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Association of IS*1016* with the *hia* Adhesin Gene and Biotypes V and I in Invasive Nontypeable *Haemophilus influenzae*[▽]

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A subset of invasive nontypeable *Haemophilus influenzae* (NTHI) strains has evidence of IS*1016*, an insertion element associated with division I *H. influenzae* capsule serotypes. We examined IS*1016*-positive invasive NTHI isolates collected as part of Active Bacterial Core Surveillance within the Georgia Emerging Infections Program for the presence or absence of *hmw1* and *hmw2* (two related adhesin genes that are common in NTHI but absent in encapsulated *H. influenzae*) and *hia* (homologue of *hsf*, an encapsulated *H. influenzae* adhesin gene). Isolates were serotyped using slide agglutination, confirmed as NTHI strains using PCR capsule typing, and biotyped. Two hundred twenty-nine invasive NTHI isolates collected between August 1998 and December 2006 were screened for IS*1016*; 22/229 (9.6%) were positive. Nineteen of 201 previously identified IS*1016*-positive invasive NTHI isolates collected between January 1989 and July 1998 were also examined. Forty-one IS*1016*-positive and 56 randomly selected IS*1016*-negative invasive NTHI strains were examined. The *hia* adhesin was present in 39 of 41 (95%) IS*1016*-positive NTHI strains and 1 of 56 (1.8%) IS*1016*-negative NTHI strains tested; *hmw* (*hmw1*, *hmw2*, or both) was present in 50 of 56 (89%) IS*1016*-negative NTHI isolates but in only 5 of 41 (12%; all *hmw2*) IS*1016*-positive NTHI isolates. IS*1016*-positive NTHI strains were more often biotype V ($P < 0.001$) or biotype I ($P = 0.04$) than IS*1016*-negative NTHI strains, which were most often biotype II. Pulsed-field gel electrophoresis revealed the expected genetic diversity of NTHI with some clustering based on IS*1016*, *hmw* or *hia*, and biotypes. A significant association of IS*1016* with biotypes V and I and the presence of *hia* adhesins was found among invasive NTHI. IS*1016*-positive NTHI strains may represent a unique subset of NTHI strains, with characteristics more closely resembling those of encapsulated *H. influenzae*.

Haemophilus influenzae is a gram-negative exclusive human pathogen whose pathogenic potential may vary, in part, depending on the presence or absence of a polysaccharide capsule. Encapsulated (typeable) *H. influenzae* bacteria are often associated with systemic invasive diseases, such as meningitis, whereas nonencapsulated *H. influenzae* (nontypeable *H. influenzae* [NTHI]) bacteria are usually associated with disease at respiratory mucosal sites, such as the sinuses, lung, or middle ear. Although invasive pediatric *H. influenzae* disease has declined with routine infant use of *H. influenzae* serotype b (Hib) conjugate vaccines, serious disease caused by non-type b encapsulated and NTHI strains remains important in children and adults (1, 25, 46). In the United States in 2006, 63% of invasive *H. influenzae* disease in children 0 to 17 years old and 65% of invasive *H. influenzae* disease in adults of ≥18 years old was due to NTHI (Active Bacterial Core Surveillance Report, Emerging Infections Network, *Haemophilus influenzae*, http://www.cdc.gov/ncidod/dbmd/abcs/survreports.htm) (18). Among children in the developed world, NTHI is a leading cause of bacterial ear infections (16, 51), with significant socioeconomic consequences (3, 4), and NTHI strains are responsible for a substantial number of pediatric deaths caused by pneumonia in the developing world (27).

Encapsulated *H. influenzae* strains possess *cap* genes necessary for production and transport of their respective polysaccharide capsules (39). In division I encapsulated *H. influenzae* strains, the *cap* locus is flanked by direct repeats of the IS*1016* insertion element; division II encapsulated *H. influenzae* strains contain one or more IS*1016* elements that are unassociated with *cap* genes. A number of investigators have shown that while IS*1016* elements are generally absent in NTHI, they may be present in a small (but varying) proportion of NTHI isolates from the respiratory tract and invasive disease (53, 59, 60). It has been suggested that the presence of IS*1016* in NTHI may be a marker for a more recent evolution from encapsulated *H. influenzae* (59, 60).

NTHI may differ from encapsulated *H. influenzae* in ways other than the lack of capsule, including a variety of adhesive structures. In studies designed to understand the mechanisms of respiratory tract colonization by NTHI, two high-molecular-weight adhesins, HMW1 and HMW2, expressed by the majority of NTHI strains and found to be immunogenic in natural infection, were identified (6, 10, 58). HMW1 adhesins mediate high levels of attachment to most human epithelial cell lines via a sialylated glycoprotein receptor (10, 58, 61); HMW2 adhesins mediate attachment to only a subset of human epithelial cell lines via an unknown receptor (29). Both are generally absent in encapsulated strains. Hia, a unique autotransporter adhesin protein that mediates attachment to cultured human epithelial cells (6, 57), is found in many non-Hib encapsulated *H. influ-*

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enzae (serotypes a, e, and f) and NTHI strains that lack HMW adhesins (52). *hia* is a homologue of the *hsf* gene that is ubiquitous among Hib strains, again suggesting a closer relationship between *hia*-containing NTHI and encapsulated *H. influenzae* (60).

Efforts to develop vaccines targeting NTHI disease have focused on a variety of surface-exposed antigens known to be targets of the immune system, including bacterial adhesion proteins (4); such efforts have been hampered by the heterogeneity of NTHI strains. Despite the heterogeneity, in a recent study using multilocus sequence typing to characterize 322 NTHI isolates, Erwin et al. demonstrated well-defined phylogenetic groups of NTHI that differ in genetic content (21). Better characterization of clinically relevant NTHI strains with shared features will improve understanding of NTHI and may inform vaccine development. In this study, using a large, well-characterized isolate collection, we sought to identify additional IS*1016*-positive, invasive NTHI isolates and to compare the adhesin gene profiles, biotypes, and restriction patterns in IS*1016*-positive and IS*1016*-negative NTHI isolates to assess relatedness to each other and to patterns previously recognized among encapsulated strains.

MATERIALS AND METHODS

Surveillance and case characteristics. A total of 734 invasive *H. influenzae* isolates were collected as part of the Active Bacterial Core Surveillance (54) of the Georgia Emerging Infections Program from January 1989 through December 2006; 437 were PCR-confirmed NTHI isolates, 430 of which were available for further testing. All isolates were obtained from normally sterile body sites, such as blood and cerebrospinal fluid. Basic clinical and demographic information, including patient age, race, gender, clinical syndrome, and disease outcome, was collected for each case.

Bacterial strains and growth. Characteristics (biotype, IS*1016* screening, and selected pulsed-field gel electrophoresis [PFGE] typing) of 201 NTHI strains collected between January 1989 through July 1998 have been previously reported (53). The remaining 229 NTHI isolates collected from August 1998 through December 2006 were PCR capsule typed as previously described (53) with the following exception: new *bexA* primers were designed based on additional sequence information (bexA101-121, 5′-CAATTTTGAGYTAMAAAAAGG-3′; bexA646-627, 5′-TCCATRTCTTCAAAATGRTG-3′, where Y is C or T, M is A or C, and R is A or G). All bacteria were grown on chocolate II solid medium or in brain heart infusion broth supplemented with 10 μg/ml hemin and 2 μg/ml NAD (Becton Dickinson Microbiology Systems, Cockeysville, MD).

Southern blot hybridization. Chromosomal DNA was extracted from 229 PCR-confirmed NTHI strains collected between August 1998 and December 2006 (9) and hybridized with digoxigenin-labeled pUO38 (39), pBR322 (8), or IS*1016* as previously described (53).

Screen for *hmw* and *hia* adhesin genes. All IS*1016*-postive NTHI strains from 1989 to 2006 and randomly selected IS*1016*-negative NTHI strains were screened for the presence of *hmw1*, *hmw2*, and *hia* using PCR on genomic DNA or single colony lysates as described by Erwin et al. (21) with updated primer sequences (A. L. Erwin et al., personal communication). Primers hmw1-F (5′CAAAGC CATCAGGTTGTTGTGC-3′) and hmw1-R (5′-CCTATTTGGTCTTGCTACG AGTGG-3′) correspond to Rd genome coordinates 1746545 to 1751277, and when *hmw1* is present, these primers amplify a 13,928-bp product. Primers hmw2-F (5′-CCGCACTTTCTTCTCGTTCTTCT-3′) and hmw2-R (5′-GCTAT TCGGTTAGGTAATGCAGATCC-3′) correspond to Rd genome coordinates 1665719 to 1667371, and when *hmw2* is present, these primers amplify a 12,717-bp product (26). *hia* flanking primers Hia F (5′-CCGAAAGCACAAGG ATATGGACG-3′) and Hia R (5′-CAGATAAATCCTGACCTCGCTCTC-3′) are located between Rd genome coordinates 1804356 to 1810590 and amplify a product of 6,235 bp if *hia* is present (26). PCR results, both negative and positive, were confirmed with Southern blot hybridization using *hmw1A*, *hmw2A*, and *hia* probes as previously described (19).

PFGE and composite cluster analysis. PFGE was performed as previously described (53). Cluster analysis was applied to a composite data set combining the PFGE fingerprint data, biotypes, and the categorical data (with and without IS*1016*, *hmw*, and *hia*). A similarity matrix of the composite data was created and displayed on a dendrogram by averaging similarity matrices from the individual experiments, keeping internal weights equal and using the unweighted pair group method of arithmetic averages (UPGMA) (BioNumeric method manual 5.0, Applied Maths, Kortrijk, Belgium).

Biotyping. Biochemical assays to determine biotype were performed at either the CDC or the Georgia Public Health Laboratory (Atlanta, GA) using the method of Kilian (37) and API 20E biochemical test strips purchased from bioMerieux (St. Louis, MO) (35).

Statistics. Frequency data were evaluated by the chi-square test or, where appropriate, Fisher's exact probability test, and means were evaluated by Student's *t* test using SAS 9.1 (The SAS Institute, Inc., Cary, NC). A *P* value of <0.05 was considered statistically significant.

TABLE 1. Distribution of biotypes of PCR-confirmed NTHI isolates from 1989 to 2006 and association with IS*1016*[a]

Isolate	No. of NTHI isolates with the following biotype:						
	I	II	III	IV	V	VI	VII
NTHI with IS*1016*	9	2	7	4	16	2	1
NTHI without IS*1016*	40	213	92	15	4	6	3
NTHI IS*1016* N/A[b]	2	1	1	3	0	0	0
Total	51	216	100	22	20	8	4

[a] 213 PCR-confirmed NTHI isolates collected from 1 August 1998 to 31 December 2006 have been added to 208 previously reported NTHI isolates (Table 4 in the article by Satola et al. [53]).

[b] NTHI IS*1016* N/A, NTHI isolates for which the IS*1016* status is not available (unknown).

RESULTS

Four hundred thirty PCR-confirmed, invasive NTHI isolates collected between 1989 and 2006 in metropolitan Atlanta, GA, were available for testing. Results of biotyping, IS*1016* screening, and selected PFGE typing have been previously reported for 201 isolates collected between 1989 and 1998 (53). In this study, 229 isolates were biotyped, screened for IS*1016*, and PFGE typed, and all 430 isolates were screened for *hia* and *hmw*. A combined dendrogram was generated.

IS*1016*-positive NTHI. Southern blot hybridization with pUO38, pBR322, and IS*1016* was performed on the 229 PCR-confirmed NTHI isolates (collected August 1998 to December 2006) to look for residual capsule-specific DNA as previously described (53). Plasmid pUO38 contains a complete *cap* locus from a division I Hib strain, including a copy of the insertion sequence IS*1016* and the beta-lactamase (*bla*) gene from the plasmid backbone, pBR322 (39). No capsule-specific DNA was found among the 229 NTHI isolates probed with pUO38, 69% were *bla* positive, and 22/229 (9.6%) of the strains hybridized to IS*1016* with a single band ranging between ~5 and 10 kb (data not shown). The proportion of invasive NTHI isolates containing IS*1016* was nearly identical to the 9.5% (19/201) rate noted previously among isolates collected between January 1989 and July 1998 (53); the combined IS*1016*-positive total was 41/430 (9.5%).

Biotypes. The biotypes of all NTHI isolates collected between 1989 and 2006 have been combined and are shown in Table 1. Among the invasive NTHI isolates with known biotypes, 51/421 (12.1%) were biotype I, 216/421 (51.3%) were biotype II, 100/421 (23.7%) were biotype III, 22/421 (5.2%) were biotype IV, 20/421 (4.8%) were biotype V, 8/421 (1.9%) were biotype VI, and 4/421 (l%) were biotype VII. More than

TABLE 2. Prevalence of *hmw* and *hia* adhesin genes in PCR-confirmed NTHI isolates and association with IS*1016*

Isolate	No. of strains possessing the following gene(s)/total no. of strains (%)				
	hmw1 only	*hmw2* only	Both *hmw1* and *hmw2*	*hmw1* or *hmw2* or both	*hia*
NTHI with IS*1016*	0/41	5/41 (12)	0/41	5/41 (12)	39/41 (95.1)
NTHI without IS*1016*	10/56 (18)	11/56 (19)	29/56 (52)	50/56 (89)	1/56 (1.8)

half of the IS*1016*-positive NTHI isolates were biotypes I and V, and compared with IS*1016*-negative NTHI, a significant association was noted between the presence of IS*1016* and biotype V ($P < 0.001$) and biotype I ($P = 0.04$). Eighty percent (16/20) of isolates with the uncommon biotype V were IS*1016* positive.

Correlation of HMW and Hia adhesins with IS*1016* in NTHI. All 41 IS*1016*-positive NTHI isolates collected from 1989 to 2006 and 56 randomly selected IS*1016*-negative invasive NTHI isolates (also collected between 1989 and 2006) were examined for the presence or absence of the adhesin genes *hmw1*, *hmw2*, and *hia* by PCR and Southern blot hybridization. None of the 41 IS*1016*-positive NTHI strains had evidence of *hmw1*, and 5 of 41 (12%) were *hmw2* positive (Table 2). The majority (39/41 [95%]) of IS*1016*-positive NTHI strains contained *hia* (Table 2). In contrast, most (50/56 [89%]) IS*1016*-negative NTHI isolates tested contained *hmw*, 10/56 (18%) isolates tested contained *hmw1*, 11/56 (19%) isolates tested contained *hmw2*, and 29/56 (52%) isolates tested contained both *hmw1* and *hmw2* (Table 2). The presence of *hia* in IS*1016*-negative NTHI was rare (1/56 [1.8%]) (Table 2). The majority (36/41 [87.8%]) of IS*1016*-positive NTHI isolates were *hmw* negative and *hia* positive, and the majority (49/56 [87.5%]) of IS*1016*-negative NTHI were *hmw* positive and *hia* negative; 6 strains were negative for both *hmw* and *hia*, and 4 were positive for both by PCR (Table 3). The association of *hia* with IS*1016*-positive NTHI was significant ($P < 0.001$).

Epidemiological characteristics of IS*1016*-positive NTHI. For the combined group of 430 cases of invasive NTHI disease, patients ranged in age between 0 and 100 years (mean age of 46.5 years; 75.8% adults were ≥18 years old). Cases of invasive NTHI disease due to IS*1016*-positive ($n = 41$) and IS*1016*-negative isolates ($n = 389$) were compared for significant differences in patient age, race, gender, clinical syndrome, and disease outcome (Table 4). A statistically significant association was observed between younger age and the presence of IS*1016* in invasive NTHI disease; the association between older age (≥18 years) and the absence of IS*1016* in invasive NTHI disease was nearly significant ($P = 0.05$). Among the 41 NTHI isolates positive for IS*1016*, one case occurred in a neonate (<1 month of age), 10 cases in children aged 1 month to 4 years old, 4 cases in children 5 to 17 years old, and 26 cases in adults 18 years old or older. Ten of 41 (24.4%) of the IS*1016*-positive NTHI strains were from children 1 month to 4 years of age compared to 10.6% of IS*1016*-negative NTHI ($P = 0.01$).

TABLE 3. *hmw* and *hia* adhesin gene profiles in PCR-confirmed NTHI isolates and association with IS*1016*

Isolate	No. of NTHI strains with the adhesin gene(s)/total no. of strains (%)			
	hmw negative and *hia* negative	*hmw* negative and *hia* positive	*hmw* positive and *hia* negative	*hmw* positive and *hia* positive
NTHI with IS*1016*	0/41	36/41 (87.8)	2/41 (4.9)	3/41 (7.3)
NTHI without IS*1016*	6/56 (10.7)	0/56	49/56 (87.5)	1/56 (1.3)

TABLE 4. Epidemiological characteristics of PCR-confirmed NTHI and association with IS*1016*

Variable	% NTHI isolates		*P* value
	With IS*1016* ($n = 41$)	Without IS*1016* ($n = 389$)	
Mean age (yr)	38	47	0.06
Age groups			
<1 mo	2.4	6.2	0.21
1 mo-4 yrs	24.4	10.6	0.01[a]
5–17 yrs	9.8	5.7	0.14
≥18 yrs	63.4	77.5	0.05
Race			
White	63.4	56.3	0.38
Black	31.7	40.4	0.28
Other	4.9	3.3	0.26
Gender			
Male	49	45	0.67
Female	51	55	0.67
Clinical syndrome			
Meningitis	5	9	0.50
Bacteremia	68	66	0.82
Pneumonia	27	25	0.86
Disease outcome (survived)	93	82	0.09

[a] The percentage of NTHI isolates with IS*1016* compared to the percentage of NTHI isolates without IS*1016* was significantly different for 1-month- to 4-year-old children.

PFGE and cluster analysis of composite data set. We compared the PFGE patterns of genomic DNA from 37 IS*1016*-positive and 55 IS*1016*-negative NTHI isolates. As expected, genetic diversity among the NTHI strains was relatively high, and 90 distinct restriction patterns were identified among the 92 strains available for PFGE (data not shown). However, when a composite data set was examined, clustering with respect to PFGE patterns, biotypes, and the presence or absence of IS*1016*, *hmw*, and *hia* was noted (Fig. 1). Using a dendrogram generated by BioNumerics' Cluster Analysis program, combined with the Cluster Cutoff method using the composite data set (PFGE, biotype, and presence/absence of IS*1016*, *hmw*, and *hia*), strains were grouped into three clusters. Clusters I and III contained the majority (92%) of IS*1016*-positive strains; 100% of cluster I strains and 75% (22/29) of cluster III strains were IS*1016* and *hia* positive (Fig. 1). In addition, 11/13 biotype I strains fell within clusters I and III, and all biotype V strains were in cluster III.

DISCUSSION

Since the introduction of the *H. influenzae* type b conjugate vaccine, the epidemiological characteristics of invasive *Haemophilus influenzae* disease have changed. Serious pediatric

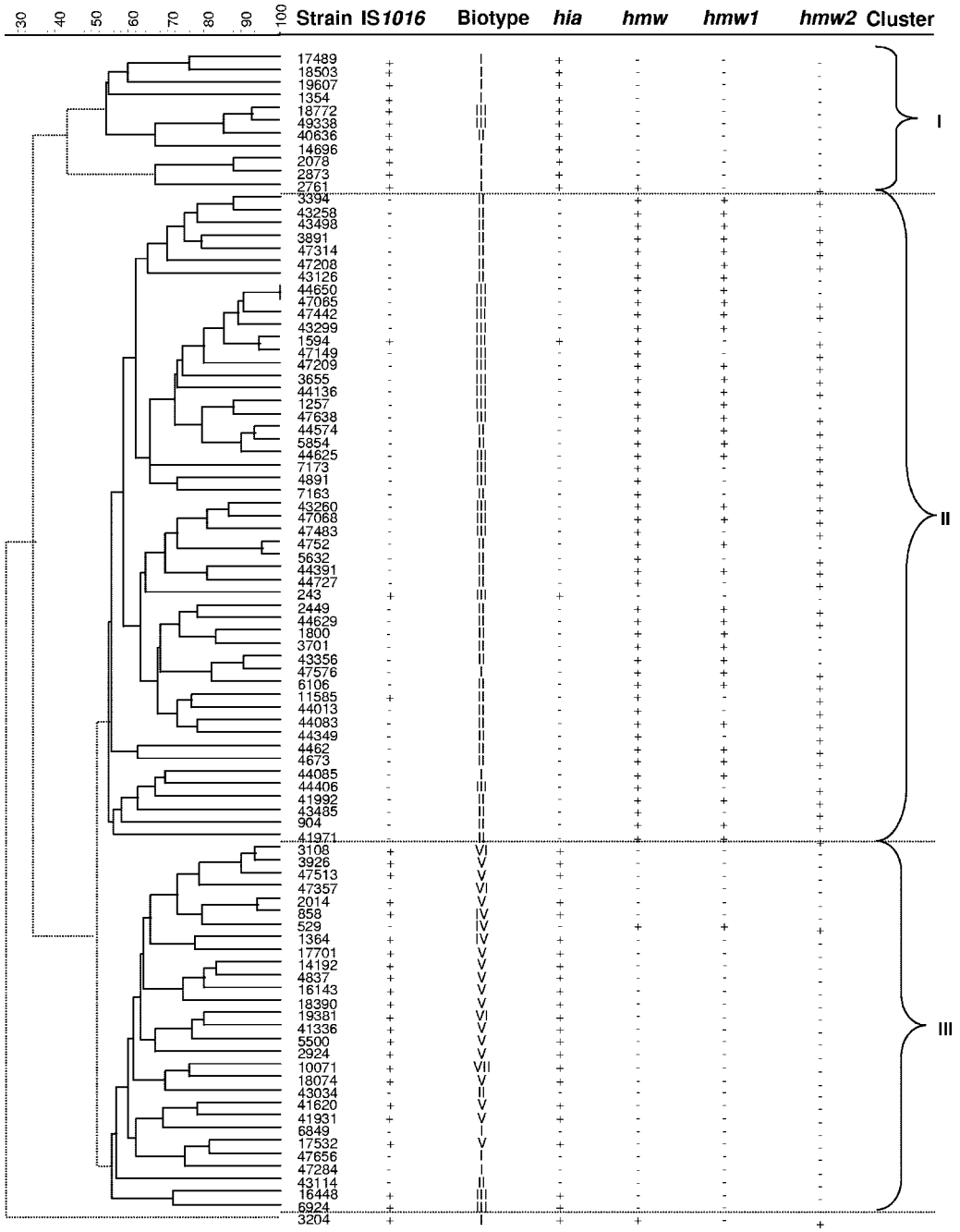

FIG. 1. Evolutionary analysis of invasive NTHI strains. The dendrogram was generated by BioNumerics software showing the genetic relationships between 17 IS*1016*-positive and 19 IS*1016*-negative NTHI strains. The dendrogram was constructed by cluster analysis of PFGE patterns (data not shown) obtained after digestion of chromosomal DNA with SmaI enzyme, using the unweighted pair group method of arithmetic averages. The strain designation, capsule type by PCR, biotype, and presence (+) or absence (−) of IS*1016*, *hmw*, and *hia* are shown. Three different clusters of branches of PFGE patterns are identified.

disease due to serotype b has been largely eliminated, while adult disease remains and is dominated by nontypeable strains. In addition to the significant burden of adult invasive NTHI disease, NTHI can occasionally cause bacteremia and meningitis in otherwise healthy newborns and older children (11, 15, 46, 48). NTHI bacteria remain important causes of localized respiratory tract infections (44).

Efforts to better understand the virulence factors responsible for NTHI disease pathogenesis have been increasing (23, 33, 62), and current evidence suggests that NTHI bacteria rely on multiple factors to colonize the human mucosal surfaces and ultimately spread to other sites (56). Genomic DNA-based microarray and bioinformatics approaches were used to compare the genomes of 1885MEE and 86-02NP, middle ear and nasopharyngeal NTHI isolates from children with otitis media, with the genome of Rd. These analyses suggested that the

genomes of the two NTHI strains are more similar to each other than to Rd, a formerly encapsulated strain. A number of genes were identified in the NTHI strains that were absent in Rd, including proteins with possible roles in adhesion, biofilm formation, pH regulation, protection against reactive oxygen species, and a member of a family of virulence-associated autotransporters (43).

Although extensive heterogeneity among NTHI isolates compared with Hib and non-Hib encapsulated strains has been well described (41, 45), nonrandom associations of a number of genetic loci have been noted in a subset of NTHI strains (21, 22). *H. influenzae* strains differ in protein adhesins (reviewed in reference 57). Most NTHI strains express one or both of two high-molecular-weight adhesins, HMW1 and HMW2. St. Geme et al. reported that IS*1016* was often present in *hmw*-negative NTHI strains and uniformly absent in *hmw*-positive NTHI strains and speculated that *hmw*-negative NTHI strains evolved more recently from an encapsulated ancestor (57). Furthermore, NTHI strains that are *hmw* negative often express the adhesin Hia, a homologue of the Hib adhesin, Hsf. Although the prevalence and distribution of *hmw* and *hia* genes do not define distinct divisions among NTHI, the *hmw-hia* dichotomy suggests that *hmw*-deficient strains may form subgroups within the larger NTHI population (21, 52, 60). Because *hia* is present in a number of non-Hib encapsulated *H. influenzae* strains and is a homologue of the Hib gene *hsf*, it has also been speculated that *hia*-containing NTHI strains may have evolved more recently from an encapsulated ancestor.

The findings that the majority of our IS*1016*-positive NTHI strains were *hmw* negative and *hia* positive and the majority of those without IS*1016* were *hmw* positive and *hia* negative suggest that the presence or absence of IS*1016* in NTHI may be a linked marker for two distinct lineages. It is important to note that since *hmw* and *hia* were detected by PCR amplification and Southern blot hybridization, we cannot be certain that these strains actually express HMW or Hia adhesin. Although it is very uncommon, we found four strains positive for both *hmw* and *hia*. Erwin et al. and O'Neill et al. each identified a single NTHI isolate positive for both *hmw* and *hia* (21, 48). We also observed six NTHI strains that lacked *hmw1* and *hmw2* as well as *hia* by PCR and Southern blotting. Although it is possible that strains found to be negative by PCR may be due to variation within the sequence at the primer binding sites, the results of Southern blot hybridization were in agreement with the PCR results in all cases. Rao et al. reported that 25% of NTHI strains lack proteins belonging to HMW1/HMW2 family of adhesins, yet nearly all such strains remain capable of efficient in vitro adherence, independent of piliation, suggesting the existence of other nonpilus adhesins (50).

An appreciable number of NTHI isolates show partial hybridization with *cap* locus sequences, most notably *cap*-associated IS*1016*, suggesting a relationship to encapsulated ancestors (36, 53, 60). Residual IS*1016* sequence was found in a highly virulent NTHI strain, R2866 (INT1) (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi) isolated from a child with meningitis (20, 21, 46). This invasive, IS*1016*-positive, NTHI demonstrated unusual resistance to killing by normal human serum, and DNA isolated from strain INT1 was able to transform a nonvirulent NTHI strain and cause bacteremia and meningitis in infant rats (46, 64). Also, strain R2866 has several genetic loci not found in strain Rd KW20 (21). These include the gene for an autotransporter, *lav* (17), the lysogenic bacteriophage HP2 (63), the *tna* cluster (40), and a 53-kb plasmid similar to other large integrative plasmids of *Haemophilus* spp. (42).

The complete genome sequences of several NTHI strains isolated from various sites have demonstrated that each of these strains contains 30 to 40 genetic loci not present in the previously sequenced Rd KW20 strain (34). We chose to focus our characterization of IS*1016*-positive NTHI on the association with *hmw* and *hia* adhesin genes. Future studies to evaluate the association of other NTHI genetic features with the presence of IS*1016* will be of interest. However, alternatively, the presence of IS*1016* in NTHI may instead be a marker for genetic characteristics that more closely resemble encapsulated *H. influenzae*, such as lipopolysaccharide biosynthesis, iron uptake, and other genes identified using a position-based scanning (7) on the Hib genome and lacking in the laboratory-passaged, unencapsulated, and avirulent Rd KW20.

Seven of the eight currently recognized *H. influenzae* biotypes were present in both IS*1016*-positive and -negative strains. Since the development of Kilian's assay for biotyping (37), several laboratories have examined the relationship between biotype and other characteristics, such as anatomic site of isolation, serotype, or pattern of antimicrobial susceptibility (2, 30, 38, 47). In past surveys, most encapsulated *H. influenzae* (serotypes a, b, and f) strains were biotypes I and II, serotype e strains were biotype IV, and NTHI strains were biotypes II and III (37). Biotype distribution among invasive disease and noninvasive disease isolates has been well documented. However, the relationship between biotype and pathogenicity has produced some conflicting conclusions (32). Although previous studies showed that the majority of invasive disease isolates from cases of meningitis and septicemia were biotype I and noninvasive isolates were biotype II, the data were derived from the pre-Hib vaccine era and likely reflect the predominance of biotype I Hib clonal groups in systemic disease.

We previously noted the association of IS*1016*-positive NTHI with biotype V, an otherwise very uncommon *H. influenzae* biotype (53). The more recent invasive *H. influenzae* isolates (1998 to 2006) also show the same significant association of biotype V with the presence of IS*1016*. The highly virulent, IS*1016*-positive NTHI strain INT1/R2866 belongs to biotype V and is also *hmw* negative (20, 46). Biotype V strains have been most frequently reported as occurring in association with acute otitis media or as respiratory tract commensals (37, 45). Although uncommon, a small proportion of Hib strains belong to biotype V, and at least one clinical study found that an unusually large percentage (14/28 [50%]) of NTHI strains were biotype V and nearly 50% of these caused invasive disease (31).

An obvious question is whether IS*1016*-positive NTHI strains differ in pathogenic potential or in the characteristics of clinical disease. Despite the association of IS*1016*-containing NTHI with certain biochemical and adhesin characteristics of encapsulated *H. influenzae*, no association between the presence of IS*1016* and clinical syndrome, race, or disease outcome were noted in our comparison. However, a significant association was observed between the presence of IS*1016* and younger patient age. Although of unclear significance, it is of

interest that the capsule-associated IS*1016* element was more often present in NTHI disease affecting children in the age group previously at highest risk for Hib disease. Host factors may play a significant role in the pathogenesis of invasive NTHI disease in adults, and microbiologic characteristics important to invasive disease may differ from those important for respiratory tract disease. A particular strength of this study is the analysis of a large series of invasive NTHI cases and isolates collected by active, population-based surveillance over a 19-year period. However, the findings may reflect regional characteristics that may not be generalizable to all invasive NTHI disease.

Since the introduction of the 7-valent pneumococcal vaccine, NTHI has emerged as a leading cause of acute otitis media in children (12). The NTHI antibody response that develops during the course of infection has been associated with a decrease in bacteria in middle ear fluid (24) and more efficient resolution of infection (55), suggesting a vaccine with NTHI bacterial components may be feasible (13). Outer membrane proteins are the major focus of NTHI vaccine development efforts (14, 49). However, despite excellent preclinical studies, the optimal antigen(s) for vaccine development remains unclear (28). HMW proteins are important targets of serum antibody in children who have had otitis media (5), and antibodies produced following immunization of chinchillas with HMW proteins are opsonophagocytic (65). A report from the Eighth International Otitis Media Research Conference recommended continued preclinical evaluation of this family of proteins (28). It has been estimated that HMW proteins are present in approximately 75% of unrelated NTHI strains (59). Our data would suggest that this number might be somewhat higher among invasive NTHI.

In summary, we found that a subset of invasive NTHI isolates possessed DNA homologous to the insertion sequence IS*1016*, an element often associated with the *H. influenzae* capsule gene cluster. The significant association of IS*1016* with biotypes V and I, the presence of *hia* adhesin and the absence of *hmw* adhesins in NTHI may represent a unique subgroup of NTHI with characteristics more closely resembling those of encapsulated *H. influenzae*. Future characterization and comparison of systematically collected isolates from invasive disease, respiratory disease, and nasopharyngeal carriage may provide a more complete understanding of the prevalence and clinical relevance of IS*1016*-positive NTHI.

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