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# HAEMOPHILUS INFLUENZAE – HUMAN SPECIFIC BACTERIA

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## 1. ABSTRACT

*Haemophilus influenzae* is both a commensal and a pathogen specific to humans. Here we review this bacterium with special emphasis on characteristics that may be involved in virulence.

## 2. HISTORICAL PERSPECTIVE

*Haemophilus influenzae* are Gram negative, pleomorphic rods first described by Pfeiffer in 1892 (1). The genus name reflects its absolute requirement for heme (haemophilus = blood-loving) and the species name reflects the erroneous initial belief that this organism was the causative agent of influenza. Since its discovery, a number of key biologic or technical paradigms have been established through scientific inquiry of *H. influenzae*. In early work defining bacterial capsules, Margaret Pittman observed that, when grown on clear agar, Hi colonies exhibited two distinct morphologies -- translucent, as had previously been described, or iridescent when viewed by oblique light. In 1930, she described the six serotypes (a through f) of the

iridescent strains and recognized that the serotypic specificity was due to capsular polysaccharide (2).

The rough, translucent isolates that possess no capsule and fail to react with a-f typing sera are called "non-typeable". The pioneering work of Fothergill and Wright (3) documented that the protective effect of blood against type b encapsulated *H. influenzae* (later shown to be mediated by anticapsular bactericidal antibodies) was age related. This observation explained the strong association of age (2 months to 2-3 years) with risk of *H. influenzae* meningitis; neonates were protected by transplacental maternal antibody and older children were protected by natural antibodies. In 1980, Hamilton Smith and his colleagues documented the natural mechanisms by which exogenous *H. influenzae* DNA is "transformed" into "competent" *H. influenzae* cells, thus defining and characterizing the process of transformation at the molecular level (4). About the same time, Anderson (5) and Schneerson and Robbins (6) demonstrated that bacterial capsules conjugated to a carrier protein generated a strong, protective and sustained immune response, even in children two months of age. These observations heralded the use of purified bacterial products as vaccine antigens and led to the development of conjugated bacterial capsule vaccines. More recently, *H. influenzae* was selected as the first complete organism for sequence of the entire genome (7), thus demonstrating the ability of modern techniques to generate large scale genome sequences and the utility of the complete sequence in understanding many biologic processes of a bacterial organism.

## 3. EPIDEMIOLOGY

*H. influenzae* live exclusively in humans, and although they may cause disease in a variety of body sites (such as the central nervous system, joints, skin, lungs, bronchi and genito-urinary tract), they are most commonly isolated from the nasopharynx, where they are carried asymptomatically (8). Throat or nasopharyngeal culture surveys of both healthy and ill individuals reveal the carriage rate to be between 20 and 85%, and the majority of colonizing strains lack a capsule. Recent studies using molecular epidemiologic techniques demonstrate that both adults and children may carry more than one strain at a time and that the week-to-week turnover of unique strains is high (9, 10). Person to person transmission of *H. influenzae* is assumed to occur by contact with infected respiratory droplets and has resulted in clusters of *H. influenzae* type b (Hib) invasive infections among children in households and day care centers.

## 4. CLINICAL MANIFESTATIONS

Invasive *H. influenzae* infections, such as bacteremia, meningitis, pneumonia in children, epiglottis, and septic arthritis, are usually caused by *H. influenzae* strains possessing the type b capsule (11, 12). Furthermore, these infections occur much more frequently in non-immune children between ages 2 months and 5 years, and this age-related susceptibility is inversely correlated with the bactericidal capacity of the blood (3), which is mediated by antibodies directed against the type b capsule.

Newborns are protected by transplacental maternal type b antibodies and older children and adults are protected by "natural antibodies", which are *H. influenzae* specific antibodies in a host who has not had a known *H. influenzae* infection. Most likely these antibodies result from subclinical infection or asymptomatic colonization. The introduction of broadly effective vaccines against *H. influenzae* type b in the United States in 1988 has resulted in dramatic reductions in all infections caused by type b strains (13).

Although non-typable *H. influenzae* rarely cause invasive infections in normal hosts, they are important causes of respiratory infections, such as bronchitis and pneumonia in adults with underlying pulmonary disease (14) and otitis media and sinusitis in healthy individuals. Recent studies show that *H. influenzae* cause 23-27% of acute otitis media in children, the same incidence as *S. pneumoniae* (15, 16).

A subset of *H. influenzae* strains have been associated with genital, neonatal, and puerperal infections (17). These isolates typically are classified as biotype IV strains and appear to comprise a cryptic genospecies of *H. influenzae* (18). Another unique group of *H. influenzae* isolates are the Brazilian purpuric fever strains, a subset of biogroup aegyptius strains, that, although they do not possess a polysaccharide capsule, cause bacteremic infections that often lead to vascular collapse and death (19). The bacterial factor(s) that explains the unusual pathogenicity of these non-encapsulated strains remains unknown.

## 5. CHEMOTHERAPY AND ANTIBIOTIC RESISTANCE

Many antibiotics are effective in treating *H. influenzae* infections. Because approximately 40% of strains carry β-lactamase producing plasmids, (20), serious infections are treated with parentally administered, β-lactamase stable antibiotics such as third generation cephalosporins (21). During outbreaks or potential outbreaks of invasive infections among non-immunized children, rifampin prophylaxis has been shown to decrease the *H. influenzae* carriage rate among close contacts of susceptible children (22). Many oral antimicrobial agents, such as combination drugs that contain sulfa, second and third generation cephalosporins, extended spectrum macrolides, and β-lactam antibiotics that also contain a β-lactamase inhibitor, effectively treat β-lactamase producing non-typeable strains that cause respiratory infections (23).

## 6. GENETIC VARIATION BETWEEN STRAINS

### 6.1. Biotypes

The species *Haemophilus influenzae* has been subdivided into biotypes or biovars on the basis of urease, ornithine decarboxylase activities and indole production (24, 25). Currently, *H. influenzae* can be subdivided into eight different biotypes (table 1, 26).

**Table 1.** Biotype characterization of *H. influenzae*

| *H. influenzae* biotype | Indole production | Urease activity | Ornithine decarboxylase activity |
|---|---|---|---|
| I | + | + | + |
| II | + | + | - |
| III | - | + | - |
| IV | - | + | + |
| V | + | - | + |
| VI | - | - | + |
| VII | + | - | - |
| VIII | - | - | - |

The majority of *H. influenzae* isolates found in clinical labs are typed as biotype I, II, or III (26, 27). Invasive capsular type b strains are typically biotype I (28). Strains isolated from the respiratory tract are predominantly biotypes I, II or III. Approximately 20% of the nontypeable *H. influenzae* strains isolated from urogenital or neonatal infections are biotype IV and comprise a cryptic genospecies (17, 29, 30).

### 6.2. Phylogeny

The *Haemophilus* genus is part of the family *Pasteurellaceae*, which also includes the genera *Actinobacillus* and *Pasteurella* (31, 32, 33). The primary attempts to group strains into a phylogenetic pattern within the *H. influenzae* species were carried out in the 1980's using multilocus enzyme electrophoresis (MLEE). Using 17 metabolic enzyme loci, Musser *et al*. (34) organized 2,209 encapsulated isolates (mostly type b) into 280 distinct electrophoretic types (ETs). Similar approaches showed that most nontypeable *H. influenzae* (NTHi) were genetically quite distinct from type b (Hib) strains and more heterogeneous (35, 36). More recently, ribotyping (37) and ERIC (enterobacterial repetitive intergenic consensus) PCR (38) have been used to relate strains. The ribotyping method suggested a population structure different from that using MLEE (37). Horizontal exchange of *H. influenzae* genetic loci between strains due to natural DNA transformation complicates traditional phylogenetic analysis, which assumes clonality based on vertical transmission of phylogenetic features, and might explain the difference seen using different methods.

Musser *et al*. (36) proposed that the ancestor of *H. influenzae* was encapsulated and the nontypeable clones arose by convergent evolutionary loss of the ability to synthesize or extracellularly express a polysaccharide capsule. However, the wide heterogeneity of nontypeable strains, the more clonal features of typeable strains and the evidence detailed in the next section that most type b specific genetic regions are flanked by repeat sequences, and thus may represent acquisition of foreign genetic elements, make it more likely that an unencapsulated ancestral *H. influenzae* strain acquired these elements and became more virulent.

## 7. GENOME STRUCTURE

In 1995, *H. influenzae* strain Rd (a laboratory strain that is a "rough" derivative of a type d strain) became the first bacteria to have its complete genome sequence determined (7). The strain Rd genome has just over 1.8 million base pairs (Mb) and initially 1743 predicted coding regions were identified. Six ribosomal RNA operons were identified as were 24 tRNA genes. Additional potential Rd genes have since been described (39, 40, 41). The Rd genome lacks many virulence genes found in other strains, including pili, Hap and HMW 1 and 2, and is estimated to be 270 Kb smaller than that of type b strain Eagan (42). Capsule type b (43), pili (*hif*) (44), tryptophanase (*tna*) (45), and haemocin genes (46) are present in Eagan and other type b strains and are not found in Rd. The cap b, *hif*, and *tna* loci are each flanked by direct repeats. The cap b gene cluster, containing a duplication of two ~18 kb segments, lies between direct repeats of *IS1016* in most isolates (43, 47). Recently we reported that Eagan contains a ~16 kb genetic island, HiGI1, with 18 potential genes that is found in all type b strains tested, but not in Rd or most other non-type b strains tested (48).

Only three, closely related bacteriophages of *H. influenzae* have been characterized at the molecular level. HP1 has been completely sequenced (49) and S2 type A has been partially sequenced (50). S2 type B appears to be a hybrid recombinant between HP1 and S2 type A (50).

*H. influenzae* tend to have fewer plasmids than many other bacteria. The main exception to this is the increasing presence of large conjugative plasmids containing TEM-1 β-lactamase genes (20, 51).

## 8. NATURAL DNA TRANSFORMATION

*H. influenzae* is one of several bacteria that have genetically determined systems for the uptake and integration of exogenous DNA. Interestingly, other human specific pathogens such as *Neisseria gonorrhoeae* and *Streptococcus pneumoniae* also have such systems. Why these rather complex systems exist is a matter of some debate. It has been proposed that these systems might exist to promote improved repair of damaged DNA by providing wild-type template DNA to recombine and remove mutated regions of a damaged host chromosome. However, both experimental (52) and theoretical (53) evidence argues against this being the primary function. Alternatively, the strong selective pressure placed on these bacteria by the adaptive immune response may select for bacteria that can most readily create immunologically distinct versions of surface proteins and other molecules. In addition to the intragenomic variation methods, recombination between different strains of *H. influenzae* may create antigenically distinct molecules. Kroll *et al*. (54) report evidence that at some time in the past natural transformation from *H. influenzae* into *Neisseria meningitidis* has occurred.

A recent review details what is known currently about the mechanics of *H. influenzae* DNA uptake (55) and an earlier review also covers the recombination process (56). In brief, the bacterial cells must become "competent" in order to be able to take up exogenous DNA. Competence development occurs primarily during the

stationary phase of growth, and may be facilitated by growth conditions (57). Competent cells specifically bind and absorb double stranded DNA, and this process is *H. influenzae* specific, as foreign DNAs bind to the cell surface, but are not taken up and do not compete with homologous DNA. DNA uptake signal sequences (USSs) are required for uptake of exogenous DNA and have been recently reviewed (58). In *H. influenzae* USSs are 29 bp in length with the consensus sequence being 5'-aAAGTGCGGT .rwwwww..... rwwwww-3', with uppercase letters representing conserved bases, lowercase letters are bases that occur in >50% of the USSs, a dot is any base, r is A or G, and w is A or T. A total of 1465 copies of USS were found in the sequence of *Haemophilus influenzae* Rd, while based on the A/T base composition only 8 would occur by chance (59). Surface vesicles (transformasomes) appear to carry the ds donor DNA into the cell, bound to its surface. During the uptake process, the *H. influenzae* DNA remains double stranded, at least into the periplasm. During this transfer into the cell the DNA is protected from cellular nucleases. The DNA crosses the inner membrane and searches out a complementary sequence in the recipient. Only one strand of the donor DNA is incorporated into the host chromosome. Regions of ssDNA are found in the chromosome of competent *H. influenzae* cells.

Recombinational deficient *rec-1* (*recA* like) and *rec-2 H. influenzae* are transformed at substantially reduced frequencies. Additional genes essential for normal levels of transformation include the *comABCDEFG* operon (60, 61), the *pilABCD* gene cluster (61), the *dprABC* operon (62), and *comM* (63). Other genes involved in regulation of competence include *icc* (64), *cya* (65), *crp* (66), *atpA* (67), *sxy* (68), and *murE* (69).

## 9. PUTATIVE VIRULENCE FACTORS

### 9.1. Adherence factors

#### 9.1.1. Hemagglutinating pili

As we have recently reviewed *H. influenzae* pili (sometimes called fimbriae) elsewhere (70, 71), we will only briefly describe them here. The first well studied adherence factors of *H. influenzae*, these filamentous organelles are seen by electron microscopy distributed in a peritrichous manner on the *H. influenzae* bacterial cell surface of isolates that hemagglutinate and adhere to buccal epithelial cells, but are absent from non-hemagglutinating and non-adherent bacteria. Pilus expression is regulated by phase variation, spontaneously switching from piliated to nonpiliated and vice versa at a frequency of about $10^{-4}$ to $10^{-3}$/generation. Generally strains isolated from invasive infections do not express pili, while those colonizing the nasopharynx more commonly do. Not all *H. influenzae* have the genes for pili. One collection of NTHi is reported to only have pilus genes in 18% of the isolates (72), while in one Hib collection 88% of isolates could be enriched to express pili (73).

van Alphen *et al*. (74) have shown that piliated *H. influenzae* binds to erythrocytes possessing the Anton (AnWj) antigen. 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.

Most strains contain the pilus gene cluster in a single copy between *purE* and *pepN* composed of five genes, *hifABCDE* (44, 75). Brazilian purpuric fever isolates possess two copies of the pilus gene cluster, with one copy in the normal location and the other between *pmbA* and *hpt* in a separate region of the chromosome (76). HifA is the major pilus structural subunit (pilin), HifB is a periplasmic chaperone that binds to HifA, HifD, and HifE, protects them from degradation and transports them to HifC, an assembly platform (usher) protein that is the base of the pilus structure. HifD is located at the tip of the pilus and appears to be a linker between the HifA shaft of the pilus and HifE, which is the actual adhesin molecule at the end of the pilus (77). Divergent promoters for *hifA* and *hifB* overlap and are found in the stretch of DNA between these two genes that contains multiple TA repeats (78). Variation in the number of 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.

#### 9.1.2. High molecular weight proteins

Two secreted highly similar high-molecular-weight proteins (HMW1, HMW2) were first identified as *H. influenzae*-associated antigens by their ability to induce robust antibody responses in individuals with acute otitis media (79). Subsequent studies have revealed that these HMW proteins function as adherence factors and can facilitate the colonization of cultured human epithelial cells (80). HMW1 is a 160 kDa product of the *hmw1A* gene and HMW2 is a 155 kDa protein encoded by the *hmw2A* gene; *hmw1A* and *hmw2A* are 71% identical and 80% similar (79). To date the *hmw* genes have only been detected in nontypable strains, and a majority of HMW-positive strains retain both chromosomal loci (81). Organisms that express either HMW1 or HMW2 have different affinities for transformed cell lines, implying that each HMW protein may display a tropism for one or more eukaryotic receptor subsets (82). These particular binding characteristics have been mapped to an N-terminal 360 amino acid fragment of the mature HMW protein, which is also the region of greatest amino acid sequence dissimilarity between HMW1 and HMW2 (82). Preliminary evidence suggests that HMW1 recognizes an $\alpha$2,3 linked sialylated glycoprotein; the receptor structure for HMW2 is unknown (80, 83).

Genes *hmw1A* and *hmw2A* each exist within a three-member gene cluster that includes two downstream open reading frames (*hmwB* and *hmwC*; 81). The two *hmwB* and *hmwC* counterparts have high degrees of identity (99 and 97%, respectively), and the stepwise events leading to secretion of the HMW proteins are dependent on these proteins (84) The HMW proteins are thought to be secreted via a conserved two-component protein export pathway exemplified by the *Serratia marcescens* haemolysin (85). Once these mature HMW proteins are presented on the bacterial surface the majority remain non-covalently attached to the organism via the C-terminus (86).

Transcriptional regulation of the *hmw* genes is via phase variation, and seems to be dependent on the slipped-strand mispairing of tandem 7 base pair repeats spanning two transcriptional start sites upstream of the *hmw*1A and *hmw*2A coding regions (87). The efficiency of transcription is inversely related to the number of tandem repeats. This process presumably occurs in a random manner, resulting in the generation of a population of organisms that produce varying amounts of HMWs (87). Recent studies indicate that this regulatory mechanism may be of importance for *H. influenzae* persistence as mucosal clearance mechanisms are activated. For example, in an experimental chinchilla model of acute otitis media, immunization with the HMW2 protein results in the selection of intrabullar isolates that predominantly express HMW1 (87). Moreover, nasopharyngeal isolates recovered from children with acute otitis media contain lower numbers of base pair repeats, and express significantly higher quantities of the HMW proteins than their isogenic middle ear counterparts, suggesting that immunological selection occurs continuously in the various microenvironments the organism finds itself (87). Since HMW1 and HMW2 have different receptor specificities (82), and most HMW-positive organisms retain both chromosomal loci, the ability of an organism to differentially express these adhesins may also aid in tissue tropism.

### 9.1.3. Hia and fibrils

The Hia protein was first detected as a high molecular weight species that was a target for human serum antibody (88). The Hia gene (*hia*) is a 3.3 kb open reading frame that encodes a 115 kDa protein that has sequence similarity to another *H. influenzae* adhesin, Hsf, a 240 kDa protein associated with the formation of short pilus-like structures termed fibrils (88, 89). Interestingly, the NH2- and COOH- regions of the two proteins are highly homologous, whereas the central segment of *hsf* is a trimeric duplication of *hia*, thus accounting for the great disparity in their size (89). Expression of either Hia or fibrils confers similar adhesive properties in *E. coli* transformants, suggesting that the receptor-binding regions of these two proteins may be conserved (89).

To date, Hia has only been detected in a subset of nontypeable strains, whereas fibrils are found in the vast majority of serotypeable strains (90). Interestingly, nontypeable strains can be further sub-divided into those that express either Hia or the HMW proteins, indicating that these two adhesins may fulfill similar roles in pathogenesis (90).

Hia has homology to AIDA-1 of *E. coli* and other members of the IgA1-protease autotransporter family (91). By sequence analysis, *hia* has conserved autotransporter features, including a C-terminal region predicted to form a β-barrel secondary structure presumably through which the N-terminal passenger domain is exported (92). Interestingly, a defined protease motif is not apparent, suggesting that Hia may not have autocatalytic activity and is retained as a full-length protein on the bacterial surface after translocation (92).

### 9.1.4. Hap

Hap was first discovered by its ability to facilitate low-level adherence of DB117, a *rec* negative derivative of strain Rd, to human transformed cell lines (93). To date, Hap has been detected in all nontypeable strains examined. Hap has homology to the *H. influenzae* serine-type IgA1 protease (although it cannot use IgA1 as a substrate), and further analysis has revealed that this protein contains a 110 kDa central protease region ($Hap_s$), a downstream catalytic site, and a 45 kDa C-terminal translocator ($Hap_\beta$) retained in the outer membrane (94, 95). Recent evidence suggests that $Hap_s$ may play multiple roles in *H. influenzae* pathogenesis. For example, mutating the active site serine abolishes autoproteolysis, and results in the retention of $Hap_s$ on the bacterial cell surface. This Hap modification is associated with an increase in the organism's ability to adhere to cultured human epithelial cell lines. In further support of $Hap_s$ as the adhesive domain, gene fusion constructs with $IgA_\beta$ as an alternate outer membrane translocator demonstrate that surface localized $Hap_s$ retained similar binding characteristics as the full-length Hap protein. In addition, purified soluble $Hap_s$ adheres to transformed respiratory epithelial cells (95).

The ability of $Hap_s$ to self-assemble into multimers also facilitates inter-bacterial interactions via associative surfaces between $Hap_s$ molecules, resulting in the formation of microcolonies, and thus, may be a potential scaffolding mechanism for the large-scale construction of mucosal biofilms (95). In this context, it seems paradoxical that the bacterium would readily liberate this important domain through autoproteolysis. Interestingly, human serine leukocyte protease inhibitor (SLPI), a protein commonly found in respiratory secretions (96), abrogates Hap autoproteolysis (95). SLPI normally functions to inhibit the activity of neutrophil elastase and assists in the containment of proteolytic enzymes that can lead to significant tissue damage during inflammation (97). Therefore, Hap may be a means of synchronizing processes such as the establishment of colonization with specific respiratory environments suited for these events. Conversely, the removal of SLPI from the pulmonary microenvironment can trigger the release of individual organisms, presumably to aid their dissemination to new niches within the respiratory tract. On another note, liberation of the $Hap_s$ domain may aid in immunological evasion by acting as a sump for specific anti-Hap IgA antibodies (95).

### 9.1.5. P5 and P5-related fimbrin

P5 is a 37 kDa major outer membrane protein (OMP) expressed by virtually all nontypeable and serotypeable *H. influenzae* strains, and has 50% identity and 65% similarity to the OmpA porin of *E. coli* (98). P5 has been of great interest as a model for antigenic drift, a countermeasure to specific mucosal immune responses (99). This is due to the observation that nontypeable strains recovered longitudinally from the lower airways of chronically infected patients demonstrate a wide variability in the amino acid content of their P5 proteins. Sequence analysis reveals that those segments with greatest amino acid diversity were largely restricted to predicted extracellular loop regions of the P5 proteins (99).

Early studies suggest that the P2 and P5 OMPs mediate bacterial attachment to a complement of sialylated oligosaccharides within respiratory mucins (100). More recently, it has been established that P5 binds to a member of the carcinoembryonic antigen family (CEACAM1) (101, 102), a glycoprotein expressed by respiratory epithelial cells and up-regulated during inflammation (103). Interestingly, this OMP can also form thin fiber-like structures composed of repeating monomers of P5 (fimbrin is an equivalent designation for these subunits; 104). These composite "fimbriae", not to be confused with hemagglutinating pili which are also described as fimbriae, were first detected by transmission electron microscopy as peritrichously displayed surface-localized structures on nontypeable bacteria (104). Fimbriae are thought to mediate adherence to oropharyngeal cells, mucins, and respiratory syncytial virus-infected A549 cells (105). Anti-fimbrin antibodies are protective in animal challenge models (106), and fimbrin gene inactivation reduced adherence to eukaryotic cells and attenuated virulence in a chinchilla otitis media model (105). Current studies are largely focused on the utility of conserved regions of this protein as a vaccine antigen (106).

**9.1.6. Lipooligosaccharide**

In addition to presenting individual adhesin proteins and complex adhesive organelles on the cell surface, *H. influenzae* can also modify intrinsic structural components of the cell envelope to function as attachment factors. A striking example of this is recent work demonstrating that specific glyco-modifications of *H. influenzae* lipooligosaccharide (LOS) confer an ability to adhere to human epithelial cells (107). Previous studies have established that a significant number of complex sugars decorate the LOS core, and their incorporation is dependent on the phase variable expression of one or more biosynthetic enzymes (see LOS section). Recently, it was observed that nontypeable strains that lacked the LOS oligosaccharide side-chain (? *pgm*B mutants) were less able to bind to and invade respiratory epithelial cells than their wild-type counterparts (107). In addition, soluble sugars that included the phosphorylcholine (ChoP) moiety were able to inhibit the adherence of organisms that contained ChoP-positive LOS glycoforms, as well as, purified LOS coated on microspheres, to bronchial epithelial cells. In eukaryotes, the acetylated form of ChoP is the platelet-activating factor (PAF), and ChoP-positive bacteria can be co-localized with the PAF receptor (PAF-R), suggesting that ChoP-positive LOS glycoforms may be alternative ligands for this receptor. Bacterial invasion can also be competitively inhibited by pre-incubating monolayers with PAF receptor antagonists, though inexplicably this pre-treatment promoted epithelial cell adherence (107).

**9.1.7. Other *H. influenzae* adhesins**

Busse *et al*. reported the identification of a ~45 kDa protein recovered from water-extracted samples of *H. influenzae* by elution with a soybean phosphatidyl-ethanolamine (PE)-charged affinity column (108). This protein species, termed the PE-binding adhesin, also had affinity for gangliotriasylceramide (Gg3), gangliotetrasyl-ceramide (Gg4), and sulfatoxygalactosylceramide (SGC), resolved on thin layer chromatography (TLC) plates. Pre-incubation of bacteria with antibodies raised against this PE-binding adhesin significantly decreased adherence to either Hep-2 epithelial cell monolayers or immobilized glycolipids (108). In addition, briefly heat shocking organisms can elicit alterations in their affinity for these glycolipids (109). Interestingly, pre-incubating bacteria with an anti-hsp 70 antibody abrogates the heat shock induced binding of organisms to immobilized SGC, suggesting a further link between this environmental cue and the dynamic expression of *H. influenzae* adhesins (109). In an unrelated study, nontypeable strains were also noted to bind gangliosides extracted from Hep-2 cells and primary human monocytes immobilized on TLC plates, and desialylation of these gangliosides abrogated adherence (110).

**9.2. Capsules**

The most important virulence factor defining pathogenic *H. influenzae* strains is capsule, of which six serotypes, a-f, have been described. Among encapsulated strains, those possessing the type b capsule (Hib strains), which is composed of polyribosylribitol phosphate (PRP), are the most virulent, capable of causing sustained bacteremia and subsequent focal infection in non-immune, normal hosts. Like the capsules of other gram negative organisms, *H. influenzae* capsules enhance the pathogenicity of the bacteria by protecting them from phagocytosis (111). The strong negative charge of the polysaccharide capsules may provide electrostatic repulsion to phagocytic cells and the capsular material itself may sterically interfere with the binding of opsonizing antibodies and complement to the bacterial surface (112).

All encapsulated strains possess the *cap* genetic locus, a segment of approximately 17 kb comprised of a region that shows serotype specificity flanked by regions sharing homology among all capsular types (113). Most types a, c-f strains possess one copy of the *cap* locus. Most type b clinical isolates, on the other hand, possess two copies in tandem. The *cap* locus, either as the tandem duplication of most type b strains or the single copy of strains of other capsular types, is flanked by IS-like sequences in most strains, suggesting that the region was acquired by a primordial strain through transposition (43).

One of the conserved regions of the *cap* locus contains the four *bex* genes, which are responsible for exporting the capsular polysaccharide (114). The tandem duplication of the *cap* locus in type b strains is characterized by a mutation in the *bex*A gene of one copy. This duplication results in an unstable gene region, with relatively high potential for recombination events. During recombination of the *cap* locus, mutants may be formed that possess a single copy of the *cap* locus containing the bexA mutation, which results in non-encapsulated mutants. On the other hand, such a recombination event may generate mutants possessing additional (n) copies of the cap locus with n-1 copies of *bex*A. Such mutants are hyper-encapsulated and show increased pathogenicity in the infant rat model (115).

**9.3. IgA1 protease**

Secretory IgA fulfills many protective functions on the mucosal surface; these include the neutralization of toxins, inhibition of pathogen attachment to the epithelium, and agglutination of organisms within mucus (116). To surmount this immunological obstacle, *H. influenzae* and a number of other mucosal pathogens, such as *N. meningitidis*, *N. gonorrhoeae*, and *S. pneumoniae* constitutively secrete IgA1 proteases (117, 118, 119). These proteins are enzymes that inactivate the predominant IgA species, IgA1, by cleaving the immunoglobulin molecule at the heavy chain hinge region, resulting in the production of two fragments (Fab and Fc). Though IgA1 proteases from different bacterial genera are enzymatically diverse, (serine-, metallo-, and thiol-proteases), they all cleave at either Pro-Ser (type 1 enzymes) or Pro-Thr (type 2 enzymes) within a small region of the immunoglobulin molecule (117).

The *H. influenzae* Rd IgA1 protease has a modular structure, similar to that originally described for the *N. gonorrhoeae* protease, consisting of four domains that are sequentially processed during secretion (120, 121). These include: (1) an N-terminal signal peptide, (2) the IgA1 protease passenger domain, (3) an α-peptide, and (4) the C-terminal domain (IgAβ), which has sequence similarity to the *E. coli* porin OmpF (122). As mentioned previously, three *H. influenzae* proteins have been identified which have the characteristics of autotransporter proteins; the IgA1 protease, and the adherence factors Hap, Hia, as well as, potentially the closely related Hsf protein (87, 121). Once externalized, an active site serine within the protease domain attains catalytic activity and releases the protease from its tether and the α-peptide is subsequently clipped by further autoproteolysis (123).

In support of its role in virulence, both nontypeable and serotypeable *H. influenzae* express type 1 and type 2 IgA1 proteases, and cleavage products can be detected in nasopharygeal secretions (117, 124). In addition, neutralizing antibodies targeting these proteins can be demonstrated in convalescing patients, and human milk lactoferrin can inactivate these enzymes through prematurely extracting the protease from the outer membrane (125, 126, 127). If IgA1 proteases do play a role in pathogenesis, then targeted secretion and relatively high local concentrations may be required to counter molecular defenses and facilitate colonization. It must be emphasized, however, that IgA1 proteases have not yet been definitively shown to be virulence factors for any of the bacteria that express them.

**9.4. Lipooligosaccharide (LOS)**

*H. influenzae* produces a lipopolysaccharide structure that lacks an O-specific antigen and, instead, incorporates short oligosaccharide side chains onto three conserved heptoses and is thus termed lipooligosaccharide (LOS). A wide variety of glyco-modifications to the core LOS molecule have been detected and include the addition of glucose, galactose, lactose, phosphatidylcholine (ChoP), and sialic acid (128, 129, 130, 131). This repertoire of sugars allows the organism to mimic common eukaryotic glycolipid structures, and is presumably an adaptive strategy for subverting immune defenses (132). Individual strains can also dynamically alter the expression of these terminal constituents by phase variation of genes (*lic1A, lic2A, lic3A, lgtC*) within chromosomal loci that encode the LOS biosynthetic pathway enzymes (133, 134). This is based on slipped-strand mispairing within long stretches of tetrameric tandem repeats just within the start of the individual open reading frames, thus leading to reversible switching via translational frameshifts (129, 130, 131). Interestingly, similar stretches of tetrameric repeats lie at the start of other potential virulence genes in *H. influenzae* (134), and has been observed in the genomes of other mucosal pathogens, such as *Neisseria* spp. and *Moraxella catarrhalis* (135).

The *lic*1 locus is comprised of four genes, *licA-D*, and is responsible for adding ChoP as a terminal component to the inner core heptose sugars (136). Sequence analysis suggests that *lic1A* is a choline kinase; *lic1B*, a choline permease; *lic1C*, a pyrophosphorylase and *lic1D* is likely a choline transferase that attaches ChoP to either heptose I or III (132). Phase variation of *lic*1*A* leads to isolates with either high level or no ChoP expression (136). The *lic*2 loci and *lgtC*, which encode an a-1,4-galactosyl transferase, are necessary for the addition of Galα1-4Gal to LOS, a glycoform with similarity to the P blood group antigens (136, 137). The *lic3* locus is critical for the addition of sialic acid (*N*-acetylneuraminic acid, Neu5Ac) to lactose and other terminal sugars, and the phase variable *lic*3*A* gene is an a-2,3-sialyltransferase (137, 138, 139).

In surveillance studies, the vast majority of nontypeable isolates recovered from the human respiratory tract contain in-frame *lic*1A genes and LOS modified with ChoP (140). The choline substrate required for ChoP addition is exogenously derived, and in the microenvironment of the respiratory tract, is likely extracted from host cell membrane lipids. Consistent with the possibility that ChoP may aid in colonization of the respiratory mucosa, a strong selection for ChoP-positive bacteria was observed when organisms were sampled from the nasopharynx in a chinchilla otitis media model, and ChoP expression was associated with the development of otitis media (141). One potential role for ChoP is as an adhesin, since it can function as a ligand for the epithelial platelet activating factor receptor (see Adhesin section). The addition of ChoP to surface-localized constituents of other respiratory pathogens, such as *S. pneumoniae* and *Mycoplasma spp.*, has also been described and suggests that this modification is a broadly conserved strategy to promote bacterial persistence within the respiratory tract (132).

Though ChoP may provide an advantage in mucosal colonization, nontypeable isolates recovered from sites of disease are ChoP-negative, suggesting that this modification may be selected against during infection (136). Insights into this disparity was provided by the finding that ChoP-positive bacteria are more susceptible to C-reactive protein-mediated serum killing (140). In contrast to ChoP, the *lic*2/*lgt*C-dependent addition of Galα1-4Gal moieties to LOS is protective against complement-mediated killing in an *in vitro* setting (136). In support of the role this glyco-modification has for pathogenesis, nontypeable strains recovered from

individuals with clinical pneumonia have in-frame *lic*2A and *lgt*C genes (136). Thus, a complex interplay may take place between these multiple phase variable genes at discrete steps of *H. influenzae* pathogenesis.

Another molecule that confers a relative resistance to the bactericidal activity of normal human serum is sialic acid (143). This is a common terminal constituent of eukaryotic glycolipids and, in part, functions to protect the cellular membrane from the potential triggering of complement deposition (138). It is not surprising then that sialylation is also a strategy a number of microbes use to circumvent mucosal immunity. Both serotypeable and nontypeable strains have the capacity to express LOS glycoforms with terminal sialic acid moieties that decorate multiple sugars on the heptose extensions (137, 138, 143). Biochemical studies have indicated that the sialic acid donor CMP-Neu5Ac, a substrate for bacterial sialyltransferases, can be endogenously synthesized by the *siaB* gene, using sialic acid scavenged from the media or host cells (138). Inactivation of either *sia*B or the a-2,3-sialyltransferase *lic*3A results in a downward shift of LOS glycoforms on tricine-SDS-PAGE gels, and an increased sensitivity to killing by normal human serum (138, 139). Consistent with these observations, *H. influenzae* mutants deficient in *nan*A (*nan*; *N*-acylneuraminate operon), an aldolase necessary for sialic acid metabolism, are hypersialylated and can out-compete their wild type counterparts in an infant rat bacteremia model when combined as an intraperitoneal inoculation (138).

**9.5. Iron & heme uptake**

Unlike other gram-negative bacteria, *H. influenzae* has an absolute requirement for exogenously supplied heme for aerobic growth (144). This requirement for heme is due to the inability of *H. influenzae* to convert δ-aminolevulinic acid to protoporphyrin IX, the direct precursor to heme (145, 146). In fact, the genomic sequence of *H. influenzae* strain Rd reveals the absence of most of the pathway leading from δ-aminolevulinic to heme (147). *H. influenzae*, however, does possess the ferrochelatase enzyme (HemH) which catalyzes the reversible insertion of $Fe^{2+}$ into the protoporphyrin IX nucleus to yield heme (148). Schlor *et al*. (149) recently showed that mutations in the *hem*H gene demonstrated that HemH was not required for heme utilization *in vitro* and did not affect *H. influenzae* survival in animal model systems. *In vitro*, free hemin satisfies the porphyrin and iron requirements (145, 150). *In vivo*, free heme is toxic to the host and the body has derived ways to complex heme as well as sequester free iron molecules. *H. influenzae* has derived several mechanisms to use host heme-hemopexin, transferrin, hemoglobin-haptoglobin as sources of heme iron for growth (150, 151).

*H. influenzae* has the ability to obtain iron from human transferrin using two bacterial cell surface transferrin binding proteins, Tbp1 and Tbp2 (151, 152). Tbp1 and Tbp2 exist as outer membrane proteins in a variety of typeable and nontypeable *H. influenzae* strains and range in size from approximately 100-kDa for Tbp1 to between 70- and 90-kDa for Tbp2. Sequence analysis of the genes encoding Tbp1 and Tbp2, *tbp*A and *tbp*B, respectively, suggested that Tbp1 is a member of the TonB-dependent family of outer membrane proteins while Tbp2 was lipid modified by signal peptidase II (152). *ton*B mutant strains of *H. influenzae* were unable to use iron-charged human transferrin as the sole source of iron for *in vitro* growth (153). Mutations in either one of the *tbp* genes showed significant reduction in transferrin-binding ability whereas mutations in both genes showed a complete loss in transferrin-binding capacity (152). Vogel *et al*. (154) recently described the use of human lactoferrin as an iron source for a variety of nontypeable *H. influenzae* strains.

In order to use iron from transferrin or iron chelates, *H. influenzae* must have a system to transport the iron into the cell cytoplasm. Sanders *et al*. (155) isolated three genes (*hit*A/*fbp*A, *hit*B/*fbp*B, *hit*C/*fbp*C) that are necessary for the utilization of iron bound to transferrin or iron chelates. The *fbp*ABC (*hit*ABC) operon is part of an ABC-type ferric transport system (reviewed in 151). Mutants in *fbp*A/*hit*A were unable to use iron from human transferrin and were impaired in their ability to grow on media containing ferric citrate as an iron source. The *fbp*ABC (*hit*ABC) gene cluster has been found in both type b and nontypeable *H. influenzae* strains (7, 153, 155).

Both type b and nontypeable *H. influenzae* utilize heme bound to hemopexin via the products of the *hux*ABC operon (156). A second heme-hemopexin-binding protein has been described in type b and nontypeable *H. influenzae* strains (157). This 57-kDa protein outer membrane protein has been shown to play a role in the acquisition of heme from hemopexin.

Four different outer membrane receptors have been described that bind either hemoglobin or hemoglobin-haptoglobin complexes. These four proteins (HgpA, HgpB, HgpC, and HhuA) range between 115- and 120-kDa in size and exhibited common features typical of TonB-dependent outer membrane receptors. A striking feature of all of the genes encoding these proteins is the presence of a four-nucleotide (CCAA) repeat motif near the start of their open reading frames (for review, 158). These sequences are thought to play a role in the regulation of these genes and the phase variation of these proteins (for review, 159).

A recent report showed that elemental iron, heme, and protoporphyrin IX can each regulate *H. influenzae* transferrin, hemopexin, and hemoglobin receptor expression *in vitro* (160). Transcripts corresponding to *tbp*A, *tbp*B, *hxu*A, and *hgp*A have been found in the middle ear fluid of patients with acute otitis media. These data suggested that iron and/or heme acquisition genes are transcribed during otitis media and potentially could play a role in *H. influenzae* survival in the middle ear fluid (161).

**9.6. Immunomodulation**

The various respiratory and invasive diseases caused by *H. influenzae* are characterized by an acute inflammatory response and the accumulation of polymorphonuclear leukocytes (PMN). The initial events

leading to respiratory inflammation are thought to involve cellular interactions between infecting bacteria and respiratory epithelial cells; these bacterial-epithelial interactions stimulate the release of inflammatory mediators (i.e. cytokines and chemokines) (162, 163).

Several respiratory pathogens such as *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *S. pneumoniae*, and *Bordetella pertussis* produce a variety of molecules that interact with epithelial cells and cause a secretion of proinflammatory cytokines (for reviews 162, 164, 165). These interactions involve bacterial cell-associated and extracellular products (i.e. modulins) that are capable of perturbing host-cell cytokine networks (162, 165). In response to bacterial modulins, airway cells produce inflammatory mediators such as interleukin (IL)-6, IL-1α, IL-1β, and tumor necrosis factor (TNF)-α along with the chemokines IL-8, and macrophage chemotactic protein (MCP)-1 which stimulate the activation and influx of neutrophils, monocytes, and macrophages (162, 164, 165, 166).

Studies to identify *H. influenzae* modulins have focused on lipooligosaccharide (LOS), the LPS equivalent in *H. influenzae*, as a major stimulator of proinflammatory cytokines. The proinflammatory cytokines TNF and IL-1β were found in the cerebrospinal fluid (CSF) of patients with Hib bacterial meningitis (167). Purified Hib lipooligosaccharide (LOS) and outer membrane vesicles stimulated meningeal inflammation in the rabbit meningitis model (167, 168).

Recent *in vitro* studies with LOS from NTHi stimulated the release of IL-6, IL-8, and TNF-α from cultured, primary human bronchial epithelial cells (HBEC) and TNF-α, IL-1β, and IL-6 from human monocytes (169, 170, 171). Nichols *et al*. (171) showed that *H. influenzae htr*B mutants that lack secondary (non-hydroxylated) acyl groups in lipid A were less virulent in a rat model of invasive disease and were attenuated in their stimulation of a TNF-α response from cultured human epithelial and monocytic cells. In addition, NTHi LOS stimulated the expression of intercellular adhesion molecule-1 (ICAM-1) on the surface of HBECs, which could be blocked with the LOS-binding antibiotic polymyxin B (170). Swords *et al*. (107) demonstrated that ChoP-containing LOS mediated bacterial adherence to platelet-activating factor (PAF) receptors on host cells; this interaction resulted in the internalization of the *H. influenzae* by the host epithelial cells. PAF has also been shown to augment meningeal inflammation caused by *H. influenzae* LOS in the rat model of meningitis (172).

We have recently presented evidence that modulins beyond LOS are present in both Hib and NTHi strains. Intact NTHi cells stimulated the secretion of cytokines IL-6, IL-8, TNF-α, and MCP-1 from cultured human tracheal epithelial cells. Stimulation with whole NTHi cells was greater than that seen with an equivalent amount of purified LOS, and polymyxin B showed only partial inhibition of cytokine secretion. Moreover, no correlation was seen between NTHi adherence to epithelial cells mediated by hemagglutinating pili, Hia, HMW1, HMW2, and Hap and epithelial cytokine secretion (173).

Adherent, intact NTHi bacterial cells have been shown to stimulate the release of TNF-α, IL-1β, and IL-6 from cultured human macrophages and IL-6 and IL-8 from respiratory syncytial virus (RSV)-infected human respiratory epithelial cells, *in vitro* (174). Frick *et al*. (175) showed that intimate contact between Hib cells or NTHi cells and respiratory epithelial cells stimulated the release of IL-8 and the expression of ICAM-1. In an *in vivo* murine model of airway infection with *H. influenzae*, increased epithelial cell ICAM-1 expression coincided with increased chemokine levels and neutrophil recruitment in the airway (175). Bresser *et al*. (176) have recently identified a heat-stable fraction from NTHi capable of stimulating IL-6 and IL-8 secretion from a human epithelial cell line.

*In vivo*, middle ear effusions from patients with NTHi otitis media have been shown to contain TNF, IL-8, and IL-1β (177, 178, 179). In the rat model of otitis media, NTHi cells stimulated the expression of cytokines IL-6, IL-1α, TNF-α, and IL-10 (180). Intranasal immunization of mice with the *H. influenzae* outer membrane protein P6 followed by subsequent challenge with NTHi showed lower levels of TNF-α in the middle ear effusions when compared to non-immune mice under the same NTHi challenge conditions (181). Finally, in the rat model of meningitis, Hib porin P2 stimulated the simultaneous expression of IL-1α, TNF-α, and macrophage inflammatory protein 2 (MIP-2) (182).

### 9.7. Protein D

Protein D (*hpd*) is a conserved 42-kDa lipoprotein originally identified based on its affinity for a complement of human IgD myeloma proteins (183). Protein D has 67% amino acid sequence identity with *E. coli* GlpQ, a periplasmic glycerophosphodiester phosphodiesterase, and retains similar enzymatic activity (184). Mutations in protein D significantly attenuate the ability of organisms to induce otitis media in a rat experimental model (185). One clue to its role in pathogenesis may lie in observations made with a human nasopharyngeal tissue culture model (186). Infection by wild type nontypeable *H. influenzae* was associated with a progressive decline in ciliary beat frequency and subsequent loss of ciliated epithelial cells, whereas isogenic protein D-inactivated counterparts did not elicit the same impairment of ciliary function (186). Interestingly, the ability of these organisms to adhere and invade in this tissue model was unchanged (186). Thus, compromise of the mucociliary escalator may be one means by which protein D aids in bacterial colonization of the respiratory mucosa.

## 10. ANIMAL MODELS OF DISEASE AND IMMUNITY

The infant rat model of invasive Hib disease played a substantial role in the identification of Hib virulence factors. The model allows for respiratory tract colonization in rats less than three weeks of age by inhalation of only a few organisms. Colonization is

followed closely by bacteremia and meningitis. Therefore, this model mimics the invasive disease seen in children (187, 188, 189). Older rats (up to three months of age) can also develop meningitis through intraperitoneal injection of Hib and subsequent bacteremia. Rabbits have also been used for experimental Hib infections and have contributed to understanding the pathophysiology of meningitis and in the design of treatment regiments. Rabbits require direct inoculation into the CNS or the perturbation of the CNS prior to i.v. injection (190, 191).

While animal models for Hib infection are relatively simple in technique and useful for monitoring disease outcome and prevention, the difficulty increases for finding models that will display the subtleties of NTHi nasopharyngeal colonization, persistence, dissemination, pathology, and immune responses in the middle ear, sinus, or lung.

Experimental infection studies for otitis media most often employ the chinchilla but the gerbil and rat have also been used (192, 193, 194). In the chinchilla, NTHi are often inoculated directly into the bulla and disease is monitored over a two- to eight-week period for fluid pressure changes using otoscopy and tympanometry. Tympanocentesis and nasopharyngeal lavage are performed to assess colonization and immune responses present in the middle ear (195, 196, 197). Direct inoculation of the middle ear results in 100% infectivity but bacterial and host factors involved in initial nasopharyngeal colonization cannot be studied through this route. While NTHi can colonize the nasopharynx of chinchillas after experimental inoculation, colonization does not consistently lead to otitis media. An otitis media model has, however, been developed that allows for initial colonization with NTHi. Chinchillas are first infected with adenovirus, and then NTHi, administered nasally, will colonize the nasopharynx and sometimes result in otitis media. This scenario mimics development of otitis media in children. This method has been used to assess the role of bacterial components in colonization and their potential as vaccine candidates (104, 198).

Pulmonary infection models used for vaccine efficacy tests have utilized both mice and rats (199, 200). In these models, animals are immunized intravenously or at the mucosal surface and then challenged by direct administration of NTHi to the lungs. Bacterial clearance is then assessed a few hours after challenge by culturing homogenized lung tissue or lavage fluid. These pulmonary clearance models, however, do not allow for the study of persistent colonization as is seen with chronic infections in the lungs of adults with COPD. Attempts have been made to reflect this scenario in rats by administering NTHi encapsulated within agarose beads. This method can lead to infections that persist for weeks resulting in pathology and immune responses that parallel those seen in humans (201, 202).

## 11. VACCINES

Prior to licensing of the conjugated Hib vaccine in 1987 and its widespread use by 1990, Hib was estimated to cause 27,000 annual cases of meningitis in developed countries (approximately 12,000 in the U.S.) and 330,000 in developing countries (203, 206). The number of invasive Hib cases in the U.S. has dropped severely with 54 cases reported in 1998 and similar success occurred in other countries having an active vaccine program (13).

Since the 1960s, the serotype b polysaccharide capsule of *H. influenzae* was recognized as a major virulence factor in invasive disease due to the capsule's role in deterring opsonization and phagocytosis. Numerous studies pointed to the type b capsule as a primary target for vaccine development. For example, the majority of invasive *H. influenzae* disease was due to serotype b strains, transplacental antibodies specific for type b capsule were found to be protective during the first 6 months of life, and convalescent patients developed a specific immune response to type b capsule (205). These studies led to the use of purified type b capsule, or polyribosylribitol phosphate (PRP), as a vaccine. In 1977, a prospective study performed in Finland demonstrated a 90% efficacy for this vaccine in children 18-71 months (204). The study, however, revealed a lack of success in younger children due to the vaccine antigen's T-cell independent nature and consequent inability to elicit a memory, or booster, immune response. This problem was resolved by conjugating PRP to various protein carriers. The resulting conjugate polysaccharide antigens facilitated interactions between B lymphocytes specific for PRP and T lymphocytes specific for the protein carrier resulting in improved antibody production (6).

PRP has been conjugated to various protein carriers such as diphtheria toxoid (PRP-D), diphtheria CRM197 protein (HbOC), tetanus toxoid (PRP-T), and meningococcal outer membrane proteins (PRP-OMP). While these vaccines vary in their immunogenicity, as defined by antibody titers, the net effect (with the possible exception of PRP-D) is the generation of protective immunity in most vaccinated individuals (207). In addition, combinations of vaccines have been developed to help minimize the number of inoculations given to children, also with varying results in antibody titers but high degrees of efficacy (reviewed in 208).

The continued decline of invasive Hib disease has prompted discussions of possible eradication in countries with active immunization programs. This level of success is, in part, due to a dramatic decrease in carriage of Hib by individuals who received the conjugated vaccine (209, 210) and has led to a decrease in Hib transmission and carriage in vaccinated populations (13). Eradication, however, is far from discussion in developing nations where use of the conjugated vaccines is limited or non-existent due to inaccessible medical care and high vaccine costs (206).

In contrast to Hib, development of a vaccine for prevention of otitis media and lower respiratory infections caused by NTHi is more difficult due to the genetic and protein heterogeneity between strains. Reflecting this heterogeneity, immune responses directed at NTHi appear to be strain specific (211, 212). Stable vaccine targets are further reduced by antigenic variation of outer membrane

and surface-associated proteins (P1, P5, pili, and IgA protease), antigenic drift (P2), phase variation (pili), or protein redundancy (heme-binding proteins) (213).

Despite these pitfalls, immunologic protection against NTHi can be achieved. Antibody titers to P6, a conserved OMP of NTHi, were found to be lower in otitis prone children than in children who were less prone (214). Furthermore, protective immunity and reduced NTHi carriage could be achieved in adults susceptible to recurrent, chronic respiratory infections when they were immunized orally with killed NTHi (215, 216, 217). Because there is no dominant virulence factor for NTHi, vaccine efforts are directed toward identifying conserved antigens that will facilitate clearance of bacteria by the immune system, thereby reducing colonization in individuals and carriage within the population. As described above, numerous surface molecules are currently under study for their vaccine potential.

## 12. PERSPECTIVE

Studies of *H. influenzae* over the past 100 plus years have increased our fundamental knowledge about important bacterial processes such as transformation and genomic organization. Antigenic variation of many *H. influenzae* surface molecules through horizontal gene transfer and phase variation serves as a paradigm for how a bacteria can be maintained successfully within a narrow host range (i.e. human specific). Increased understanding about *H. influenzae* pathogenesis has lead to a very successful Hib vaccine that has greatly reduced invasive disease morbidity and mortality. Hopefully further insights will lead to prevention of otitis media and other diseases caused by NTHi.

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