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Characterization of Genetic and Phenotypic Diversity of Invasive Nontypeable *Haemophilus influenzae*

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The ability of unencapsulated (nontypeable) *Haemophilus influenzae* (NTHi) to cause systemic disease in healthy children has been recognized only in the past decade. To determine the extent of similarity among invasive nontypeable isolates, we compared strain R2866 with 16 additional NTHi isolates from blood and spinal fluid, 17 nasopharyngeal or throat isolates from healthy children, and 19 isolates from middle ear aspirates. The strains were evaluated for the presence of several genetic loci that affect bacterial surface structures and for biochemical reactions that are known to differ among *H. influenzae* strains. Eight strains, including four blood isolates, shared several properties with R2866: they were biotype V (indole and ornithine decarboxylase positive, urease negative), contained sequence from the adhesin gene *hia*, and lacked a genetic island flanked by the *infA* and *ksgA* genes. Multilocus sequence typing showed that most biotype V isolates belonged to the same phylogenetic cluster as strain R2866. When present, the *infA-ksgA* island contains lipopolysaccharide biosynthetic genes, either *lic2B* and *lic2C* or homologs of the *losA* and *losB* genes described for *Haemophilus ducreyi*. The island was found in most nasopharyngeal and otitis isolates but was absent from 40% of invasive isolates. Overall, the 33 *hmw*-negative isolates were much more likely than *hmw*-containing isolates to have tryptophanase, ornithine decarboxylase, or lysine decarboxylase activity or to contain the *hif* genes. We conclude (i) that invasive isolates are genetically and phenotypically diverse and (ii) that certain genetic loci of NTHi are frequently found in association among NTHi strains.

Haemophilus influenzae is a small, fastidious gram-negative coccobacillus that colonizes the human nasopharynx, usually without causing symptoms. When disease occurs, it is usually limited to local infections of the respiratory tract such as otitis media in young children, bronchitis or sinusitis in adults, or pneumonia secondary to chronic pulmonary disease. Invasive disease such as bacteremia or meningitis is currently uncommon in the developed world and is nearly always associated with strains possessing polysaccharide capsules. The six serotypes of *H. influenzae* have capsules with different carbohydrate structures, with serotype b being the most common. Since the introduction of conjugate vaccines against the type b capsular polysaccharide, the incidence of life-threatening *H. influenzae* infection has decreased substantially. As expected, the vaccine has not affected the incidence of infections due to unencapsulated (nontypeable) *H. influenzae* (NTHi). It was once thought that invasive NTHi disease occurred only in children with immunologic or anatomical defects that predispose them to bacterial infections. More recently it has become apparent that NTHi can cause bacteremia and meningitis in otherwise healthy children. One of the first well-documented cases of invasive NTHi was reported in 1996 (33), and since then there have been several publications describing additional cases (5, 6, 8, 34). It is not known whether NTHi isolates from invasive infections possess virulence determinants not shared with isolates associated with otitis media or isolates asymptomatically colonizing the nasopharynx. It is important to determine whether NTHi strains isolated from invasive infections represent distinct lineages or have novel virulence genes not shared with commensals or isolates from localized infections.

The genetic diversity of encapsulated *H. influenzae* and NTHi has been studied using ribotyping, restriction fragment length polymorphism (RFLP), multilocus enzyme electrophoresis, and multilocus sequence typing (MLST) (4, 26, 32, 43). Phylogenetic trees generated using these methods have

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shown that whereas encapsulated isolates cluster by serotype, NTHi isolates are much more diverse, with less evidence of clonality (26, 32). The extent to which disease isolates of NTHi are phylogenetically similar has not been studied extensively. van Alphen et al. reported that isolates from acute disease (otitis media or meningitis) are less phylogenetically diverse than isolates from chronic infection (cystic fibrosis or chronic bronchitis) (43). Classically, *H. influenzae* strains were grouped into "biotypes" by biochemical assays. Kilian (23) described a system for biotyping *H. influenzae* based on the presence or absence of three enzymatic activities: urease, ornithine decarboxylase, and the production of indole from tryptophan. Biotyping was useful because of weak correlations with pathogenicity. Serotype b strains, particularly those isolated from systemic disease, usually possess all three activities, making them biotype I; conjunctival isolates are usually biotype III and urogenital isolates type IV. Mucosal isolates, whether from healthy children or associated with disease, are more diverse in biotype (23). Indole production, associated with a tryptophanase (*tna*) gene cluster and with several biotypes, is more frequent in isolates from mucosal and invasive disease than in commensals or in conjunctivitis (25).

Respiratory tract isolates differ in the fermentation of fructose, maltose, and xylose (22, 41). The API 20E system, developed for testing multiple biochemical reactions in clinical isolates of *Enterobacteriaceae*, has shown several additional activities to be present in some *H. influenzae* strains but not in others, including arginine dihydrolase and lysine decarboxylase activities and arabinose fermentation (18). For NTHi, none of these biochemical activities has been shown to be strongly associated with a specific disease or with asymptomatic carriage, though it has been noted that lower respiratory tract isolates from patients with cystic fibrosis are more likely to decarboxylate lysine than are other isolates (17).

Surface molecules of both NTHi strains and encapsulated *H. influenzae* strains have been shown to be heterogeneous in a variety of ways that may affect pathogenesis. The major outer membrane proteins known as P1, P2, and P5 are particularly diverse in sequence (2). Several genes involved in lipopolysaccharide biosynthesis are variably present in *H. influenzae* strains, and some of these are phase variable. The resulting heterogeneity of lipopolysaccharide structure affects susceptibility to complement-mediated serum killing and the interaction of bacteria with host cells (40). *H. influenzae* isolates also differ in protein adhesins: most contain genes for one of the adhesins known as Hia and HMW, but not both, and some strains are also able to synthesize fimbriae (38). A recent study reported that otitis isolates are more likely than throat isolates to contain *hmw* genes, while throat isolates were more likely than isolates from blood or the middle ear to hybridize with a probe for the *hifBC* genes (required for synthesis of fimbriae) (10).

We undertook these studies to determine whether isolates of NTHi associated with invasive disease differ phenotypically or genetically from isolates cultured from healthy children or associated with acute otitis media. We previously reported that the invasive NTHi strain Int1 (later R2866) differs from most NTHi strains in being unusually resistant to the bactericidal activity of normal adult human serum (49). We have also found that strain R2866 contains several genetic loci that are absent from the sequenced strain Rd KW20 and from several nontypeable strains. These include the gene for an autotransporter, *lav*, located between *holB* and *tmk* (9), the lysogenic bacteriophage HP2 (48), the *tna* cluster (25), and a 53-kb plasmid similar to other large integrative plasmids of *Haemophilus* spp. (28). In the present work a collection of 17 NTHi isolates associated with invasive disease were compared with isolates from healthy children or associated with acute otitis media in order to determine whether invasive isolates are genetically or phylogenetically distinct. We assayed the susceptibility of each strain to complement-mediated killing by pooled normal human serum and tested each in a panel of biochemical assays. In addition, we used PCR to screen for the presence of genes for HP2, Lav, fimbriae, and the adhesins Hia and HMW, the 53-kb plasmid, and the lipopolysaccharide biosynthetic locus flanked by *infA* and *ksgA*. Invasive strains were genetically heterogeneous and did not differ significantly in the range of serum sensitivity from nasopharyngeal commensals or otitis media isolates. However, several genetic characteristics are not assorted randomly but are strongly associated with one another. In particular, several biochemical and genetic traits were found primarily in strains lacking genes for the *hmw* adhesin. Within the *hmw*-negative group we identified a cluster of eight isolates that resembled R2866 in being biotype V and *hia* positive, lacking an *infA-ksgA* island, and having the urease locus replaced by a homolog of the gonococcal membrane protein *mtrF*. These strains were shown by MLST to be members of a phylogenetic cluster.

MATERIALS AND METHODS

Bacterial strains and growth. *H. influenzae* strains are listed in Table 1. We studied three types of isolates: nasopharyngeal or throat isolates from healthy children, strains cultured from middle ear aspirates from children with otitis media, and blood or cerebrospinal fluid isolates obtained from children with invasive disease. For simplicity, isolates are referred to below as "throat," "otitis," or "invasive." Strains were isolated between 1995 and 2004, at different locations within the United States, Canada, and Europe. For invasive isolates, we limited the study to isolates from children who were considered by the treating physician to have normal anatomy and immune function. Neonates were excluded. Otitis isolates included the recently sequenced strains 86-028NP (31) and R2846 (strain 12). Invasive isolates included R2866 (Int1), which has also been sequenced recently. Bacteria were cultivated at 37°C on chocolate agar supplemented with 1% IsovitaleX (Becton Dickinson and Co., Sparks, MD) or in Difco brain heart infusion broth (Becton Dickinson and Co.) supplemented (sBHI) with hemin (10 μg/ml) and β-NAD (10 μg/ml) and with agar for solid sBHI plates. The requirement of all strains for X and V factors was confirmed by disk diffusion. All strains were negative for *bexA* by PCR, indicating the inability to express capsular polysaccharide. The serotype b strain Eagan was used as a positive control for *bexA* PCR.

Biochemical assays. Bacteria were suspended at 10^8 CFU/ml in 0.9% NaCl containing hemin (10 μg/ml) and β-NAD (10 μg/ml) and were used to inoculate API 20E biochemical test strips purchased from bioMerieux (St. Louis, MO) (19). Enzyme activities and fermentation reactions were recorded after 24 h of incubation at 37°C in air. Indole, ornithine decarboxylase, and urease activities were used for biotyping (23).

Screen for genetic markers by PCR. Genomic DNA was prepared using the DNeasy tissue kit (QIAGEN, Inc., Valencia, CA). Primers (Table 2) were synthesized by Integrated DNA Technologies, Coralville, IA. For *lav*, we amplified across the *holB-tmk* junction (HI0455 to HI0456 in Rd KW20). Positive strains yielded a product of 2 to 2.2 kb. Partial sequencing confirmed that all were related to the published sequence of *lav*. The remaining isolates did not yield an amplicon or else produced a 150-bp product consistent with no inserted gene. For detection of bacteriophage HP2, we used PCR to amplify regions of *rep* (common to both phage HP1 and HP2) and *orf10* (specific for HP2). For the plasmid, PCR with the primers listed as topoF and topoR gave a product of about 450 bp, and primers AF and AR gave a product of about 500 bp. We used

TABLE 1. Bacterial strains

Clinical source and strain no. (previous strain designation)	Yr isolated; source[a] (reference)
Nasopharyngeal	
C500	1978; CHMC, Boston, MA
R3019 (A840162), R3020 (A930066), R3021 (A950077), R3022 (A950078), R3023 (A950084), R3024 (A950085)	1984–1995; L. van Alphen, Amsterdam, The Netherlands
R3261 (32), R3262 (11), R3263 (18), R3264 (153), R3265 (510)	1996–1997; J. Campos, Madrid, Spain
R3266 (K42-44), R3367 (K35-48), R3368 (K29-43), R3369 (K56-132), R3370 (K157-51)	1972–1982; F. Henderson, University of North Carolina
Middle ear	
C68, C69, C447, C464, C470, C476, C486, C505, C507, C544, C1049, C1344, C1626	1977–1984; CHMC, Boston, MA, and CHRMC, Seattle, WA
R2846 (strain 12)	S. J. Barenkamp, St. Louis, MO (3)
R3140 (LKP1 P860295), R3141 (LKP4 P861249), R3142 (LKP5 P861384)	B. A. Green, Wyeth Vaccines
R3157 (1128)	L. O. Bakaletz, Columbus, OH (37)
R3642 (86-028NP)	R. S. Munson, Columbus, OH (1, 31)
Invasive—set 1[b]	
C2965 (2)	1995; K. Muhlemann (29)
R2866 (Int1)	1996; CHMC, Seattle, WA (33)
R3565	1999; M. M. Farley, Atlanta, GA (8)
R3566 (GA14939), R3567 (GA16143), R3568 (GA16385), R3569 (GA18074), R3570 (GA18390), R3571 (GA18503)	2000–2001; M. M. Farley, Atlanta, GA[c]
R3572 (765), R3577 (774), R3579 (776), R3583 (784), R3588 (789), R3590 (794), R3601 (772)	1993–2000; R. F. Jacobs, Little Rock, AR (34)
R3631	2004; R. F. Jacobs, Little Rock, AR
Invasive—set 2	
R3595 (799), R3596 (800)	1993–2000; R. F. Jacobs, Little Rock, AR (34)
R3169 (15), R3170 (52), R3171 (129), R3172 (134), R3173 (253), R3174 (330), R3175 (378), R3176 (447), R3177 (455)	1995; K. Muhlemann (29)
R3042 (Z1082), R3046 (Z1030), R3049 (Z202), R3050 (Z343), R3052 (Z1210), R3055 (Z378), R3060 (Z241), R3064 (Z960)	1995–1996; C. Shaw, Vancouver, BC, Canada
Bronchitis[d]	
R3101 (6173)	L. van Alphen, Amsterdam, The Netherlands (12)

[a] CHMC, Children's Hospital and Medical Center; CHRMC, Children's Hospital and Regional Medical Center; BC, British Columbia.

[b] The 17 isolates listed as "Invasive—set 1" were used for the data presented in Fig. 1 and in Tables 3, 4, and 5. The 19 isolates listed as "Invasive—set 2" were used only for the data reported in the section of Results describing the *infA-ksgA* island.

[c] Isolates R3566 to R3571 were collected as part of the Active Bacterial Core Surveillance of the Georgia Emerging Infections Program, funded by the Centers for Disease Control and Prevention.

[d] R3101 was isolated from a patient with chronic bronchitis. Data on this isolate are included in Table 3 and in the section of Results describing PstI restriction patterns of the *hif* locus.

primers designed to amplify conserved regions of the adhesin genes *hmwA* and *hia* (36, 44). PCR with the *hmw* primers gave a product of approximately 1.2 kb. Occasional faint products were sequenced and were scored negative if the sequences were not related to *hmwA*. *hia* products were usually 3 to 4 kb but ranged from approximately 0.75 kb to 10 kb. The basis of this size difference was not evaluated. Ten of the *hia* amplicons were sequenced and found to be similar to the published *hia* sequence. Of four strains yielding *hia* products of only 0.75 kb, two were sequenced and found to be homologous to the 3′ end of the *hia* gene. All of these *hia*-related sequences were scored as positive for *hia*. The presence and size of the *hif* locus, required for synthesis of fimbriae, was evaluated by amplification across the *purE-pepN* junction. Isolates with a complete *hif* locus produced a product of approximately 7 kb. These PCR fragments were sequenced using the *purE* primer to detect the presence of *hicA* and *hicB* in addition to the *hif* genes. As seen previously (27), the remaining isolates contained either a shorter *purE-pepN* insert, reflected by a product of approximately 0.5 to 1 kb (presumed to include *hicAB*), or no insert, reflected by a PCR product of approximately 250 bp. The genetic island flanked by *infA* and *ksgA* was detected by PCR across the *infA-ksgA* junction. The contents of the 2.2-kb island were determined either by sequencing or by PCR using primers *infA* and *losB*-R to detect *losAB* and primers *lic2B*-F and *lic2B*A to detect *lic2BC*. Routine PCR was carried out using the Biolase DNA polymerase (Bioline USA, Randolph, MA), with the following amplification protocol: reaction tubes were incubated at 94°C for 2 min, then for 36 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min per kb of predicted product. The Expand Long Template PCR system (Roche Diagnostics Company, Indianapolis, IN) was used as directed for amplification of the *hif* and *hia* loci. Following electrophoresis, ethidium bromide-stained agarose gels were visualized and photographed using the Fluorochem 8900 digital imaging system (Alpha Innotech Corporation, San Leandro, CA). PCR products to be sequenced were processed using the Qiaquick PCR purification kit (QIAGEN, Inc.) and submitted to the core sequencing facility at Seattle Biomedical Research Institute. Sequences were aligned using the BioEdit Sequence Alignment Editor (Isis Pharmaceuticals, Inc., Carlsbad, CA).

MLST. PCR for the housekeeping genes *adk*, *atpG*, *adk*, *atpG*, *frdB*, *fucK*, *mdh*, *pgi*, and *recA* was carried out using the primers and methods described in reference 26. Sequences were submitted to the MLST website (www.mlst.net) for allele and sequence type (ST) assignment.

Serum bactericidal activity. Log-phase bacteria (2,000 CFU/ml) were incubated for 30 min at 37°C with pooled normal human serum diluted in 10 mM phosphate-buffered saline containing 4 mM KCl and 0.1% gelatin and were then plated to determine bacterial survival. The concentration of serum that killed 50% of bacteria was calculated using XLfit 4.1 (ID Business Solutions, Guildford, United Kingdom) and is referred to as the IC_{50} of the serum for that strain.

Statistics. Groups were compared using the Fisher exact probability test. Calculations were performed online using VassarStats: Web Site for Statistical Computation (http://faculty.vassar.edu/lowry/VassarStats.html).

Sequence data. Sequences for the genomes of *H. influenzae* strains Rd KW20, R2846, R2866, and 86-028NP and of *Neisseria gonorrhoeae* FA1090 and *Neisseria meningitidis* strains MC58 and Z2491 were accessed through the Microbial Genomes pages at the website of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). The sequences

TABLE 2. PCR primers

Genetic locus: region amplified (reference)	Forward primer	Reverse primer
bexA (this study)	5′-ATGATTCGCGTAAATAATGTATGTAAGAAG-3′	5′-TTGCGTCTCGTTGTAGTATTGATAC-3′
Integrative plasmid (this study)		
topoF and topoR	5′-GAGACTCACAAAGCGACAACC-3′	5′-GGCTTGAGGTGCGTCATCATCTTC-3′
primers AF and AR	5′-CGGTTCCAGTGTTATATTCACG-3′	5′-GAATGTGATCGGTGAGAAGC-3′
Bacteriophage HP2 (48)		
rep, HP2p10	5′-AAGAAATGGTTGGGGGTGA-3′	5′-TTTGCCGCTAAGATTCGGCT-3′
orf10, HP2p11	5′-GCCGAATCTTAGCAGCAAAA-3′	5′-GACCCACTACACCACCGATAA-3′
Autotransporter Lav: *holB- tmk* junction (9)	5′-CTTTAATCAGCACAGCATGATGCC-3′	5′-TGTTCAAGCTGATATTGAAAGTGC-3′
hif locus: *purE-pepN* junction (27)	5′-GACCCCATCACAACGCGAATTTGTG-3′	5′-CTGTGACCGTAAAATCTGGTTGTTTGTAATC-3′
Adhesin Hia (36)	5′-TAAATTGCCGTTCCCTTTTGCCTAAAACCTGCTT-3′	5′-CCAAACTTTACCACTGGTAACCAACACCAGCTGC-3′
Adhesin HMW (44)	5′-GCGTCGACGAGGGAGCTGAACGAACG-3′	5′-GCCCCACACAATAGCGCG-3′
infA-ksgA genetic island (this study)		
infA-ksgA junction (this study)	5′-TATGTGCAGTAACCACGTGACCGT-3′	5′-TCGACAAGTTCACCAACAGGCTCT-3′
lic2B-F (35), *lic2BA* (20)	5′-TAAGTATGATCCTCAAATGCAT-3′	5′-CAATTTAGCGATGAGTTCC-3′
losB-R (this study)		5′-GGTTGAGCCATCTTGTGGAATGTC-3′
Urease/MtrF: *aspA-groEL* (this study)	5′-TAGTTGTACCGTCACCTGCTGCAT-3′	5′-CAACAGTCATTGGCACCGCATCTT-3′

of the *mtrF* (strain R2866) and *losAB* (strain R2846) loci were submitted to NCBI and were assigned accession numbers DQ007025 and DQ007026, respectively.

RESULTS

Biotyping and other biochemical characterization. For *H. influenzae* type b, invasive isolates nearly always belong to biotype I (positive for all three of the biotyping reactions), with biotype II (negative for ornithine decarboxylase only) seen occasionally (14). In contrast, of the invasive NTHi isolates studied here, only one-third belonged to biotype I and about one-fourth belonged to biotype V (negative for urease only) (Table 3). We noted heterogeneity among strains in several other biochemical reactions. Arginine dihydrolase and arabinose fermentation were seen predominantly for throat isolates, while lysine decarboxylase and growth on citrate were seen less often for middle ear isolates than for the other two groups (Table 3). In most cases these correlations were not statistically significant.

Correlation of the *hmwA* gene with biochemical reactions and genes for other surface molecules. We evaluated strains for the presence of genes encoding the HMW adhesins by PCR amplification of a conserved region of *hmw1A* and *hmw2A*. The results correlated strongly with the biochemical reactions discussed above and with the presence of genes encoding other surface molecules (Table 3). *hmw*-negative isolates were much more likely than *hmw*-positive isolates to be positive for indole, ornithine decarboxylase, lysine decarboxylase, and arginine dihydrolase activities, to be negative for urease activity, and to ferment arabinose and grow on citrate. *hmw*-negative isolates were nearly always positive for *hia*-related sequences, as reported previously (39). One isolate, R3579, contained sequences for both *hmw* and *hia*. This result was confirmed by sequencing the products and by carrying out PCR on five separate colonies. Each of the five colonies yielded both a *hia* product (approximately 3 kb) and a *hmw* product, indicating that the culture was not a mixture of a *hia*-positive and a *hmw*-positive strain. We used PCR to determine the presence and size of the fimbrial gene cluster flanked by *purE* and *pepN*. We found that 11 isolates contained a 7-kb *purE-pepN* insert consistent with a complete fimbrial biosynthetic locus; 10 of these isolates were *hmw* negative. Of 15 isolates lacking a *purE-pepN* insert, 14 were also *hmw* negative. Of the 20 *hmw*-positive isolates, 18 had a *purE-pepN* insert of 0.8 to 1 kb. Such inserts have been reported to contain two genes of unknown function, *hicA* and *hicB*. This correlation between *hmw* and the fimbrial gene cluster has not been reported previously.

Lipopolysaccharide biosynthetic island flanked by *infA* and *ksgA*. The lipopolysaccharide biosynthetic genes now known as *lic2B* and *lic2C* were initially described for a type b strain, RM7004 (originally strain 760705 from the van Alphen laboratory, isolated from cerebrospinal fluid). In this strain they are flanked by *infA* and *ksgA* (homologs of HI0548 and HI0549 in Rd KW20) (16, 20). Comparison of the *infA-ksgA* region in the available *H. influenzae* genome sequences showed that in strains Rd KW20 and R2866, the *infA* and *ksgA* genes are adjacent. Strain 86-028NP contains a single gene, *lic2C*, separating *infA* and *ksgA*. In strain R2846, *infA* and *ksgA* are separated by two genes that are unrelated to *lic2B* and *lic2C*.

TABLE 3. Biochemical and genetic characterization of isolates and association with clinical source of isolates or with *hmw* genes[a]

Phenotype or genotype	Total no. of strains with the characteristic (%)	Association with:						
		Clinical source				*hmw* gene		
		No. of strains with the characteristic			*P*, throat vs not throat	No. of strains with the characteristic		*P* (−*hmw* vs +*thaw*)
		Throat (n = 17)	Otitis (n = 19)	Invasive (n = 17)		−*hmw* (n = 33)	+*hmw* (n = 20)	
Clinical source								
Throat	17 (32.1)					12	5	
Otitis	19 (35.8)					10	9	
Invasive	17 (32.1)					11	6	
Biochemical reactions								
Indole	42 (79.2)	14	15	13		30	12	0.012
Urease	44 (83.0)	16	16	12		24	20	0.008
Omithine decarboxylase	24 (45.3)	8	5	11		23	1	<0.0001
Lysine decarboxylase	16 (30.2)	7	3	6		16	0	0.0001
Arginine dihydrolase	3 (5.6)	3	0	0		2	1	
Arabinose fermentation	9 (17.0)	7	1	1	0.003	8	1	
Growth on citrate	5 (9.4)	3	0	2		4	1	
Autotransporter *lav*	16 (30.2)	1	11	4	0.010	8	8	
Bacteriophage HP2	4 (7.5)	0	1	3		4	0	
Adhesin *hia*	29 (54.7)	9	8	12		28	1	<0.0001
Genetic island flanked by *purE* and *pepN*								
7-kb *hif* (pilus) only	2 (9.4)	0	2	0		1	1	
hicAB only	27 (50.9)	7	10	10		9	18	<0.0001
hicAB and *hif*	9 (17.0)	4	3	2		9	0	0.019
No island	15 (28.3)	6	4	5		14	1	0.004
Genetic island flanked by *infA* and *ksgA*								
lic2BC or *lic2C*	31 (58.5)	10	11	10		17	14	
losAB	10 (18.9)	5	4	1		6	4	
No island	12 (22.6)	2	4	6		10	2	

[a] As described in the text, strains were scored for the presence of *hmw* and other genes based on the results of PCR amplification. The nature of the sequence amplified was confirmed by sequencing for a subset of strains. Strains that scored positive for a genetic locus may not contain the complete locus and may not express the gene product(s). −*hmw*, absence of *hmw*; +*hmw*, presence of *hmw*.

These genes are strongly homologous to *losA* and *losB* (also known as *lbgB* and *lbgA*), characterized in *Haemophilus ducreyi* as encoding β-1,4-galactosyltransferase and D-*glycero*-D-*manno*-heptosyltransferase, respectively (13, 42). The *infA-ksgA* junction thus appears to be a site of substantial genetic heterogeneity, with at least four complements of genes seen in different isolates (Fig. 1).

We used PCR across the *infA-ksgA* junction to evaluate the diversity of this region in the same 53 isolates that were used for the biochemical studies described above. Most of the throat and mucosal-disease isolates in our study contained a genetic island of ~2.2 kb. Of 12 isolates lacking the island, 6 were invasive, 4 were otitis isolates, and only 2 were throat isolates. Partial sequencing of the amplified *ksg-infA* regions showed that the distribution of *losAB* and *lic2BC*-related sequences differed among the three groups: only one invasive isolate, but about one-quarter of throat and otitis isolates, contained *losAB*-related sequences. Most of the remaining strains contained *lic2B* and *lic2C*, but four contained only *lic2C* (Table 4).

These data suggest that invasive isolates of NTHi may be less likely than other NTHi isolates to contain an island flanked by *infA* and *ksgA*, and also less likely to contain an island consisting of *losAB*. However, this is based on a fairly small number of isolates. We used PCR to investigate the *infA-ksgA* regions of 19 additional invasive isolates of NTHi received from laboratories in the United States, Canada, and Switzerland. Nine of these isolates lacked the *infA-ksgA* island. Thus, of the 72 isolates studied, we identified 21 isolates that lack the island. Of these, 15 were invasive ($P = 0.04$). The 19 additional invasive isolates also included 7 with sequences related to *lic2BC* and 3 with sequences related to *losAB*, consistent with the earlier observation that *losAB* is relatively rare among invasive isolates.

Identification of group of closely related isolates. The presence or absence of the *infA-ksgA* island and its content correlate with several of the genetic and biochemical features known to be variably present among *H. influenzae* strains (Table 4). Isolates lacking the *infA-ksgA* island were more likely than other isolates to contain *lav* or the HP2 bacteriophage. Two-thirds of the isolates lacking this island were urease negative but positive for indole and ornithine decarboxylase, making them members of the relatively rare biotype V (23). All biotype V strains were *hia* positive ($P = 0.007$). The similarities of the biotype V isolates led us to look carefully for differences among them (Table 5). Although these isolates have several features in common, they are heterogeneous with regard to the presence of lysine decarboxylase activity, production of nitrite from nitrate, arabinose fermentation, and the presence of the *lav* and HP2 genes. It thus seems unlikely that they represent a recently derived clone. We used MLST to determine the STs for 11 strains that are biotype V, *hmw* negative, and negative for the *infA-ksgA* island and for 13 strains of biotypes I, II, and III. Eight of the biotype V strains formed a more distinct cluster than any of the other strains, while one belonged to a

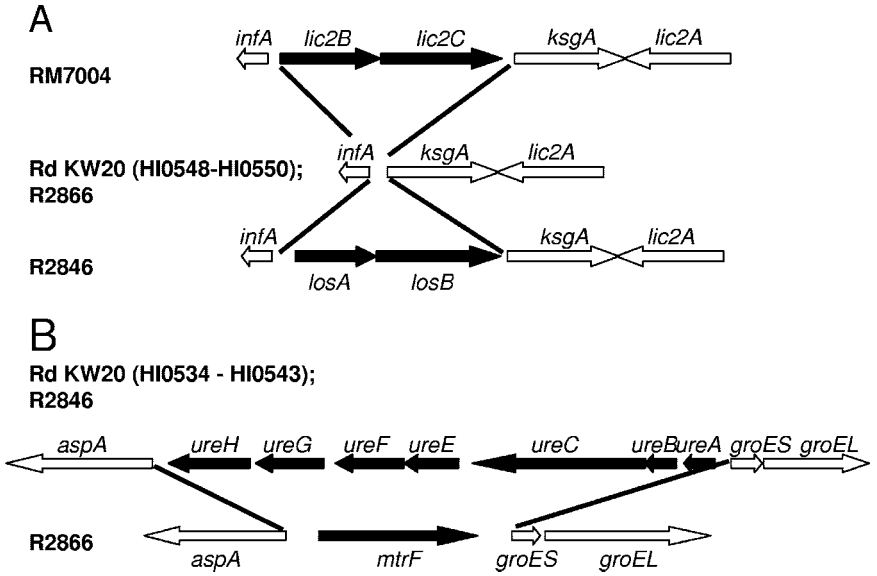

FIG. 1. Gene arrangement at two variable loci. (A) Variable content of the genetic island between *infA* and *ksgA*. Of the 99 NTHi isolates studied, 31 lack this island, as depicted for Rd KW20 and R2866. Forty-five isolates contain *lic2B* and *lic2C*, as shown for RM7004 (16, 20). Seventeen contain *losA* and *losB*, as shown for R2846. Six isolates contain *lic2C* without *lic2B* (not shown). (B) The urease locus of Rd KW20 and R2846 is replaced in R2866 by *mtrF*. We amplified the region between *aspA* and *groES* of 12 urease-negative isolates, and all yielded a 4-kb product consistent with *mtrF*. Homology with *mtrF* was confirmed by sequencing for seven of the isolates.

neighboring lineage; the remaining two biotype V strains were closely related to each other but not to other strains (Fig. 2).

Replacement of the urease locus by an *mtrF* homolog. The strong correlation between urease activity and the presence of an *infA-ksgA* island ($P < 0.0005$) may be due to genetic linkage, since these two loci are separated by only 4 kb. A search of the available *H. influenzae* genome sequences revealed that strains Rd KW20, R2846, and 86-028NP each contain a cluster of seven genes (designated HI0535 to HI0541 in Rd KW20) with homology to the well-characterized urease genes of *Klebsiella aerogenes* (30). In each case this locus is flanked by homologs of *aspA* and *groES*. In strain R2866, which is urease negative, the seven-gene cluster is replaced by a single gene with homology to the gonococcal gene *mtrF* (Fig. 1). Using PCR primers that hybridized to the *aspA* and *groES* genes, we were able to amplify the expected 9-kb product from genomic DNA of each of four urease-positive strains. Partial sequencing of the PCR products showed that each was similar to the urease locus of strain Rd KW20. The same PCR primers yielded a 4-kb product from each of nine urease-negative strains, consistent with the sequence of R2866. Partial sequencing of these 4-kb products showed that all were very similar to that of R2866.

TABLE 4. Association of the *infA-ksgA* island with other variable regions

Region or feature	No. of isolates with the characteristic			
	No island ($n = 12$)	*losAB* ($n = 10$)	*lic2BC* ($n = 27$)	*lic2C* only ($n = 4$)
Integrative plasmid	3	2	3	1
Bacteriophage HP2	3	0	1	0
lav-related sequence[a]	7	2	6	1
Genetic island flanked by *purE* and *pepN*				
7-kb *hif* (pilus) only	0	0	1	1
hicAB only	5	3	17	2
hicAB and *hif*	4	3	2	0
No island	3	4	7	1
hia and *hmw*-related sequences				
hia only	9	4	14	1
hmw only	2	4	10	3
Both *hia* and *hmw*	0	0	1	0
Neither	1	2	2	0
Urease negative, *mtrF* positive	8	0	1	0

[a] Isolates lacking an *infA-ksgA* island are more likely to contain *lav*-related sequences than other isolates ($P = 0.02$).

RFLP analysis of the *hif* locus. In previous work (T. Mhlanga-Mutangadura and M. Golomb, unpublished) on the heterogeneity of the *hif* locus, we had identified three NTHi isolates, R3101, R3151, and R3157, that had *hifA* sequences nearly identical to that of R2866 and in which the 7-kb *hif* locus had a PstI RFLP pattern similar to that of R2866. We now extended that work to the strains found in this study to have a 7-kb *hif* locus. Consistent with previous work, several patterns were identified. R3265, a throat isolate, had the same RFLP pattern as R2866. All of the isolates with this pattern belonged to biotype V and lacked the *infA-ksgA* island.

Serum resistance. Our prototype invasive nontypeable strain, R2866 (Int1), had previously been shown to survive in 40% normal human serum much longer than the laboratory strain Rd KW20 or the nontypeable strain U11 (49). We hypothesized that the in vitro serum resistance of R2866 reflected a virulence trait that might be shared with other invasive NTHi isolates. In the present study we used a more quantitative assay to evaluate the serum resistance of the 53 clinical isolates described above. We found that the range of IC_{50}s for clinical NTHi isolates was nearly as wide as the difference between

TABLE 5. Characteristics of biotype V isolates

Characteristic	Result[a] for the following isolate:											
	Throat (R3265)	Bronchial (R3101)	Otitis			Invasive						
			C1344	C470	R3157	R2866	R3174	R3176	R3567	R3569	R3631	R3570
Geographic region	SP	NL	WA	MA	OH	WA	SW	SW	GA	GA	AR	GA
ST	239	240	234	187	238	99	241	242	236	237	236	ND
Serum IC_{50}	2.75	2.72	10.47	12.93	2.01	11.52	ND	ND	3.41	7.88	2.01	3.40
Biochemical reactions												
Lysine decarboxylase	−	−	+	−	−	−	−	+	+	−	+	−
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	−	+
Arabinose fermentation	+	−	−	−	−	−	−	−	−	−	−	−
Growth on citrate	−	−	−	−	−	−	−	−	−	−	−	−
PCR data												
Plasmid	−	−	−	+	−	+	ND	ND	+	−	−	−
Autotransporter *lav*	−	−	+	+	+	+	ND	ND	−	+	−	+
Bacteriophage HP2	−	−	−	−	+	+	ND	ND	+	−	−	+
Adhesin *hmw*	−	−	−	−	−	−	−	−	−	−	−	−
Adhesin *hia*	3.8 kb	3.5 kb	3.5 kb	3.5 kb	4 kb	4 kb	6 kb	6 kb	3.5 kb	3.5 kb	5 kb	3.5 kb
purE-pepN island	*hic-hif*	*hic-hif*	*hic*	*hic-hif*	*hic-hif*	*hic-hif*	ND	ND	−	*hic*	−	*hic*
infA-ksgA island	−	−	−	−	−	−	−	−	−	−	−	*lic2BC*
aspA-groES island	*mtrF*	*mtrF*	*mtrF*	*mtrF*	*mtrF*	*mtrF*	*mtrF*	*mtrF*	*mtrF*	*mtrF*	*mtrF*	*mtrF*

[a] The table summarizes data for 12 biotype V isolates (indole positive, ornithine decarboxylase positive, urease negative). Nine of these were identified in the study for which results are given in Table 3, and three (R3101, R3567, R3569) were identified from separate studies. All of the biotype V isolates were also *hmw* negative and *hia* positive, and all except one (R3570) lacked a genetic island between *infA* and *ksgA*. Of the 53 isolates described in Table 3, the 9 biotype V isolates were more likely than the 44 non-biotype V isolates to contain a sequence related to the gene for the autotransporter Lav ($P = 0.061$) or bacteriophage HP2 ($P < 0.0005$). They were also more likely to have been isolated from blood, but the difference is not statistically significant ($P = 0.109$). Such clustering of traits is so unusual in *H. influenzae* as to raise the question of whether these isolates are clonal. These strains are from different parts of the world (SP, Spain; NL, The Netherlands; WA, Washington state; MA, Massachusetts; SW, Switzerland; OH, Ohio; GA, Georgia) and differ in sugar fermentation and other biochemical reactions. Phylogenetic analysis of 11 of these strains showed that they are related (Fig. 2). ND, not determined.

encapsulated and unencapsulated strains and that R2866 was one of the most resistant strains in our collection. However, the 16 additional invasive isolates that we tested were not overall more serum resistant than otitis isolates and were only slightly more serum resistant than throat isolates. The IC_{50} range for the invasive isolates was broad, from 1.9% to 13.3%, and this range was similar to that for the other two groups. It is clear that in vitro serum resistance is neither common to all invasive isolates nor a specific characteristic of this group (Fig. 3).

DISCUSSION

While it is well known that NTHi isolates are more heterogeneous than type b isolates, the extent to which the heterogeneous characteristics are correlated with each other or with the anatomic site of isolation is not well understood. We had previously identified genes encoding the autotransporter Lav and bacteriophage HP2 in the invasive isolate R2866 and hypothesized that they might be involved in the unusual virulence of this strain (9, 48). In this study we found that the *lav* gene is indeed rare in throat isolates but is present in many otitis isolates and in only about one-fourth of invasive isolates. Only 4 of the 53 isolates studied in this work contained HP2; 3 of these are invasive. R2866 also contains a 53-kb region that appears to be an integrated plasmid similar to those described for other *Haemophilus* strains (28). We identified sequences characteristic of the plasmid in 11 isolates, only 1 of which was a throat isolate. None of the genetic loci or biochemical reactions we studied were found to be consistently present in invasive isolates. A genetic island flanked by *infA* and *ksgA* was identified in most throat and otitis isolates but was absent in 40% of invasive isolates. When present, this island contains lipopolysaccharide biosynthetic genes, as discussed below.

The ease with which *H. influenzae* strains exchange DNA and the observed genetic heterogeneity among NTHi isolates might have suggested that variable genes would occur randomly. A high rate of recombination in otitis media isolates has been reported (7). However, we found that certain genetic loci occurred together much more often than would occur by chance. Many of the characteristics we examined were significantly associated with the presence of sequences related to *hmw* or *hia* genes. Because we detected these genes by PCR amplification of an internal portion of each gene, we cannot be sure that the strains we scored positive for an adhesin actually contain the complete genetic locus required to express HMW or Hia adhesins. Indeed, some strains appeared to contained only a fragment of the *hia* gene. It is also possible that some of the strains we scored negative contained gene variants that differed slightly at one of the primer binding sites. St. Geme et al. reported that nearly all *H. influenzae* strains contain genes encoding either the Hia adhesin or the HMW adhesins (39). *hia* is an allele of the *hsf* gene found in encapsulated strains. This led to the hypothesis that *hia*-containing strains may have evolved from an encapsulated ancestor. In support of this idea, the insertion element IS*1016*, which is found in the *cap* locus of encapsulated strains, was found to hybridize to genomic DNA of 6 *hia*-containing NTHi isolates out of 9 isolates tested but not to hybridize to any of the 47 *hmw*-containing isolates that were tested (39). Our observation that several genetic and biochemical traits are correlated with the presence of a sequence related to *hmw* or *hia* is consistent with the idea that these genes are found in two distinct lineages. Indole, ornithine decarboxylase, and lysine decarboxylase were all significantly

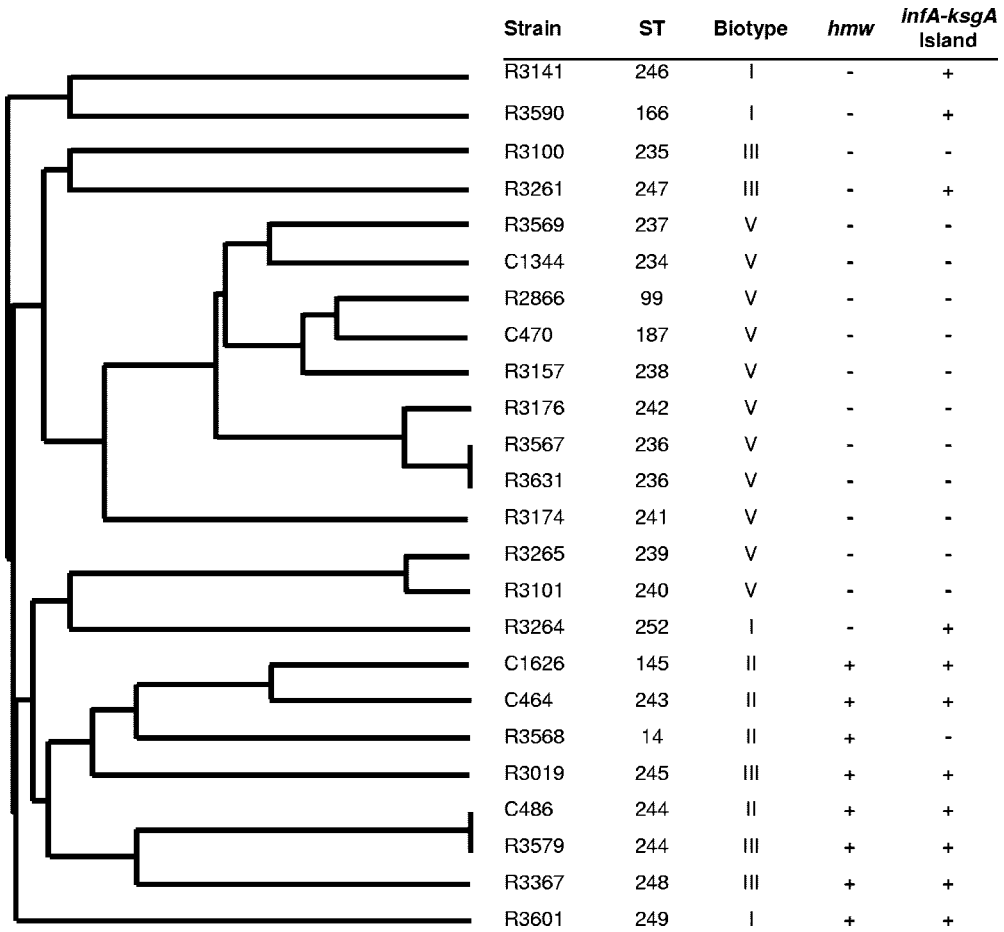

FIG. 2. UPGMA (unweighted pair-group method with arithmetic averages) dendrogram based on the pairwise differences in the MLST allelic profiles of 24 NTHi isolates. Eleven strains that share the properties of belonging to biotype V, lacking *hmw*-related sequence, and lacking a genetic island flanked by *infA* and *ksgA* are clustered on the dendrogram. Of these, eight are most closely related: R3569, C1344, R2866, C470, R3157, R3176, R3567, and R3631. R3174 is less closely related, and R3265 and R3101 are closely related to each other but not to the other biotype V isolates. The remaining isolates for which MLST was determined belong to biotype I, II, or III. Those containing *hmw*-related sequence are separated on the dendrogram from the strains lacking *hmw*.

more prevalent among *hia*-containing isolates than among *hmw*-containing isolates. The *hmw*-containing isolates in our study were uniformly urease positive, while nearly one-third of the *hia*-containing isolates were urease negative.

Within the *hmw*-negative, *hia*-positive isolates, we identified a group of 11 biotype V isolates that shared several characteristics of the invasive strain R2866 (Table 5). All the biotype V strains except one lacked the genetic island between *ksgA* and *infA*. Some of the isolates lacking an *infA-ksgA* island contained a *hif* locus with a characteristic RFLP pattern. Isolates lacking an *infA-ksgA* island were also more likely than other isolates to contain *lav* genes. Bacteriophage HP2 was found only in biotype V strains.

With the exception of the *hmw-hia* dichotomy (39), associations among variable genetic loci of *H. influenzae* have not been previously noted. Because natural transformation and other means of genetic exchange readily disrupt linkages, gene associations must be explained by a recent shared lineage or by shared adaptive strategies or requirements for virulence. The urease locus and the *infA-ksgA* locus are linked within 4 kb and might have persisted in linkage disequilibrium. However, the associations with the *hia* gene and the *hif* RFLP pattern cannot be explained in this way. It is possible that the isolates lacking an *infA-ksgA* island are closely related to each other, although they were derived from different parts of the United States as well as from Canada and Europe. As noted above, the isolates in this group were more likely to have been cultured from blood than from ear aspirates or nasopharyngeal samples. This suggests that they may share virulence determinants. Phylogenetic analysis by MLST showed that 9 of 11 biotype V isolates formed a single cluster, while 2 other biotype V isolates were closely related to each other but not to the cluster of 9. Typing of 13 strains of other biotypes showed some tendency for strains to cluster by biotype and the by presence of an *hmw*-related sequence. Further evaluation of the correlation between MLST and other characteristics of NTHi is ongoing.

We found that in the urease-negative isolates we studied, the urease locus was invariably replaced by a gene with homology to the *mtrF* gene found in *N. gonorrhoeae* and *N. meningitidis*. In gonococci, *mtrF* encodes a predicted membrane protein that has been reported to be involved in resistance to detergents and hydrophobic antibiotics, in conjunction with the resis-

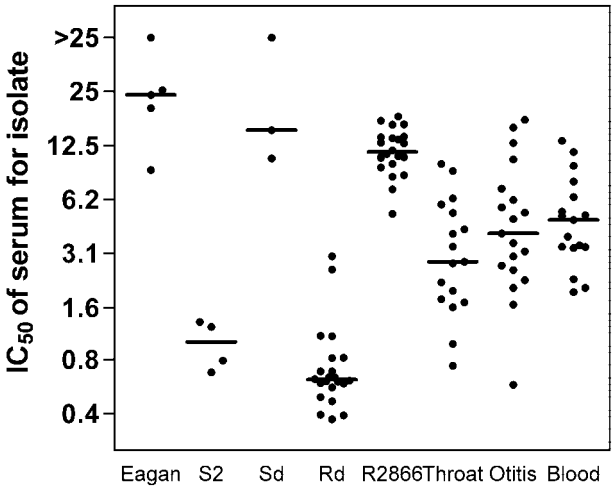

FIG. 3. Bactericidal activity of pooled normal human serum for throat, middle ear, and invasive isolates of NTHi compared with that for laboratory isolates. Strain Eagan is an encapsulated type b strain, and S2 is an unencapsulated mutant derived from Eagan. Sd is the encapsulated type d strain (Garf) from which Rd KW20 was derived. A total of 17 throat, 19 otitis, and 17 invasive isolates were incubated with normal human serum, and IC_{50}s were calculated as described in Materials and Methods. The results of 22 replicate IC_{50} determinations for strains Rd KW20 and R2866 are shown to illustrate the reproducibility of the assay. Horizontal lines indicate the median IC_{50} for each group of data points. The geometric mean IC_{50}s for the clinical isolates were 2.97% for the throat isolates, 4.27% for the otitis media isolates, and 4.66% for the invasive isolates.

tance-nodulation-cell division (RND) pump MtrCDE (45), which does not appear to be present in any of the *H. influenzae* strains that have been sequenced. A less closely related homolog, *abgT*, is thought to encode a transporter that allows an *Escherichia coli* mutant that is defective in *p*-aminobenzoate synthesis to utilize *p*-aminobenzoyl-glutamate (21). It thus seems likely that the *H. influenzae* MtrF homolog is involved in transport, but the substrate cannot be determined without experimental data.

We found that when the *infA-ksgA* region contains an insert, the locus may consist of *lic2BC*, as previously seen for type b isolates (15), of *lic2C* only, or of two genes with homology to *losA* and *losB* of *H. ducreyi*. The prevalence of *lic2B* and *lic2C* has been studied previously, though not in invasive isolates. Hood et al. reported that 13 of 27 NTHi strains studied contained *lic2C* (20). Pettigrew et al. examined 90 throat and 48 otitis isolates and found *lic2B* in one-half of the otitis isolates but only 14% of the throat isolates (35). In contrast, we found for throat, otitis, and invasive isolates that about half in each group contained *lic2BC*. However, it is possible that our small sample of throat isolates (17 isolates) had an atypical distribution of genes at this locus. The *losAB* genes have not been studied previously in *H. influenzae*. We found that the *infA-ksgA* island contained *losAB* in about one-third of our throat and otitis isolates but in very few invasive isolates.

Without further study, it is not possible to determine whether the variation we see in the structure of the *infA-ksgA* region has a direct role in *H. influenzae* biology. Certainly the *lic2BC* and *losAB* genes are likely to affect the structure of lipopolysaccharide and thereby the interaction of bacteria with the host. It is also possible that these genes are markers for other characteristics that collectively affect the ability of a given strain to colonize or cause disease. We noted that the genome sequences of both R2846 and R2866 contain homologs of *losAB* at sites distant from the *infA-ksgA* region. In R2846, the second *losA* homolog contains a frameshift and appears unlikely to encode an active product.

NTHi strains isolated from the blood of ill children were as likely to be sensitive to serum as throat and otitis isolates. One explanation for this unexpected finding is that our laboratory stocks differ genetically from the bacterial population in the patients during infection. *H. influenzae* is subject to high-frequency, reversible gain or loss of phenotypes such as hemagglutination (reflecting piliation), colony morphology, or reactivity of lipopolysaccharide with monoclonal antibodies (11, 24, 46). The molecular basis of this phase variation is variation in the length of tandem repeat regions, often within the coding region of genes (47). A growing culture of any *H. influenzae* strain thus contains a large number of variants that differ from each other in the expression of one or more genes. A change in environmental conditions may result in selection of a different population of variants, and a culture that has been derived from a single colony (as is likely to happen during isolation in a clinical laboratory) and then propagated in the lab may contain a mixture of variants very different from that in the patient's blood. Although R2866, the first invasive NTHi strain studied, has retained serum resistance in the lab, this may be the result of a fortuitous subculture of a resistant colony. The range of serum resistance observed for other clinical isolates may reflect true differences among these isolates but may also indicate that for some isolates we are working with serum-sensitive variants and for other isolates our laboratory stocks happen to consist largely of serum-resistant variants.

Overall, our data indicate that NTHi isolates from invasive disease are not homogeneous: they differ in genotype and in resistance to human serum. It is possible that invasiveness can result from more than one combination of biologic capabilities and that different invasive strains have different sets of genes mediating these functions. It is also possible that all invasive isolates share a gene or group of genes that has not yet been discovered. Several of the biochemical and genetic traits we studied were correlated with each other and particularly with the *hia* and *hmw* genes, supporting the idea that NTHi strains may cluster into distinct lineages, although the lines may be blurred by genetic exchange. We have recently determined the complete genome sequence of the invasive strain R2866 and an otitis strain, R2846 (strain 12). Each of these strains contains 30 to 40 genetic loci not present in the previously sequenced strain, Rd KW20. Analysis of these novel loci will add to our understanding of the genetic relationships among NTHi isolates and may identify genetic determinants characteristic of strains associated with invasive disease and with otitis media. This will aid in the understanding of the virulence mechanisms required for nasopharyngeal bacteria to cause mucosal or systemic disease.

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ADDENDUM IN PROOF

Since acceptance of this paper, we discovered that the *bexA* primers listed in Table 2 are able to amplify *bexA* from serotype b, c and d strains but do not reliably amplify *bexA* from serotype a or e strains. On testing the strains listed in Table 1 for *bexA* using the primers described by Falla et al. (J. Clin Microbiol. **32:** 2382–2386, 1994), strains R3049, R3172, R3173, R3368, and R3595 were found to be positive. Strain R3368 was biotype I and negative for lysine decarboxylase, arginine dihydrolase, citrate, and arabinose. It was positive for *hia* and *losAB* sequences and negative for *hmw, lav*, HP2, plasmid, *hif*, and *hic* sequences. Strains R3049 and R3173 contain the *lic2BC* genes, and R3172 and R3595 contain *losAB*. Elimination of R3368-derived data from Table 3 increased each of the reported *P* values slightly, with each *P* value remaining <0.02. Eliminating data on the remaining strains from the section entitled "Lipopolysaccharide biosynthetic island flanked by *infA* and *ksgA*" did not alter the conclusions.

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