Comparative Profile of Heme Acquisition Genes in Disease-Causing and Colonizing Nontypeable *Haemophilus influenzae* and *Haemophilus haemolyticus*

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Nontypeable *Haemophilus influenzae* (NTHI) are Gram-negative bacteria that colonize the human pharynx and can cause respiratory tract infections, such as acute otitis media (AOM). Since NTHI require iron from their hosts for aerobic growth, the heme acquisition genes may play a significant role in avoiding host nutritional immunity and determining virulence. Therefore, we employed a hybridization-based technique to compare the prevalence of five heme acquisition genes (*hxA*, *hxB*, *hxC*, *hemR*, and *hup*) between 514 middle ear strains from children with AOM and 235 throat strains from healthy children. We also investigated their prevalences in 148 *Haemophilus haemolyticus* strains, a closely related species that colonizes the human pharynx and is considered to be nonpathogenic. Four out of five genes (*hxA*, *hxB*, *hxC*, and *hemR*) were significantly more prevalent in the middle ear strains (96%, 100%, 100%, and 97%, respectively) than in throat strains (80%, 92%, 93%, and 85%, respectively) of NTHI, suggesting that strains possessing these genes have a virulence advantage over those lacking them. All five genes were dramatically more prevalent in NTHI strains than in *H. haemolyticus*, with 91% versus 9% *hxA*, 98% versus 11% *hxB*, 98% versus 11% *hxC*, 93% versus 20% *hemR*, and 97% versus 34% *hup*, supporting their potential role in virulence and highlighting their possibility to serve as biomarkers to distinguish *H. influenzae* from *H. haemolyticus*. In summary, this study demonstrates that heme acquisition genes are more prevalent in disease-causing NTHI strains isolated from the middle ear than in colonizing NTHI strains and *H. haemolyticus* isolated from the pharynx.

Nontypeable *Haemophilus influenzae* (NTHI) are small nonencapsulated Gram-negative coccobacilli that lack a polysaccharide capsule and commonly colonize the human pharynx. They also cause respiratory tract infections and are now responsible for more than half of the cases of otitis media (OM) (1), a very common bacterial infection of the middle ear space in young children. OM may result in substantial sequelae including deafness and suppurative complications (2), significant antibiotic use (2), and OM with effusion (OME) (3), an important cause of transient, moderately severe hearing loss during a critical period for language development (4).

Although *Haemophilus influenzae* type b (Hib) vaccines have been highly effective in preventing invasive infections caused by *H. influenzae* that possess the type b capsule, they have no effect on preventing infections caused by *H. influenzae* that possess a non-b capsule or no capsule, i.e., NTHI. Identification of optimal vaccine candidates for NTHI has been challenging because of the high variability of their surface antigens; therefore, novel approaches to disease prevention are needed. Identification of genes that have been preserved by natural selection among NTHI strains causing respiratory infection but may not be essential for pharyngeal colonization provide new potential strategies to prevent NTHI infection.

Iron catalyzes many enzymatic reactions and thus is an essential element in all living organisms. *H. influenzae*, like many other bacteria, obtain iron that is sequestered in their human host in the form of iron-binding proteins, including heme, which is available as free heme, hemoglobin, ferritin, or the hemoglobin-haptoglobin, heme-hemopexin, and heme-albumin complexes (5). NTHI lack classic siderophores to competitively bind iron contained in host proteins (6). Rather, they have developed highly redundant mechanisms to obtain heme-sequestered iron from host sources

using TonB-dependent outer membrane proteins that function in heme uptake and as transporters through the periplasmic space and across the cytoplasmic membrane (7).

Given this absolute requirement of heme from their host, NTHI heme acquisition genes may play a significant role in avoiding host nutritional immunity and, thus, in determining virulence, as has been elucidated for *Neisseria* spp. (5), which also have an absolute requirement for an exogenous heme source. Heme acquisition through the heme-hemopexin utilization gene cluster (*hxCBA* [NTHI_RS01760, NTHI_RS01755, and NTHI_RS01750]) and from serum albumin through *hxC* has been shown to be important in sustaining bacteremia and mediating mortality in Hib-infected infant rats (8). However, the roles of other heme acquisition genes in human infection and disease

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TABLE 1 PCR primers used in this study

Target gene	Primer	Primer sequence	Expected amplicon size (bp)	Reference/source
<i>iga</i>	iga-F	GTTCCACCACCTGCGCCTGCTAC	812	14, 18
	iga-R	GTTATATTGCCCCCTCGTTATTCA		
<i>lgtC</i>	lgtC-F	CGGACTGTCAGTCAGACAAATG	840	14
	lgtC-R	CTCAAAATGATCATACCAAGATG		
<i>bexA</i>	HI-1	CGTTTGTATGATGTTGATCCAGAC	343	13
	HI-2	TGTCCATGTCCTTCAAAATGATG		
<i>bexB</i>	bexB-F	GGTGATTAACGCGTTGCTTATGCG	567	12
	bexB-R	TTGTGCCTGTGCTGGAAGGTTATG		
<i>hxA</i>	hxA-F	TCTCATCAGGGTTACACCTT	516	This study
	hxA-R	TTCAACATCTAAGCTACTCACTAA		
<i>hxB</i>	hxB-F	CTAATACAACAGTGAATACGTCCG	611	This study
	hxB-R	AATAAGATTCCAAGAACCGTCAA		
<i>hxC</i>	hxC-F	GAATTAGACTCTATCAACGT	505	This study
	hxC-R	CACTAATAAGAACATCGAATTTG		
<i>hemR</i>	hemR-F	CTTCTAATTCTAATAGAGAGGAGTT	875	This study
	hemR-R	GCTGAAGTGAAGTAGATAAGTTAAT		
<i>hup</i>	hup-F	ACGATTACACAAGCTCAAGC	602	This study
	hup-R	ACTTCACGTTGGCTATAACC		

remain less clear. To address this question, we employed an epidemiological approach to virulence gene identification by comparing the relative prevalences of several outer membrane heme acquisition genes, namely, the *hxA* gene cluster, *hemR* (NTHI_RS00945, which encodes a probable heme receptor and has been identified as more prevalent among OM isolates than among colonizing strains in our laboratory) (9, 10), and *hup* (NTHI_RS06565, which encodes a protein that utilizes heme from various sources) (11), among disease-causing NTHI to their prevalences among colonizing NTHI from the throats of healthy children.

In addition, we compared the prevalences of heme acquisition genes among colonizing NTHI and *H. haemolyticus*, a closely related, generally nonpathogenic species that also colonizes the human pharynx.

MATERIALS AND METHODS

Bacterial strains. Isolates previously collected under approval from the University of Michigan Institutional Review Board and identified as *H. influenzae* using standard microbiological techniques from the middle ear ($n = 534$) and throat ($n = 421$) were grown on chocolate agar plates (BD, Franklin Lakes, NJ) at 37°C with 5% CO₂. Strains were collected between 1980 and 2001 from middle ear samples of children with acute OM and throat samples from healthy children in the United States, Israel, and Finland. Additional throat strains, 63 of 421 samples, were obtained from adults in the United States within the same period. All strains were tested by PCR for the capsule region genes *bexA* and *bexB* as previously described (12, 13), and NTHI were defined as *bexA* and *bexB* negative. All strains were also tested by PCR for *lgtC* and *iga* as previously described (14). *H. haemolyticus* strains were defined as negative for *lgtC* and *iga*, while NTHI strains were defined as positive for *lgtC* and *iga*. Isolates with discordant *lgtC* and *iga* genes were classified as variant strains. Specimens from middle ears were considered disease isolates, and those from the throat were

considered commensal isolates. Controls comprised 10 *bexA*- and *bexB*-positive (typeable) *H. influenzae* strains, 12 NTHI strains whose genomes were fully or partially sequenced and contained no *bexA* or *bexB*, 6 NTHI biotype IV strains possessing variant P6 proteins (15), 1 *H. haemolyticus* strain (ATCC 33390), 1 *H. parainfluenzae* strain, and 1 *Pasteurella multocida* strain. DNA was extracted using the MasterPure DNA purification kit from Epicentre Biotechnologies (Madison, WI) and placed on hybridization slides using the library-on-a-slide technique as previously described (16).

Probe preparation. Fluorescein-labeled probes were prepared using PCR products obtained from strain 86-028NP (NTHI isolates from the nasopharynx of a child with chronic OME) as the template for heme acquisition genes (17), type b strain Eagan for the variable region of *iga*, and strain RdKW20 for *lgtC*; primer sequences, based on sequence data available in GenBank, were used to construct the probes and are described in Table 1. Positions of the heme acquisition genes studied in the 86-028NP chromosome and the corresponding probes are illustrated in Fig. 1. DNA was obtained from the bacterial isolates by using a Wizard genomic DNA purification kit from Promega Co. (Madison, WI). PCR amplification for heme acquisition genes was performed with 25-μl reaction mixtures consisting of 20 pmol of each relevant oligonucleotide primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μM (each) deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), 1 U of recombinant *Taq* DNA polymerase from New England BioLabs (Ipswich, MA), and 50 ng template. The mixture was subjected to 30 amplification cycles, including 30 s at 94°C for denaturation, 30 s at 55°C for primer annealing, and 1 min at 72°C for chain extension. For *lgtC*, *iga*, *bexA*, and *bexB*, PCR amplification was performed as previously described by our laboratory (12, 14). The sequences of PCR amplicons were confirmed by the University of Michigan DNA Sequencing Core, and the probes were validated by dot blot hybridization assay, using the control strains whose genomes had been sequenced (Table 2) and the same method implemented in the hybridization studies.

FIG 1 Position and size of heme acquisition genes and probes in 86-028NP. Dots and thick bars, 86-028NP chromosome; arrows, heme acquisition gene; small bars, *hxA* probe from 361298 to 361813, *hxB* probe from 357967 to 358577, *hxC* probe from 355320 to 355824, *hemR* probe from 186157 to 187031, *hup* probe from 1319679 to 1320280; *n.n.*, starting and ending base pair positions of the gene.

Hybridization studies. The library-on-a-slide technique enables entire bacterial genomes from multiple bacterial isolates to be arrayed on a single slide and then probed for the presence or absence of specific genes and/or their alleles (16). Each isolate used in this study was tested in duplicate on the array. Hybridization for heme acquisition genes, *iga*, *lgtC*, and capsule locus genes *bexA* and *bexB* was performed as previously described by our laboratory (18, 19).

PCR studies to determine gene presence in strains with indeterminate results on hybridization studies. Isolates with indeterminate results for heme acquisition genes by hybridization assay were tested by PCR as described above for presence of these genes. Primer sequences are included in Table 1.

Statistical analysis. Prevalence ratios were calculated for each strain type as the ratio of heme acquisition gene prevalence of NTHI middle ear strains compared to the heme acquisition gene prevalence of NTHI throat strains. Similar calculations were made comparing total NTHI strains, *H. haemolyticus* strains, and variant strains. Prevalence ratios, *P* values, odds ratios, and 95% confidence intervals (CI) were calculated using the online OpenEpi program (http://www.openepi.com/Menu/OE_Menu.htm). A *P* value of <0.05 was considered statistically significant.

RESULTS

Classification of NTHI and *H. haemolyticus* strains. Nineteen isolates (from a total of 955) were noted to have indeterminate results for *iga* or *lgtC* even after individual PCR studies were performed and were therefore excluded from analysis. Three additional isolates with indeterminate hybridization results were not available for PCR studies and were also excluded. Ninety-four strains isolated from the throats of healthy children had already been identified as *H. haemolyticus* based on a previous study from this laboratory (14). An additional 51 strains from throat samples and 3 strains from middle ear samples were identified as *H. haemolyticus* based on *iga* and *lgtC* hybridization results in this study, bringing the total number of *H. haemolyticus* strains tested to 148. Eleven strains from the middle ear and 25 from the throat were

designated variants due to discordance in the presence of their *iga* and *lgtC* genes, highlighting the variability of biomarkers used to distinguish *H. influenzae* from *H. haemolyticus*. Thus, a total of 749 NTHI strains were included in this study: 514 disease-causing strains from middle ear samples and 235 commensal strains from throat samples.

Genes present in NTHI strains compared to those in *H. haemolyticus* strains. All heme acquisition genes were significantly more prevalent in NTHI strains than in *H. haemolyticus* strains (Table 3). NTHI strains were 10.3 times more likely than *H. haemolyticus* strains to have the *hxA* gene, 8.5 times more likely to have the *hxB* and *hxC* genes, 4.6 times more likely to have the *hemR* gene, and 2.9 times more likely to have the *hup* gene. Compared to variant strains, NTHI strains were more likely and *H. haemolyticus* were less likely to hybridize with the probes of the heme acquisition genes examined in this study. The difference remains significant even if *H. haemolyticus* and variant strains are collapsed into a single non-NTHI group (data not shown).

The hybridization results from the control strains were consistent with those of the validation dot blot assay for the five heme acquisition genes studied (data not shown). PCR analysis to clarify indeterminate hybridization results was only needed in a small portion of total samples (19 to 32 isolates for each gene examined).

Genes present in NTHI OM strains compared to NTHI throat strains. With the exception of *hup*, the heme acquisition genes were significantly more prevalent in NTHI OM strains than in NTHI throat strains (Table 4). Notably, OM strains were 1.188 times more likely to have the *hxA* gene and 1.14 times more likely to have the *hemR* gene than were throat strains.

The small number (3) of *H. haemolyticus* isolates among the OM strains precluded a statistical comparison between *H. haemolyticus* OM strains and throat strains. However, the significant differences in heme acquisition gene prevalences remained when OM variant strains were compared to throat variant strains (Table 5), such that, considering the presence of the iron acquisition genes under study, the variant OM strains looked more like NTHI strains and the variant throat strains looked more like *H. haemolyticus* strains.

Concordance of genes. Most strains that contained the *hxA* gene cluster contained all of the studied genes, and NTHI throat isolates were more likely than NTHI OM isolates to have an in-

TABLE 2 Strains used in dot blot protocol to validate probes for heme acquisition genes

Strain	Source
86-028NP ^a	Nasopharynx of a child with otitis media with effusion
PittAA, ^a PittEE, ^a PittHH, ^a PittII ^a	Middle ear effusion of children with chronic otitis media
PittGG ^a	External ear discharge of a child with otorrhea
RdKW20 ^a	Encapsulated type-d strain missing the capsule-encoding genes
R2846 ^a	Middle ear fluid of a child with acute otitis media
R2866 ^a	Blood of a child with meningitis
2.21-21, ^a 2.24-21 ^a	Nasopharynx of healthy children
<i>H. haemolyticus</i>	ATCC 33390
<i>H. parainfluenzae</i>	Laboratory collection
<i>Pasteurella multocida</i>	Laboratory collection
(negative control)	

^a Strains confirmed for heme acquisition genes by DNA sequence analysis in GenBank.

TABLE 3 Heme acquisition gene prevalence in NTHI, *H. haemolyticus*, and variant strains

Gene	NTHI (<i>n</i> = 749)		<i>H. haemolyticus</i> (<i>n</i> = 148)		NTHI/ <i>H. haemolyticus</i> prevalence ratio ^a (95% CI)	Variant (<i>n</i> = 36)		NTHI/variant prevalence ratio ^a (95% CI)
	No.	%	No.	%		No.	%	
<i>hxA</i>	680	91	13	9	10.34 (6.15–17.38)	11	31	2.97 (1.82–4.86)
<i>hxB</i>	731	98	17	11	8.50 (5.43–13.29)	15	42	2.34 (1.59–3.45)
<i>hxC</i>	733	98	17	11	8.52 (5.48–13.33)	15	42	2.35 (1.60–3.46)
<i>hemR</i>	696	93	30	20	4.58 (3.33–6.31)	17	47	1.97 (1.39–2.78)
<i>hup</i>	726	97	50	34	2.87 (2.29–3.60)	16	44	2.18 (1.51–3.14)

^a *P* < 0.0001.

complete cluster. However, 79.6% of NTHI throat strains contained the complete *hxA* gene cluster, while 90.3% of *H. haemolyticus* strains did not have the cluster at all (Table 6). When a strain had an incomplete *hxCBA* gene cluster, the *hxA* gene was most likely to be missing.

DISCUSSION

As an essential element in numerous metabolic pathways, iron is required by bacteria to survive. High-affinity iron uptake systems are important in the pathogenesis of various bacteria (20). Because free iron is toxic, extracellular iron in humans is incorporated into heme in the form of free heme or heme-containing proteins (5, 21, 22) or is sequestered in iron-binding proteins. Therefore, bacteria must employ sophisticated mechanisms to acquire iron, especially heme, from those sources in their hosts. A common mechanism used by many bacteria is to secrete proteins that chelate iron or heme from the extracellular environment (21). Such proteins, siderophores, are low-molecular-weight iron ligands that, once they are loaded with iron, bind to receptors on the bacterial surface and are taken up through the outer membrane (22). Hemophores function similarly to siderophores by taking up free heme or extracting heme from hemoproteins in the extracellular medium and delivering it to a TonB-dependent hemophore-specific outer membrane receptor. A less common mechanism for obtaining iron is direct binding of heme or heme-containing proteins to specific outer membrane receptors (21, 22). Bacteria can also steal iron from their hosts in the form of nonheme proteins, such as transferrin, lactoferrin, or ferritin, using specific outer membrane receptors.

H. influenzae do not secrete siderophores, although they have been shown to possess at least one siderophore receptor, suggesting that *H. influenzae* scavenge iron using siderophores produced by other bacteria in the pharynx (6). *HxA* has been suggested as

part of a hemophore system in *H. influenzae* as it was proposed to be released from the bacterial outer membrane by *HxB* in a heme-hemopexin utilization process with *HxC* as its receptor (8, 21, 23). While *HxA* and *HxB* are involved only in heme-hemopexin utilization, *HxC* is thought to also play a role in heme acquisition in the form of free heme and heme-human serum albumin. Several other TonB-dependent outer membrane proteins play a role in acquiring heme either as free heme or in the form of heme-containing proteins, including *HemR* and *Hup*, which were examined in this study. *HemR* acquires heme in its free form, while *Hup* can obtain it as either hemoglobin or its free form (7, 11). In addition to heme or heme-containing proteins, *H. influenzae* strains possess outer membrane proteins to acquire iron in nonheme forms, such as transferrin by the transferrin binding protein system (5, 7).

The sequences of the five heme acquisition genes examined in this study (*hxA*, *hxB*, *hxC*, *hemR*, and *hup*) were shown to be highly conserved in *H. influenzae* control strains (Table 2). Specifically for control strains with completely sequenced genomes (86-028NP, RdKW20, PittEE, PittGG, R2846, and R2866), the sequences have 88% to 97% similarity over 94% to 100% of the length of the studied genes in 86-028NP, according to BLAST nucleotide analysis. Therefore, we do not expect the results of this study to be distorted by strain heterogeneity of these gene sequences.

In this study, four of the five heme acquisition genes were more prevalent among NTHI OM isolates than among NTHI commensal isolates. This is consistent with previous reports from our laboratory of increased *hemR* (NTHI_RS00945) and *hgpB* prevalence in NTHI isolated from the middle ears of children with acute OM compared to those isolated from the throats of healthy children (10). While a clear illustration of heme/iron availability and sources in the middle ear space dur-

TABLE 4 Heme acquisition gene prevalence in NTHI OM strains compared to NTHI throat strains

Gene	NTHI OM strains (<i>n</i> = 514)		NTHI throat strains (<i>n</i> = 235)		Prevalence ratio (95% CI)	<i>P</i> value
	No.	%	No.	%		
<i>hxA</i>	491	96	189	80	1.19 (1.11–1.27)	<0.0001
<i>hxB</i>	514	100	217	92	1.09 (1.05–1.13)	<0.0001
<i>hxC</i>	514	100	219	93	1.08 (1.04–1.11)	<0.0001
<i>hemR</i>	497	97	199	85	1.14 (1.08–1.21)	<0.0001
<i>hup</i>	500	97	226	96	1.01 (0.98–1.04)	0.5469

TABLE 5 Heme acquisition gene prevalence in variant strains in OM compared to throat colonization

Gene	OM variant strains (<i>n</i> = 11)		Throat variant strains (<i>n</i> = 25)		Prevalence ratio (95% CI)	<i>P</i> value
	No.	%	No.	%		
<i>hxA</i>	8	73	3	12	6.06 (1.98–18.60)	0.0013
<i>hxB</i>	11	100	4	16	4.99 (2.23–11.17)	<0.0001
<i>hxC</i>	11	100	4	16	4.99 (2.23–11.17)	<0.0001
<i>hemR</i>	10	91	7	28	3.25 (1.69–6.26)	0.0013
<i>hup</i>	10	91	6	24	3.79 (1.84–7.80)	0.0005

TABLE 6 Concordance of heme acquisition genes

No. of genes ^a or type	No. (%) of NTHI strains in:		No. (%) of <i>H. haemolyticus</i> strains in throat (n = 145)
	Middle ear (n = 514)	Throat (n = 235)	
0	0	1 (0.4)	92 (63.4)
1	0	7 (3.0)	29 (20)
2	4 (0.8)	9 (3.8)	10 (6.9)
3	7 (1.4)	17 (7.2)	1 (0.7)
4	28 (5.4)	31 (13.2)	3 (2.1)
5	475 (92.4)	170 (72.3)	10 (6.9)
No <i>hxu</i>	0	16 (6.8)	131 (90.3)
<i>hxA</i> only	0	0	0
<i>hxB</i> only	0	0	0
<i>hxC</i> only	0	0	0
<i>hxA</i> and <i>hxB</i> only	0	0	0
<i>hxA</i> and <i>hxC</i> only	0	2 (0.9)	0
<i>hxB</i> and <i>hxC</i> only	23 (4.5)	30 (12.8)	2 (1.4)
All <i>hxu</i>	491 (95.5)	187 (79.6)	12 (8.3)

^a Genes studied: *hxA*, *hxB*, *hxC*, *hemR*, and *hup*.

ing OM has not been delineated, OM effusion was shown to derive predominantly from serum transudates and refluxed nasal secretions (24). Hemopexin, haptoglobin, and human serum albumin are among the proteins known as heme transportation vehicles in the serum (21). Mucosal surfaces have also been shown to contain free heme (22). Therefore, it can be assumed that heme/iron from those sources would be available to NTHI strains in the middle ear space (24). In addition, inorganic iron complexed to lactoferrin is present on mucosal surfaces and in fluids in the infected middle ear space (22, 25). Nevertheless, the increased prevalence of heme acquisition genes among NTHI strains isolated from the middle ear described in this study suggests that these genes confer a selective advantage to survival in the middle ear space.

Interestingly, *hxB* and *hxC* were present in all middle ear NTHI strains, while *hxA* (the third member of the *hxCBA* gene cluster) was absent in 4.5% of those strains. This finding may be explained by the role of proteins encoded by these genes. HxC is a transmembrane transport channel necessary for the acquisition of heme from both heme-hemopexin and heme-albumin complexes and is one of several possible transport channels for free heme (which is present in very low levels in humans) (8). Thus, it is expected to be consistently present as a part of a universal high-affinity heme uptake pathway. *hxA* and *hxB*, on the other hand, encode a two-subunit secretion system in which HxA is exported across the outer membrane by HxB to actively acquire only exogenous heme-hemopexin to be transported intracellularly through HxC. Therefore, it is possible that the roles of HxA and HxB can be accomplished by proteins that acquire heme in different complexes to be transported through HxC. However, this explanation does not explain the similarity of *hxB* and *hxC* prevalence among NTHI strains, especially disease strains, as shown in this study. Moreover, Fournier et al. (23) found that HxA is indispensable for the acquisition of heme from the heme-hemopexin complex, while HxB is not essential in this process. Since HxA sequences are the least conserved of the *hxCBA* proteins (8), specificity of the probes used in this study may provide an explanation for this result.

One possible explanation for the apparent concurrence of the *hxu* genes among OM strains is that they are located close to each other on the NTHI chromosome. On the other hand, *hemR* and *hup* are relatively distant from the *hxCBA* gene cluster. Therefore, the higher prevalence of *hemR* cannot be attributed to the proximity of its position to other heme acquisition genes.

Although numerous phylogenetic studies, including one from our laboratory (14), have clearly validated the separation of *H. influenzae* and *H. haemolyticus*, these two species may be on an evolutionary continuum, which might explain the difficulty in finding one biomarker gene to distinguish them for taxonomic purposes (14, 26). Strains whose genetic makeup places them intermediately between NTHI and *H. haemolyticus* likely exist. Thus, we expected that a portion of study isolates previously designated NTHI might, in fact, be *H. haemolyticus* and another portion might be variants. Using the presence or absence of *iga* and *lgtc*, we classified the strains used in this study as NTHI, *H. haemolyticus*, or variant. Since we hypothesized that NTHI strains from disease isolates would have a higher prevalence of heme acquisition genes than those from commensal isolates, we proposed that the gene prevalences would differ between NTHI and *H. haemolyticus* in a similar manner, considering the nature of *H. haemolyticus* strains as commensals.

In light of their close phylogenetic relationship and their role as commensals in the human pharynx, the dramatic differences in the prevalences of all five heme acquisition genes studied in NTHI and *H. haemolyticus* isolates are surprising. While >90% of NTHI strains contained the five genes assessed in this study, <12% of *H. haemolyticus* isolates contained the *hxCBA* cluster genes, 15% contained *hemR*, and 34% contained *hup*. When only commensal NTHI strains were compared to *H. haemolyticus* strains, the significant differences remained.

The explanation for these large differences in prevalence is unclear, but because *H. haemolyticus* strains have the same heme requirements as NTHI strains, presumably *H. haemolyticus* strains possess additional heme/iron acquisition proteins that we did not assess, such as the hemoglobin-haptoglobin binding proteins, hypothetical TonB-dependent outer membrane proteins that are paralogs to several NTHI heme/hemoglobin binding proteins (including *hxC*), transferrin binding protein, or other less-studied heme-iron acquisition proteins of *Haemophilus*.

Interestingly, among both *H. haemolyticus* and commensal NTHI strains, *hup* was the most prevalent of the five heme acquisition genes examined. *Hup*, the transmembrane protein encoded by *hup*, acquires heme from hemoglobin complex or free heme (7, 11). Both *H. haemolyticus* and commensal NTHI strains live in pharynx, and these results may indicate that heme in the form of hemoglobin and/or free heme is more readily available than other heme complexes in the throat, whereas other iron acquisition genes may play a larger role in the middle ear space.

The significant differences in heme acquisition genes between *H. influenzae* and *H. haemolyticus* demonstrated in this study indicate a potential use for these genes as biomarkers for distinguishing *H. influenzae* from *H. haemolyticus*, recognizing that the ability of *Haemophilus* of human origin to exchange genes by DNA uptake and homologous recombination results in heterogeneity of these species (14), with overlap in their taxonomy based on the genes they possess.

The prevalences of heme acquisition genes in variant strains in this study support the notion that NTHI and *H. haemolyticus*

might actually exist in an evolutionary continuum (26). The variant strains showed a lower prevalence than the NTHI strains of heme acquisition genes but a higher prevalence than found for *H. haemolyticus* strains. More interestingly, the difference in heme acquisition gene prevalences was statistically significant when we grouped variant strains into disease isolates (isolated from the middle ear) and commensals (isolated from the throat), supporting our hypothesis that these genes play a role in virulence.

In this study, we observed for the first time three strains isolated from the middle ears of children with acute OM that exhibit the *H. haemolyticus* genotype (absence of *lgtC* and *iga*). Furthermore, two of the strains had four heme acquisition genes (*hxB*, *hxC*, *hemR*, and *hup*) and one had all five. Strains exhibiting the *H. haemolyticus* genotype by a somewhat different taxonomic definition were described in invasive infections (27). Thus, these results expand our understanding of the pathogenicity of *H. haemolyticus* in humans and may indicate that the three *H. haemolyticus* OM isolates were able to cause disease, since they possessed a larger number of heme acquisition genes.

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