as *Neisseria gonorrhoeae* and *S. pneumoniae*, is naturally transformable in that it possesses specific mechanisms for importing DNA from the environment. In *H. influenzae*, natural transformation is dependent on the presence of a specific nucleotide sequence in donor DNA fragments that includes the invariate 9-bp sequence 5′-AAGTGCGGT-3′. During natural transformation, this specific site on DNA fragments binds to the bacterial cell surface, the double-stranded donor DNA is internalized, the strands are partially degraded, and single-stranded DNA is incorporated into the genome by *rec*-1-dependent homologous recombination (45). The chromosome of *H. influenzae* Rd, which has been completely sequenced, contains 1,465 copies of the uptake sequence in its 1.83-Mbp genome (74). The uptake sequences are nearly randomly distributed across the chromosome, although they are somewhat less likely to be located in open reading frames than in intragenic DNA.

This ability to incorporate donor DNA into the *H. influenzae* genome facilitates the process of horizontal gene exchange, a process by which genes are transferred between bacteria. In many other bacteria, the usual mechanism of horizontal gene exchange is through plasmids by conjugation or through bacterial viruses (phages) by transduction. These mechanisms appear to be rare in *H. influenzae*. In this organism, as well as in other bacteria that colonize mucosal tissues and whose host range is limited to humans, horizontal gene exchange could occur through specific uptake of homologous DNA that is present in the organism's immediate environment because of bacterial autolysis. Mucosal colonization with multiple strains, as is now recognized to commonly occur with *H. influenzae*, adds to the diversity of DNA available for *H. influenzae* horizontal exchange, thus facilitating antigenic diversity by this means.

Several examples of possible horizontal gene exchange among *H. influenzae* strains have been described. *H. influenzae* strains serially recovered from Aboriginal infants living in a relatively closed population in Australia possessed regions of the outer membrane porin, P2, identical to those of strains exhibiting different genetic backgrounds (75). Furthermore, these regions of P2 were in the highly variable, surface-exposed loops, which are at greatest risk of attack by the immune response. The ability of *H. influenzae* to vary its surface antigenic array by incorporating exogenous DNA that encodes antigens with different epitopic domains would decidedly convey a selection advantage in an antibody-rich environment.

In addition, sequence analysis of *H. influenzae* IgA protease genes (69) revealed polymorphisms within the variable regions that show a mosaic-like organization similar to that seen with *N. gonorrhoeae* IgA protease genes, in which horizontal gene exchange is believed to occur (37). Interestingly, the *H. influenzae* IgA protease gene is flanked by copies of the *H. influenzae* DNA uptake sequence, enhancing the likelihood of IgA polymorphisms through gene transfer by transformation and recombination. A recent report also describes copies of the *H. influenzae* DNA uptake sequence flanking the tryptophanase gene cluster of *H. influenzae* (55).

The variable regions of *hifA* and *hifE* pilus genes also show this mosaic-like organization within the variable regions (14, 56). Furthermore, restriction fragment length polymorphism (RFLP) analysis of *hifA* and *hifE* genes from a variety of nontypeable and type a through f strains revealed poor correlation

between *hifA* and *hifE* gene RFLP types, suggesting considerable mixing and matching of these two genes, even though they are located within the same gene cluster (14, 56). Nucleotide sequence analysis confirmed the validity of the RFLP analysis to accurately group organisms with similar *hifA* and *hifE* sequences. This apparent mixing and matching of genes within the same cluster are consistent with horizontal exchange of DNA. Similarly, the IgA protease serotype does not correlate well with biotype, again suggesting mixing and matching of IgA protease genes among strains with different genetic backgrounds as defined by biotype (52).

Finally, two examples of possible horizontal transfer of an *H. influenzae* capsule gene have been reported (49). The IS-*bex*A deletion previously seen in one copy of the 18-kb tandem repeat in the capsular gene region of type b strains was identified in the Cap region of two type a strains, one isolated from The Gambia in West Africa and the other from Kenya in East Africa. The presence of this mutation in one copy of the duplication is thought to stabilize the duplication, which allows for potential gene amplification, for increased capsule production, and possibly for increased virulence, and apparently resulted from DNA transfer between type a and type b strains.

Another potential mechanism for antigenic variation in bacteria is posttranslational modification, such as glycosylation, phosphorylation, sulfation, or sialylation, of surface proteins. Such posttranslational modifications have occasionally been described in procaryotic proteins but never in *H. influenzae*. Virji reported an O-linked trisaccharide as well as an alpha-glycerophosphate on the major pilin subunit of *Neisseria meningitidis* pili (85). The role of these modifications in determining pilus epitopes is unclear. Preliminary data (25a) suggest that HifA, the major pilus subunit, may also be posttranslationally modified.

SUMMARY

H. influenzae has evolved a highly adaptive means (antigenic diversity) to survive in an environment (the human nasopharynx) that is constantly bathed in antibodies. Furthermore, this organism possesses a number of unique biologic features and genetic mechanisms to assure rapid variation in its surface antigens. Cocolonization by more than one *H. influenzae* strain facilitates horizontal transfer of genes by providing a supply of potentially diverse DNA that can be taken up by the colonizing strains and incorporated into the bacterial genome. In addition, *H. influenzae* use phase variation to turn off and turn on surface antigens through regulatory mechanisms that involve nucleotide repeats. Finally, point mutations in genes encoding surface-exposed proteins may dramatically alter the conformation of antibody binding sites on these proteins. As a testimony to its versatility, *H. influenzae* appears to use two or all three genetic mechanisms to alter a single antigen, such as pili.

The antigenic diversity of only a few *H. influenzae* antigens has been well described. The full richness of this diversity will be further revealed by sequence analyses of antigens from nonclonal *H. influenzae* strains and by epidemiologic studies in which *H. influenzae* are typed according to the polymorphisms exhibited by relatively invariate genes (such as those required for essential metabolic functions) by biotyping, multilocus electrophoretic typing, or typing of PCR products generated by DNA amplification with random primers and then further characterized by polymorphisms in their variable proteins.

ACKNOWLEDGMENTS

I thank Carl Marrs, Michael Koomey, and Daniel Clemans for their review of the manuscript and valuable discussions that led to the development of this paper.

This work was supported in part by Public Health Service grant AI 125630 from the National Institute of Allergy and Infectious Diseases.

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Editor: J. T. Barbieri