

Relationships of Nontypeable *Haemophilus influenzae* Strains to Hemolytic and Nonhemolytic *Haemophilus haemolyticus* Strains[∇]

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***Haemophilus influenzae* is both a human respiratory pathogen and pharyngeal commensal, while *H. haemolyticus*, the closest phylogenetic relative of *H. influenzae*, is arguably a strict pharyngeal commensal. A hemolytic phenotype has historically differentiated *H. haemolyticus* from *H. influenzae*, but the recent recognition of significant nonhemolytic *H. haemolyticus* colonization has decreased this trait's resolvability. Given this and the potential of recombination between the species, we examined the distribution of microbiologic and molecular traits between collections of *H. influenzae* and *H. haemolyticus* strains separated within a dendrogram obtained by multilocus sequence analysis (MLSA). All strains hybridizing with a probe to *iga*, a gene encoding an immunoglobulin A protease of *H. influenzae*, clustered apart from strains that did not hybridize with the probe. Other traits also segregated significantly along this division, suggesting a separation of the species. Of note, the LOS genes *licA*, *lic2A*, and *lgtC* of *H. influenzae* were approximately 2, 6, and 54 times, respectively, more prevalent in *H. influenzae* than in *H. haemolyticus*. In contrast to species separation, interspecies recombination was evidenced by the inability of single gene sequences to phylogenetically separate the species and by the "fuzzy" distribution of some species-specific traits across the species dividing line. Together, these data support the historically accurate and pragmatic division of these species while recognizing their potential for recombination. Future comparative genomic studies identifying common and distinctive genes could be useful in evaluating their role in the commensal or virulent growth, respectively, of *H. influenzae*.**

Haemophilus influenzae is one of eight *Haemophilus* species that reside as commensal organisms in the pharyngeal cavity of humans. These other commensal *Haemophilus* species include *H. parainfluenzae*, *H. haemolyticus*, *H. parahaemolyticus*, *H. paraphrohaemolyticus*, *H. segnis*, *H. aphrophilus*, and *H. paraphrophilus*. *H. influenzae* is by far the most pathogenic member of the genus in humans. Strains possessing a type b capsule are often associated with invasive diseases, such as meningitis, sepsis, and pneumonia; and strains lacking a capsule (referred to as nontypeable [NT]) are associated with localized mucosal diseases, such as otitis media, sinusitis, and bronchitis. Other haemophili are considered to be rarely associated with disease, and *H. haemolyticus* has never been implicated as an infectious disease agent (1).

In contrast to other haemophili, *H. haemolyticus* and *H. influenzae* both depend on X (hemin) and V (NAD) growth factors, both lack the ability to ferment sucrose, both share similar G+C contents, and both are nearly indistinguishable by their colony and cellular morphologies (25). Initial phylogenetic studies performed by using DNA-DNA hybridization revealed that *H. haemolyticus* associated with *H. influenzae* in a cluster of the family *Pasteurellaceae* termed *Haemophilus sensu*

stricto (6, 41, 47), and these results were substantiated by analysis of the 16S rRNA gene (8, 9, 43), analysis of the *infB* gene (20), or multilocus sequence analysis (MLSA) (42).

Despite the close relationship of the species, only the beta-hemolytic phenotype of *H. haemolyticus* is routinely used in the clinical setting to distinguish *H. haemolyticus* from *H. influenzae* (45, 46, 53). Beta-hemolysis, however, has recently been shown to be a poor indicator of species separation due to the finding that various *H. influenzae* strain collections contained significant proportions of nonhemolytic *H. haemolyticus* strains (38, 40, 62). Murphy and colleagues found that close to 40% of the isolates in an *H. influenzae* strain collection obtained from the sputum of patients with chronic obstructive pulmonary disease (COPD) were *H. haemolyticus*, and their identities were confirmed by phylogenetic studies and amino acid sequence analysis for differences in the P6 outer membrane protein (OMP) (40). Their study also found that more than 20% of a collection of *H. influenzae* strains obtained from the throats of healthy children attending day care were *H. haemolyticus*; and this was confirmed with a larger set of strains by Xie et al. (62), who also identified nonhemolytic *H. haemolyticus* strains by their inability to hybridize with a probe specific for *iga*, an *H. influenzae* gene encoding the virulence-associated immunoglobulin A (IgA) protease (28). Similar to hemolytic *H. haemolyticus*, those studies failed to find an association of the nonhemolytic species with disease: nonhemolytic *H. haemolyticus* isolates were not present among almost 300 *H. influenzae* isolates obtained from the middle ears of children with otitis media, were not associated with new strain acquisitions during COPD exacerbation, and were not present in nearly 600

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respiratory tract isolates present in the *H. influenzae* multilocus sequence typing (MLST) database (40, 62).

Apart from hemolysis, however, a precise taxonomic division between *H. influenzae* and *H. haemolyticus* may be elusive. In 1976, Kilian (who was the first to note strains of *H. haemolyticus* that had lost their hemolytic phenotype) found that only the production of gas during glucose metabolism, H₂S production, and the lack of ornithine decarboxylase (ODC) activity reasonably differentiated *H. haemolyticus* from *H. influenzae* (25). Kilian later showed that *H. influenzae*, in contrast to nonpathogenic *Haemophilus* species, expressed an IgA protease. In a phylogenetic study of the family *Pasteurellaceae*, Hedegaard et al. (20), however, noted two *H. haemolyticus* strains (strains HK855 and HK856) that had lost their hemolytic activity but that expressed the IgA protease. One strain, HK855, produced gas during glucose metabolism and produced H₂S (*H. haemolyticus*-associated traits). Phylogenetic analysis of the *infB* gene revealed that HK865 was more related to *H. influenzae* than to *H. haemolyticus* but that HK855, the gas- and H₂S-producing strain, was intermediately related between the species. This lack of species delineation is reminiscent of the "fuzzy species" observations noted between the pathogenic bacteria *Neisseria meningitidis* and the nasopharyngeal commensal *N. lactamica* (18). These organisms were shown to have undergone some level of interspecies recombination. Similarly, species-specific trait variations in *H. influenzae* and *H. haemolyticus* might also be explained by interspecies recombination; *H. influenzae* is a naturally transformable organism, and if this property exists in *H. haemolyticus*, the species may conceivably exchange DNA.

Given that *H. haemolyticus* may be a more prevalent commensal organism than was previously thought, that *H. influenzae* and *H. haemolyticus* are close phylogenetic relatives, and that the species may potentially exchange DNA through natural transformation, we have evaluated the relationships of large collections of NT *H. influenzae* and *H. haemolyticus* (hemolytic and nonhemolytic) strains by MLSA, classical taxonomic traits, and (since *H. haemolyticus* has not been associated with disease) the distribution of the *H. influenzae* *iga* gene and three genes (*licA*, *lic2A*, and *IgtC*) contributing to virulence-associated lipooligosaccharide (LOS) structures.

MATERIALS AND METHODS

Bacterial strains and culturing. For general use, the bacteria were grown on chocolate agar plates (BD, Franklin Lakes, NJ) at 37°C with 5% CO₂. For the detection of gas production, Levinthal broth (58) was supplemented with 1% glucose as described previously (25). Liquid cultures were grown without shaking.

Four *H. influenzae* reference strains were used in this study and represent strains whose genomes have been completely or partially sequenced: Rd (KW-20, ATCC 51907), 86-028NP (an NT nasopharyngeal strain isolated from a child with otitis media), R2846 (strain 12; an NT strain from a patient with otitis media), and R2866 (INT-1 and ATCC 51997; an NT isolate from blood). One *H. haemolyticus* type strain, ATCC 33390, was also used. The remaining strains were parts of various collections obtained by the laboratory of the Department of Pediatrics and Communicable Diseases, University of Michigan Medical School, or by other laboratories in previous studies (11, 16, 29, 38, 40, 50, 54) (Table 1). Briefly, the *H. influenzae* strains were isolated from the throats of healthy children attending day care, the throats of healthy adults in a longitudinal carriage study, or the middle ear aspirates of children with otitis media. The *H. haemolyticus* strains were isolated from the throats of healthy children attending day care, the adult longitudinal carriage study, or the sputum of adults enrolled in a COPD exacerbation study (Table 1). The *H. haemolyticus* strains from COPD

TABLE 1. Population and ecological stratification of the *H. influenzae* and *H. haemolyticus* collections

Epidemiologic segregation (no. of isolates)	No. of isolates		References
	<i>H. influenzae</i> (<i>n</i> = 88 total)	<i>H. haemolyticus</i> (<i>n</i> = 109 total)	
Isolation site			
Middle ear (44)	44	0	12, 29
Healthy throat (107)	44	63	11, 38, 54
Sputum (46)	0	46	40, 50
Age groups			
Adult (67)	6	61	38, 40, 50
Child (130)	82	48	11, 54

patients were shown not to be new strain acquisitions that led to the exacerbation of COPD (40).

The *H. influenzae* and nonhemolytic *H. haemolyticus* strains were originally defined and tested again in this study by typical colony morphology on chocolate agar, X and V growth factor dependency, and no reaction in the porphyrin test (11, 16, 29, 38, 54). Hemolysis, used to differentiate hemolytic *H. haemolyticus* strains, was assessed as zones of beta-hemolysis surrounding individual colonies grown on horse blood agar (Remel, Lenexa, KS). Working stocks of each strain were made by growing the original stocks on chocolate agar (BD Biosciences, San Jose, CA) and then harvesting the overnight growth into 2 ml of sterile skim milk. Aliquots of 900 µl were then placed in duplicate 96-well plates with 1-ml wells. The plates were subsequently sealed and frozen at -80°C. Cultures were made by partially thawing one plate and pipetting 1 µl of stock onto chocolate agar. This initial growth was then subcultured as needed.

DNA template isolation and PCR. DNA was obtained from the bacterial isolates by using a Wizard genomic DNA purification kit from Promega Co. (Madison, WI). After purification, the DNA from the strains was diluted to half the original concentration, and 1 µl was aliquoted and placed into multiple unskirted 96-well thermocycler plates from Bio-Rad Laboratories, Inc. (Hercules, CA). The plates were stored at -20°C until they were needed. PCR amplification was performed with 50-µl reaction mixtures that comprised 10 pmol of each relevant oligonucleotide primer (Table 2), 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 200 µM (each) deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), and 1 U of recombinant *Taq* DNA polymerase from Invitrogen (Carlsbad, CA). The mixture was subjected to 30 amplification cycles, including 1 min at 94°C template for denaturation, 1 min at 51 to 55°C (depending on primer specificity) for primer annealing, and 1 min at 72°C for chain extension.

DNA sequencing. Partial DNA sequences of the *adh*, *pgi*, *recA*, and *infB* genes were generated for MLSA according to the strategy used by Nørskov-Lauritsen et al. (42), with the exception that the oligonucleotides used for PCR amplification of these genes in the present study were based on sequences from *H. influenzae* strain Rd (Table 2). The oligonucleotides used for PCR amplification of the partial 16S rRNA gene sequences for the differentiation of *Haemophilus* species have been described previously (32) (Table 2). After the PCR amplification was confirmed by gel electrophoresis on 1% agarose, the PCR products were purified with a Qiagen (Valencia, CA) QIAquick 96 or single-sample PCR purification kit. Automated fluorescent dideoxy-DNA sequencing was done by the University of Michigan DNA sequencing core on an ABI model 3730 sequencer. Previously published sequences for type strains of the family *Pasteurellaceae*, including *H. haemolyticus*, *H. parainfluenzae*, *H. pittmaniae*, *Actinobacillus lignieresii*, *Actinobacillus pleuropneumoniae*, *H. segnis*, *H. aphrophilus*, *H. paraphrophilus*, *Actinobacillus actinomycetemcomitans*, and *Pasteurella multocida* have been described for *adh*, *pgi*, and *recA* (42) and for *infB* (20). The 16S rRNA gene sequences for a subset of these strains (Fig. 1) have also been published previously (8, 9). Relevant sequences from *Escherichia coli* K-12 and *H. influenzae* strains Rd, 86-028NP, R2846, and R2866 were obtained from their genome sequences (4, 10, 12, 19). Sequence editing, trimming, and concatenation were done with Lasergene software (version 7.0; DNASTar, Madison, WI), and phylogenetic analysis was done with Mega software (version 3.1) (31). Bootstrap consensus, minimum-evolution dendrograms for concatenated or individual sequences were made with 1,000 replicates.

Classical taxonomy. Biochemical and phenotypic analyses of *H. influenzae* and *H. haemolyticus* traits were performed as previously described by Kilian (25, 26). Briefly, the emission of H₂S was assessed with lead acetate strips (Fisher Scien-

TABLE 2. Oligonucleotides used for PCR or DNA sequencing

Gene	Primer sequence ^a	Position in Rd genome
<i>adk</i>	F: GGTGCACCGGGTGCAGGTAA	376521
	R: CCTAAGATTTTATCTAACTC	375899
<i>pgi</i>	F: GGTGAAAAAATCAATCGTAC	1644657
	R: ATTGAAAGACCAATAGCTGA	1645246
<i>recA</i>	F: ATGGCAACTCAAGAAGAAAA	622556
	R: TTACCAAACATCACGCCCTAT	621940
<i>infB</i>	F: TGAAAATGAGCTTGAAGAA	1361550
	GCGG	
	R: GATAGTTGCCACAGGGCCAC	1362198
16S rRNA	F1: CCAGCAGCCGCGGTAATACG	624342
	R1: ATCGGYTACCTTGTTACGA	625335
	CTTC	
	F2: GCCCGCACAAAGCGGTGGAGC	624751
ATGTG		
R2: CTCGTAAGGGCCATGATGAC	625039	
TTGACG		
<i>iga</i>	F: GTTCCACCACCTGCGCCTG	1050386
	CTAC	
	R: GTTATATTGCCCTCGTTA	1049130
TTCA		
<i>licA^b</i>	F: GTAGGATTTGTAAAACCTGC	1608693
	TACAAGCC	
	R: GGCAATTCCTCTAACAGTTTA	1609579
AATGCTGCG		
<i>lic2A^b</i>	F: ATATTACATAATATAGAGGAA	571382
	TCTAG	
	R: CTACATAAAACGAACAATTC	570690
TTTACC		
<i>lgtC^b</i>	F: CGGACTGTGTCAGTCAGACAATG	289338
	R: CTCAAAATGATCATACCAA	288499
	GATG	

^a All oligonucleotides are based on the DNA sequences from *H. influenzae* strain Rd. F, forward, R, reverse.

^b The forward primers begin downstream of tetranucleotide repeats.

ific, Pittsburgh, PA) placed on the lids of chocolate agar plates inoculated with individual bacterial strains. Conversion of the strips from white to black after overnight growth was considered positive. The production of gas from glucose metabolism was assessed by the collection of gas in inverted Durham tubes immersed in Levinthal broth containing 1% glucose (Fisher Scientific). *H. influenzae* biotype reactions have been extensively described (26, 25, 39, 60). Briefly, indole production was assessed by placing a small amount of bacteria on filter paper saturated with Kovács reagent (Remel) and observing the paper for a purple color reaction; urease activity was assessed in rapid urea broth (BD), according to the manufacturer's instructions, by mixing an inoculum loop of bacteria from the plate growth and observing the plate for a color reaction after 4 h of incubation at 37°C; and ODC activity was determined with ODC broth (Remel), according to the manufacturer's instructions.

Dot blot immunoassays for MAb 7F3 recognition of P6 OMP. The detection of the P6 OMP by monoclonal antibody (MAb) 7F3 in dot blot immunoassays has been described previously (17). Briefly, membranes were prepared by harvesting bacteria directly from the growth on chocolate agar and placing them onto nitrocellulose membranes with a sterile pipette tip. The blots were immediately washed in phosphate-buffered saline (PBS) and then blocked in PBS containing 5% nonfat dry milk (BLOTTO [22]) for 1 h. MAb 7F3 was diluted 1:10 in BLOTTO, and the mixture was incubated overnight at room temperature. After the blots were washed three times in PBS alone, they were incubated in PBS containing goat anti-mouse IgA antibody conjugated to alkaline phos-

phatase (Sigma) diluted 1:5,000 for 1 h. The blots were then washed three times in PBS and exposed to nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate substrate (Pierce, Rockford, IL) for color development.

Dot blot hybridization. Membranes dotted with single-stranded genomic DNA obtained from crude lysates of each *Haemophilus* strain were made by using a modification of the method of Foxman et al. (13, 14). The bacteria were grown overnight on chocolate agar and were suspended in 4 ml of PBS to an optical density of 1.0. The bacteria were then centrifuged, and the pellets stored at -20°C until all strains were collected. The pellets were then thawed, resuspended in 8 ml of lysis solution (0.4 M NaOH and 10 mM EDTA), and heated for 30 min in a 70°C water bath. One-milliliter aliquots of each extract were placed in multiple 96-well plates with 1-ml wells. The plates were sealed and frozen at -80°C until they were needed. Dot blot membranes were made by applying 40 µl of each DNA extract to a nylon membrane in an array of 8 by 12 on a Bio-Dot microfiltration apparatus (Bio-Rad), as described previously (13, 14).

Generation of probes, hybridization, and analysis were carried out as described previously (44, 63). Oligonucleotides for the amplification of the *iga*, *licA*, *lic2A*, and *lgtC* gene probes were generated from *H. influenzae* strain Rd; and oligonucleotides were directed to the nonrepeat regions of each gene (Table 2). DNA was amplified by PCR as described above. The PCR products were purified from agarose gels by using the QIAquick gel extraction kit from Qiagen and labeled with the AlkPhos direct labeling and detection systems (GE Healthcare, Piscataway, NJ). The probes were hybridized to the dot blot membranes under stringent conditions and developed with an ECF detection system (GE Healthcare). The probe signal intensity was read with a Storm 860 phosphorimager and analyzed with ImageQuant (version 5.0) software (Molecular Dynamics/GE Healthcare).

Statistical analyses. The data were entered in Excel software (Microsoft) in binary form for the presence (which was given a value of 1) or absence (which was given a value of 0) of any given trait. The prevalence ratios of the traits for *H. influenzae* compared to the traits for *H. haemolyticus* or the prevalence ratios of the traits for nonhemolytic *H. haemolyticus* compared to the traits for hemolytic *H. haemolyticus* were calculated as a ratio of the proportions of traits among each species or collection, for example, as the proportion of all *H. influenzae* strains with a particular trait over the proportion of all *H. haemolyticus* strains with the same trait. χ^2 analysis was used to determine the significance of the differences of the trait associations between species or between nonhemolytic and hemolytic *H. haemolyticus* strains. Student's *t* test was used to assess significant differences in the distributions of the traits between different nodes of the same species. Statistical analyses were performed with SAS software (version 9.1).

Nucleotide sequence accession numbers. Partial gene sequences for each of the 88 *H. influenzae*, 109 *H. haemolyticus*, and 3 *H. parainfluenzae* strains are available from GenBank under accession numbers EU150426 to EU150625 (for *adk*), EU150626 to EU150825 (for *pgi*), EU150826 to EU151025 (for *recA*), EU151026 to EU151225 (for *infB*), and EU151226 to EU151425 (for the 16S rRNA genes).

RESULTS

Phylogenetics of *H. influenzae* and *H. haemolyticus* within the family Pasteurellaceae. Recently, Nørskov-Lauritsen et al. (42) defined the relationships of human-specific haemophili within the family Pasteurellaceae by performing MLSA with partial, concatenated *adk*, *pgi*, *recA*, and *infB* gene sequences. Since this approach revealed that both *H. influenzae* and *H. haemolyticus* were present in an *Haemophilus* sensu stricto phylogenetic cluster and were separate from other members of the family, we have used it to determine if the NT *H. influenzae* and *H. haemolyticus* (hemolytic and nonhemolytic) strains in our study also maintain the same relationships. An MLST scheme, designed to detail and track differences within the *H. influenzae* species, was not used. In addition, various MLST genes of *H. influenzae* have been shown not to be present in other members of the family Pasteurellaceae (42).

A dendrogram produced from the concatenated DNA sequences for each of the 200 strains predicted to be *H. influenzae* or *H. haemolyticus*, together with sequences from four *H. influenzae* type strains, one *H. haemolyticus* type strain, and

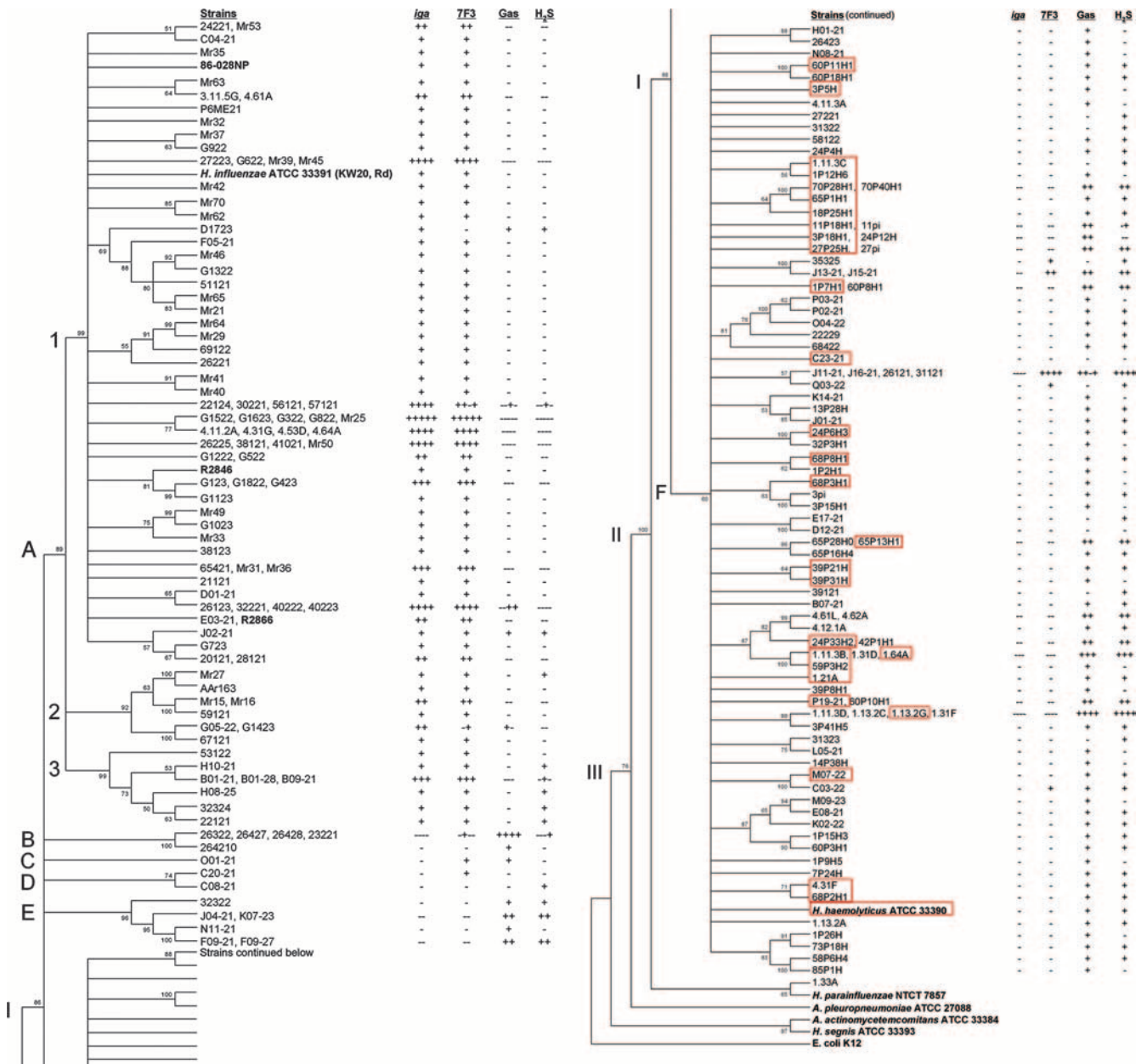


FIG. 1. Minimum-evolution dendrogram of the *Haemophilus sensu stricto* cluster. The tree, rooted by *E. coli* strain K-12, is based on concatenated *adh*, *pgi*, *recA*, *infB*, and 16S rRNA gene sequences, with bootstrap values of $\geq 50\%$ of 1,000 bootstraps indicated. Node I contains all *H. influenzae* and hemolytic and nonhemolytic *H. haemolyticus* strains within an *Haemophilus sensu stricto* cluster, while nodes II and III contain selected members of the family *Pasteurellaceae*. Strains with identical sequences are listed on the same branch. Red boxes define beta-hemolytic *H. haemolyticus* strains. Positive (+) and negative (-) results for the *iga* gene probe hybridization, P6 OMP MAb 7F3 reactivity, the production of gas during glucose metabolism, and H₂S production are shown to the right of each strain. The results are also relative to the strain order on branches with multiple strains. On the basis of the stratification of *iga* and other results (see the text and Table 2), it is proposed that node A contains all *H. influenzae* strains, while nodes B to F contain all *H. haemolyticus* strains.

nine other members of the family *Pasteurellaceae* (represented by 15 strains) (42), revealed a *Haemophilus sensu stricto* cluster containing the *H. influenzae* and *H. haemolyticus* type strains and all but 3 of the 200 test strains separate from other members of the family *Pasteurellaceae* (data not shown). The three strains not contained in the *Haemophilus sensu stricto* cluster were more related to *H. parainfluenzae* than to *H. influenzae* or *H. haemolyticus*. Since *H. parainfluenzae* grows independently

of factor X, we reassessed the X and V growth factor requirements of these three strains and found that they grew in the presence of V factor alone, indicating that they are most likely *H. parainfluenzae*. Given this result, the remaining 197 strains were reassessed for their dependence on factors X and V, and all strains were found to require both factors. These results, together with the MLSA data, support the designation of these 197 strains as either *H. influenzae* or *H. haemolyticus*.

Because 16S rRNA gene sequences were previously used to define members of the family *Pasteurellaceae* (43) and to separate the nonhemolytic form of *H. haemolyticus* from *H. influenzae* (40), partial sequences of the 16S rRNA gene were obtained from the *H. influenzae* and the *H. haemolyticus* test strains described above and concatenated with the four house-keeping gene sequences. A dendrogram created from the extended sequences of these strains, together with those of the *H. influenzae* and *H. haemolyticus* type strains and four members of the family *Pasteurellaceae* for which published 16S rRNA gene sequences were available, revealed that all *H. influenzae* and *H. haemolyticus* strains remained in an *Haemophilus* sensu stricto cluster similar to that described with the four house-keeping genes alone (Fig. 1 and 2A). Within the cluster, some branches were found to contain groups of two to five strains with identical sequences. Of the 197 strains in the collection, 88 (45%) had sequences identical to those of 1 or more strains; and the remaining strains, which had unique sequences, were found on separate branches (Fig. 1). The *Haemophilus* sensu stricto cluster emerged from node I of the dendrogram, separate from nodes II and III, which contained four other members of the family *Pasteurellaceae* (Fig. 1). The *Haemophilus* sensu stricto cluster at node I was composed of six subnodes, designated A to F; node A contained all four *H. influenzae* type strains together with 88 of the 197 test strains, and node F contained the *H. haemolyticus* type strain, ATCC 33390, together with 95 test strains. The 14 remaining strains were present on four branches that emerged from nodes B to E (Fig. 1).

Phylogenetic distribution of *iga* gene probe hybridization and P6 MAb reactivity between *Haemophilus* species. In a previous study, *H. influenzae* strains were defined as factor X- and V-dependent, nonhemolytic strains that both hybridized to an *iga* gene probe and reacted with P6 OMP MAb 7F3, an antibody previously shown to react with the conserved P6 OMP of *H. influenzae* but not with that of *H. haemolyticus* (40, 62). These strains were distinguished from nonhemolytic *H. haemolyticus* that lacked at least one of these traits (62). Therefore, the 197 strains belonging to the *Haemophilus* sensu stricto collection described above were also examined for their ability to hybridize with an *iga* gene probe by dot blot hybridization. As expected, the four *H. influenzae* reference strains hybridized with the probe, while the *H. haemolyticus* type strain, ATCC 33390, did not. All 88 strains in clades emerging from node A of the MLSA-based dendrogram hybridized with the *iga* gene probe, while the 109 strains in clades and branches emerging from nodes B to F did not hybridize to the probe (Fig. 1). Next, a dot blot immunoassay was performed for all strains with MAb 7F3. The four *H. influenzae* type strains, but not the *H. haemolyticus* type strain, reacted with MAb 7F3. All but 3 of the 88 strains (97%) that hybridized to the *iga* gene probe also reacted with MAb 7F3. In contrast, the antibody reacted with only 13 of the 109 strains (12%) that did not hybridize to the *iga* gene probe (Fig. 1; Table 3). In contrast to the clear segregation of the *iga* gene probe hybridization in the MLSA-based dendrogram, strains with discordant results for MAb 7F3 reactivity were distributed throughout the dendrogram. Thus, the species designations of the strains in this study were made primarily by MLSA and *iga* gene probe hybridization. In this context, the data suggest that node A of the MLSA-based

dendrogram contains 88 *H. influenzae* strains that hybridize with an *iga* gene probe and that predominantly react with MAb 7F3, while nodes B to F of the dendrogram contain 109 *H. haemolyticus* strains (hemolytic or nonhemolytic) that lack *iga* hybridization and that mostly lack MAb 7F3 reactivity.

Distribution of classically defined taxonomic traits between the *Haemophilus* species. On the basis of this stratification of species, we estimated the distribution of classically defined taxonomic traits such as the production of gas during glucose metabolism, H₂S emission, and the lack of ODC activity (traits previously shown to be associated with *H. haemolyticus* [25]). In addition, we examined indole production and urease activity since these markers (together with ODC) have been routinely used for *H. influenzae* biotyping and may therefore be differentially distributed among the species.

As expected, the *H. haemolyticus* type strain, ATCC 33390, was found to produce gas during glucose metabolism, emit H₂S, and lack ODC activity, while the four *H. influenzae* type strains expressed the opposite phenotypes (with the exception of strain 86-028NP, which lacked ODC activity). Gas production and H₂S emission were 69 and 88% prevalent, respectively, among the phylogenetically defined *H. haemolyticus* strains and 12 and 6% prevalent, respectively, among the phylogenetically defined *H. influenzae* strains (Table 3 and Fig. 1), suggesting that these traits were 17 and 6 times more prevalent, respectively ($P < 0.05$ for both traits), in *H. haemolyticus* than in *H. influenzae* (the prevalence ratios are the inverse of those shown in Table 3). In contrast, ODC activity was about four times more prevalent ($P < 0.05$) among the *H. influenzae* strains than among the *H. haemolyticus* strains. Indole production and urease activity were significantly different ($P < 0.05$), but they were only 1.3 and 1.2 times more prevalent, respectively, in *H. influenzae* strains than in *H. haemolyticus* strains (Table 3). Because *H. influenzae* and *H. haemolyticus* are such close phylogenetic relatives, we unconventionally assigned *H. influenzae* biotype profiles (biotypes I to VIII) to *H. haemolyticus* strains and compared the prevalence of the profiles between the species. Biotype I was almost six times more prevalent among the *H. influenzae* strains and biotype III was about two times more prevalent among the *H. haemolyticus* strains ($P < 0.05$ for both traits). The overall prevalence rates of these and other biotypes, however, were not high enough to be useful for species differentiation (Table 3). Together, the results of the classical taxonomic traits correlate well with those of a previous taxonomic study by Kilian that used nine *H. haemolyticus* strains (25), but none of the traits were able to completely differentiate *H. influenzae* from *H. haemolyticus* in our larger strain collections.

Distribution of LOS genes with tetranucleotide repeats among *H. influenzae* and *H. haemolyticus* strains. Because the presence or absence of the virulence-associated *iga* gene appears to corroborate the data obtained by MLSA in segregating *H. influenzae* from *H. haemolyticus*, we speculated that other virulence-associated genes might separate the species in a similar manner. LOS-modifying genes in *H. influenzae* that contain tetranucleotide repeats undergo slipped-strand mispairing, resulting in on-off phase variation in gene expression. These repeats allow the organism to modify its LOS structures in response to host immune pressure, and therefore, the loci are strongly suspected of facilitating virulence (2, 37). Because

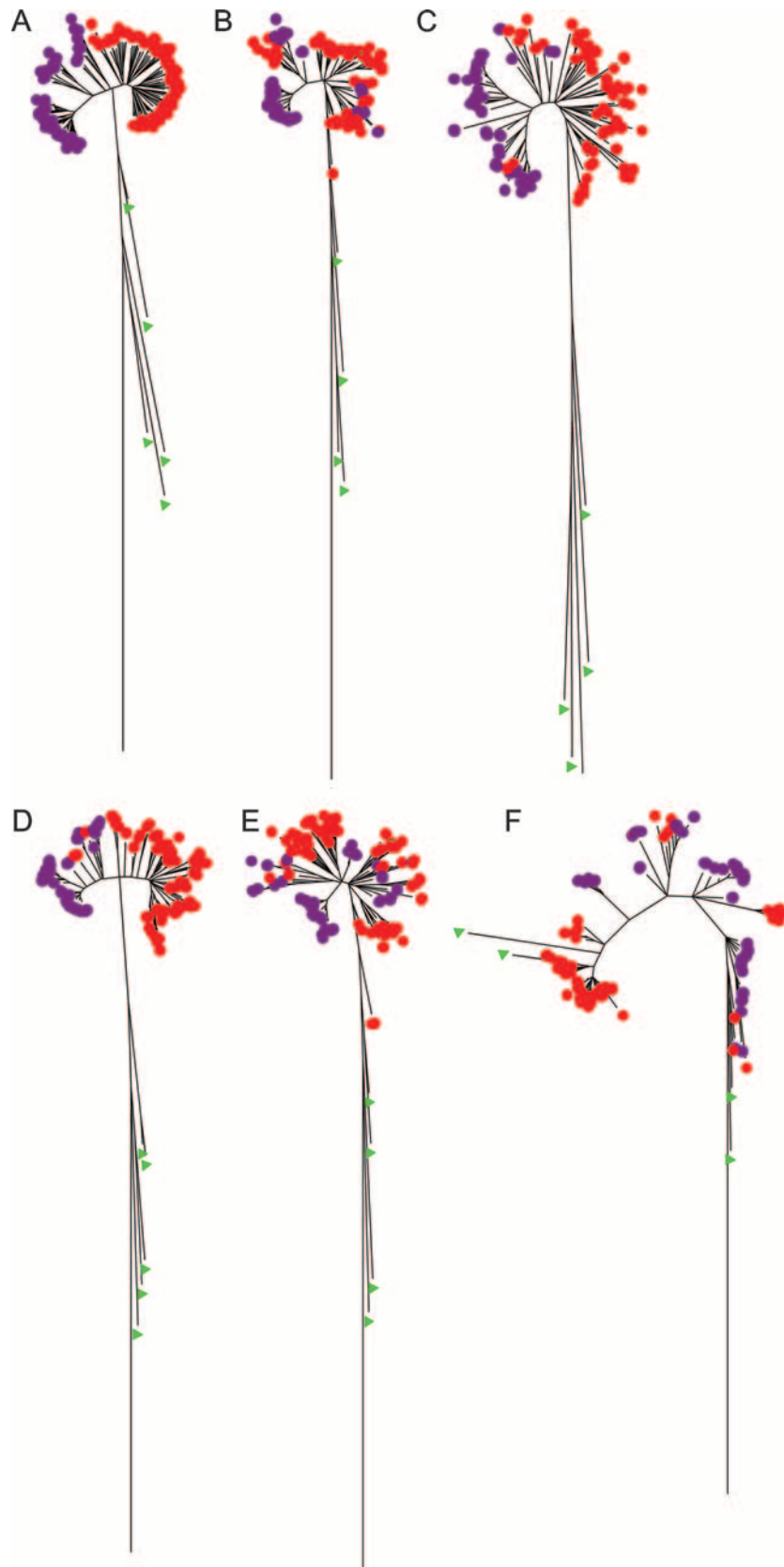


FIG. 2. Multiple and single DNA sequence segregation of *H. influenzae* and *H. haemolyticus* strains. Radiation tree views of the (A) five concatenated sequences and individual (B) *adk*, (C) *pgi*, (D) *recA*, (E) *infB*, and (F) 16S rRNA gene sequences. *H. influenzae* (purple dots) and *H. haemolyticus* (red dots) strains are shown together with other members of the family Pasteurellaceae (green triangles).

TABLE 3. Distribution of taxonomic traits among *H. influenzae* and *H. haemolyticus* isolates

Trait	No. (%) of isolates ^a		PR ^b	Result of χ^2 analysis (P value ^d)
	<i>H. influenzae</i> (n = 88)	<i>H. haemolyticus</i> (n = 109)		
Molecular-based traits				
<i>iga</i> gene probe hybridization	88 (100)	0 (0.0)	Referent	
MAb 7F3 reactivity	85 (96.6)	13 (11.9)	8.10	<0.0001
Classical traits				
H ₂ S production	11 (12.5)	75 (68.8)	0.18	<0.0001
Gas production	5 (5.7)	96 (88.1)	0.06	<0.0001
Indole production	73 (83.0)	68 (62.4)	1.33	0.0015
Urease activity	74 (84.1)	77 (70.6)	1.19	0.0265
ODC activity	24 (27.3)	8 (7.3)	3.72	0.0002
Biotype I	18 (20.5)	4 (3.7)	5.57	0.0002
Biotype II	44 (50.0)	43 (39.4)	1.27	0.1382
Biotype III	12 (13.6)	28 (25.7)	0.53	0.0366
Biotype IV	0 (0) ^c	2 (1.8)	0.31	0.2015
Biotype V	5 (5.7)	2 (1.8)	3.10	0.1470
Biotype VI	1 (1.1)	0 (0) ^c	2.48	0.2645
Biotype VII	6 (6.8)	19 (17.4)	0.39	0.0261
Biotype VIII	2 (2.3)	11 (10.1)	0.23	0.0280
LOS gene virulence traits				
<i>licA</i>	84 (95.5)	46 (42.2)	2.26	<0.0001
<i>lic2A</i>	80 (90.0)	17 (15.6)	5.83	<0.0001
<i>lgtC</i>	88 (100.0)	2 (1.8)	54.50	<0.0001

^a The species designation is based on the stratification of strains in the MLSA dendrogram of Fig. 1 and on the presence or absence of hybridization with an *iga* gene probe.

^b PR, prevalence ratio. The prevalence ratios were calculated for *H. influenzae* by using *H. haemolyticus* as the referent group.

^c Logit, 0.5 was used in place of 0 for prevalence ratio and statistical calculations.

^d A P value of <0.05 is considered statistically significant.

H. haemolyticus has never been implicated in disease, we hypothesized that LOS genes with tetranucleotide repeats might be lower in prevalence or altogether absent in *H. haemolyticus* strains compared to their abundance in *H. influenzae* strains.

Dot blot hybridization was performed with probes specific for *licA*, *lic2A*, and *lgtC*, three LOS genes that contain tetranucleotide repeats. All three genes hybridized to the four *H. influenzae* type strains but not to the *H. haemolyticus* type strain (data not shown). The *licA*, *lic2A*, and *lgtC* gene probes hybridized to 96, 90, and 100% of the *H. influenzae* strains, respectively, and to 42, 16, and 2% of the *H. haemolyticus* strains, respectively (Table 3). The prevalence ratios calculated from these values showed that the *licA*, *lic2A*, and *lgtC* genes were about 2, 6, and 54 times more prevalent, respectively ($P < 0.05$ for all associations), in *H. influenzae* than in *H. haemolyticus* (Table 3). Of the three LOS contingency genes, these data suggest that only *lgtC* segregates between the species, in similarity to the *iga* gene results. Although the prevalence rates of the *licA* and *lic2A* genes were high in the *H. influenzae* strains, they were variably present among the *H. haemolyticus* strains. In this regard, the relatively high prevalence of the *licA* gene in *H. haemolyticus* (42%) may suggest that it facilitates the normal commensal growth of *H. haemolyticus*.

Recombination between species. The *H. influenzae* and *H. haemolyticus* species designations in this study are supported by a combination of MLSA, molecular, and traditional taxonomic traits. These bacteria, however, may undergo interspecies recombination through natural transformation, a feature that has been well documented between other recombinogenic pathogens and their commensal relatives (18, 23).

To estimate interspecies recombination in *H. influenzae* and *H. haemolyticus*, we examined the utility of individual rather than concatenated gene sequences for segregation of the species. Compared to the MLSA-based dendrogram containing the concatenated sequences (shown as a radial view in Fig. 2A), dendrograms constructed from individual *adk*, *pgi*, *recA*, *infB*, and 16S rRNA gene sequences (Fig. 2B to F, respectively) failed to completely separate *H. influenzae* from *H. haemolyticus*. In addition, only the *recA* sequence was able to maintain a complete *Haemophilus sensu stricto* cluster separate from four other members (five strains) of the family *Pasteurellaceae* used in the trees. The 16S rRNA gene sequences poorly defined the cluster; and *H. parainfluenzae* strain 1.33A did not segregate from the *H. influenzae* and *H. haemolyticus* strains with individual *adk*, *pgi*, or *infB* sequences.

Next, we investigated the potential for the unequal distribution of species-specific traits among the MLSA-based nodes separating the species. For instance, strains that phylogenetically border the species dividing line in the MLSA-based dendrogram (Fig. 1) might be expected to exhibit more interspecies recombination than strains that are more distal to that division. As mentioned, node A contains all *H. influenzae* strains and is separate from nodes B to F, which contain all *H. haemolyticus* strains. In addition, node A is composed of three clades emerging at subnodes A1 (72 strains), A2 (8 strains), and A3 (8 strains). In the *H. haemolyticus* strains of nodes B to F, 14 strains were found in nodes B to E, while 95 strains were more distally positioned in the clade emerging from node F. Therefore, the trait prevalence was compared between the *H. influenzae* strains at node A1 and the combined strains (to increase the sample size) at nodes A2 and A3. Similarly, the trait prevalence was compared between the combined *H. haemolyticus* strains at nodes B to E and the *H. haemolyticus* strains at node F.

H₂S production (an *H. haemolyticus*-associated trait) was found to be significantly less prevalent among the *H. influenzae* strains at node A1 (6%) than among the *H. influenzae* strains at nodes A2 and A3 (44%), while indole production, the *licA* gene, and the *lic2A* gene (all *H. influenzae*-associated traits) were significantly more prevalent in strains at node A1 (88, 100, and 99% of strains, respectively) than in the combined strains at nodes A2 and A3 (63, 75, and 56%, respectively) ($P < 0.05$ for all associations) (Table 4). Although the differences were not statistically different, H₂S production was less prevalent in the *H. haemolyticus* strains at nodes B and E (50%) than in the *H. haemolyticus* strains at node F (72%); and indole production, the *licA* gene, and the *lic2A* gene were more common in the strains at nodes B to E (71, 57, and 21%, respectively) than in the strains at node F (61, 40, and 15%, respectively). In addition, the strains at nodes B to E were twice as likely as the strains at node F to react with MAb 7F3 (an *H. influenzae*-associated trait) (Table 4). In contrast to the results presented above, however, the possession of an *lgtC* gene and

TABLE 4. Trends of species-specific traits within a species

Trait	No. (%) of strains ^a			
	<i>H. influenzae</i> nodes		<i>H. haemolyticus</i> nodes	
	A1 (n = 72)	A2 and A3 (n = 16)	B to E (n = 14)	F (n = 95)
H ₂ S production	4 (5.6)	7 (43.8) ^c	7 (50.0)	68 (71.6)
Indole production	63 (87.5)	10 (62.5) ^c	10 (71.4)	58 (61.1)
<i>licA</i>	72 (100)	12 (75.0) ^c	8 (57.1)	38 (40.0)
<i>lic2A</i>	71 (98.6)	9 (56.3) ^c	3 (21.4)	14 (14.7)
P6 positive	70 (97.2)	15 (93.7)	3 (21.4)	10 (10.5)
<i>lgtC</i>	72 (100)	16 (100)	0 (0) ^b	2 (2.1)
Gas production	4 (5.6)	1 (6.3)	12 (85.7)	84 (88.4)
Urease activity	59 (81.9)	15 (93.8)	6 (42.8)	71 (74.7) ^c
ODC activity	16 (22.2)	8 (50.0) ^c	1 (7.1)	7 (7.4)

^a The nodes and the number of strains in each node are relative to those in the dendrogram in Fig. 1.

^b Logit, 0.5 was used instead of 0 for statistical calculations.

^c Nodes possessing a statistically significant difference ($P < 0.05$) for the trait on the basis of Student's *t* test.

gas production appeared to be equally distributed between clades within the same species, and the urease and ODC activities did not exhibit the trends described above (Table 4). Together, the results of these phylogenetic and taxonomic studies suggest the possibility of recombination between *H. influenzae* and *H. haemolyticus*.

Distribution of traits between hemolytic and nonhemolytic *H. haemolyticus* strains. The relationships between hemolytic and nonhemolytic *H. haemolyticus* strains are poorly understood. In the MLSA-based dendrogram (Fig. 1), 33 hemolytic *H. haemolyticus* strains were interdispersed among 62 nonhemolytic *H. haemolyticus* strains emerging from node F, suggesting that hemolytic and nonhemolytic *H. haemolyticus* strains do not cluster as a separate subspecies. No hemolytic strains were present in the 14 strains emerging from nodes B to E that bordered the species dividing line. Stratification of the molecular and classical taxonomic traits described above between hemolytic and nonhemolytic *H. haemolyticus* strains from all nodes revealed that the prevalence of H₂S emission, gas formation during glucose metabolism, ODC activity, and the possession of an *lgtC* gene were not significantly different ($P > 0.05$) between hemolytic and nonhemolytic *H. haemolyticus* strains (Table 5). Although urease activity was significantly more prevalent in hemolytic strains than in nonhemolytic strains, reactivity with MAb 7F3 and possession of the *licA* and *lic2A* genes were all significantly more prevalent ($P < 0.05$ for all associations) in nonhemolytic strains than in hemolytic strains (Table 5). Of the 13 total *H. haemolyticus* strains that reacted with MAb 7F3, none were hemolytic, and of the 17 total *H. haemolyticus* strains that hybridized with the *lic2A* probe, only one was hemolytic. Similarly, the *licA* gene was almost twice as prevalent in nonhemolytic *H. haemolyticus* strains than in hemolytic *H. haemolyticus* strains. To decrease the potential for interspecies recombination to contribute to these results, the prevalence of the traits was recalculated for the *H. haemolyticus* strains emerging only from node F. With the exception of urease activity (which lost its statistical significance), the associations remained very similar to those obtained when all *H. haemolyticus* strains were used (data not shown). Although hemolytic and nonhemolytic *H. haemolyticus* strains appear to be phylogenetically inseparable, the results

TABLE 5. Distribution of taxonomic traits between hemolytic and nonhemolytic *H. haemolyticus* strains

Trait	No. (%) of strains		PR ^a	Results of χ^2 analysis (<i>P</i> value ^c)
	Hemolytic (n = 33)	Nonhemolytic (n = 76)		
Molecular-based traits				
Hemolysis	33 (100)	0 (0)	Referent	
MAB 7F3	0 (0) ^b	13 (17.1)	11.3	0.0114
Traditional traits				
H ₂ S production	24 (72.7)	51 (67.1)	0.92	0.5605
Gas production	31 (93.9)	65 (85.5)	0.88	0.2131
Indole production	20 (60.6)	48 (63.2)	1.04	0.8005
Urease activity	28 (84.8)	49 (64.5)	0.76	0.0319
ODC activity	2 (6.1)	6 (7.9)	1.30	0.7358
LOS gene virulence traits				
<i>licA</i>	8 (24.2)	38 (50.0)	2.06	0.0124
<i>lic2A</i>	1 (3.0)	16 (21.1)	6.95	0.0172
<i>lgtC</i>	0 (0) ^b	2 (2.6)	1.74	0.3469

^a PR, prevalence ratio. Prevalence ratios were calculated for nonhemolytic strains by using the hemolytic strains as the referent group.

^b Logit, 0.5 was used instead of 0 for prevalence ratio and statistical calculations.

^c A *P* value of <0.05 is considered statistically significant.

suggest the possibility that some genes and traits common to *H. influenzae* exist more frequently in nonhemolytic than hemolytic *H. haemolyticus* strains.

DISCUSSION

Since its first description as "Bacillus X" in 1919, *H. haemolyticus* has been differentiated from *H. influenzae* predominantly by its ability to produce hemolysis on blood agar plates (46). Shortly after this description, arguments were made that *H. haemolyticus* was a hemolytic variant of *H. influenzae*, since the two species appeared to be closely related by their X and V growth factor dependencies and other criteria (36, 57, 59). In 1953, however, Margaret Pittman (45) empirically proposed that a separate *H. haemolyticus* species designation should continue, according to the 5th edition of *Bergey's Manual of Determinative Bacteriology*, published in 1939 (3). Numerous phylogenetic studies, including the current study, clearly validate separation of the species (8, 9, 20, 25, 42, 47). In addition, the same studies have substantiated that *H. influenzae* and *H. haemolyticus* are perhaps the closest phylogenetic relatives of each other.

The recent edition of *Bergey's Manual of Systematic Bacteriology* suggests the possible existence of novel species that are intermediately related to *H. influenzae* and *H. haemolyticus* (27, 43). These species include *H. intermedius* subsp. *gazogenes* (a nonhemolytic taxon noted for its ability to produce gas in glucose broth, a distinctive feature of the nonhemolytic *H. haemolyticus* strains in this study) and *H. intermedius* subsp. *intermedius* (a species that grows independently of factor X). These proposals of novel species, however, are based only on a few strains that have been segregated with single DNA sequences (e.g., by use of the 16S rRNA gene alone). The increased recognition of recombination within and between species, as evidenced by the tremendous diversity in the genome sequences of recombinogenic bacteria (including *H. influenzae* [10, 19]), has prompted the recommendation that the phylogenetic designation of species should include analyses of multiple strains of each species together with MLSA (7, 15, 52).

The difficulty of separating individual strains by the analysis of individual genes is seen in the current study: *H. parainfluenzae* strain 1.33A segregated from the *Haemophilus* sensu stricto phylogenetic cluster in a dendrogram containing five concatenated gene sequences but failed to segregate from *H. influenzae* and *H. haemolyticus* in trees created from most individual DNA sequences. Thus, this strain, which is dependent only on factor V, may have been classified as *H. intermedius* subsp. *intermedius* if it had been assessed by the use of only one DNA sequence. Although it is not the focus of the current study, these observations also highlight the overall poor understanding of the interspecies relationships of *H. parainfluenzae* with *H. haemolyticus* and *H. influenzae*.

The hemolytic properties of *H. haemolyticus* are largely uncharacterized. In 1920, Stillman and Bourn (53) noted that the hemolytic activity of *Haemophilus* species isolated from the throat (which were referred to as Bacillus X but which were most likely *H. parahaemolyticus* or *H. haemolyticus*) was unfilterable and stable on ice but was heat labile at 56°C. In addition, strain-to-strain variations in the hemolytic phenotype were noted. As mentioned above, some *H. haemolyticus* strains, including the type strain, ATCC 33390, are known to have lost their beta-hemolytic phenotype on passage. Interestingly, we have found strain ATCC 33390 to be consistently hemolytic on 5% horse blood agar. Similar to what was reported by Stillman and Bourn (53), the hemolytic activity of this strain was unfilterable and heat labile at 56°C. In addition, the zonal size and clarity of beta-hemolysis among hemolytic *H. haemolyticus* strains in our collection were highly variable, but beta-hemolysis was consistently present (K. W. McCreA, J. Xie, C. F. Marrs, and J. R. Gilsdorf, unpublished data). The regulation of hemolytic activity in *H. haemolyticus* is unknown. The hemolytic phenotype in other bacteria is subject to variations in oxygen tension, the concentrations of specific ions, the type of basal medium used, and the type of animal blood used for analysis (24, 48, 49). Regardless of the mechanism, however, the observations of hemolysis in this study strongly support the conclusion of Kilian in 1976 that hemolysis is not a reliable trait to be used for the differentiation of some *Haemophilus* species (24, 25).

As mentioned above, neither hemolytic nor nonhemolytic *H. haemolyticus* strains have been associated with disease (40, 62). Therefore, distinguishing *H. influenzae* from other bacterial species infecting normally sterile sites (i.e., the middle ear or blood) can be done reliably by determining X and V growth factor dependence. The differentiation of *H. influenzae* from *H. haemolyticus* in culture specimens potentially contaminated by the pharyngeal microbiome may still rely, in part, on determining the absence of beta-hemolysis, since the prevalence of hemolytic *H. haemolyticus* strains in specimen collections is highly variable and ranges from 0 to 51% (5, 30, 40, 51). In contrast, a beta-hemolytic phenotype would not be useful in differentiating nonhemolytic *H. haemolyticus* strains, which are now known to be significantly prevalent in the pharyngeal cavities of healthy children and of adults with COPD (40, 62). A rapid, clinically useful marker for species differentiation is currently not available. In our study, dot blot hybridization with *iga* and *lgtC* gene probes specifically differentiated *H. haemolyticus* from *H. influenzae*, but further work is necessary to refine their clinical applicability.

Although *H. influenzae* and *H. haemolyticus* are closely related commensal organisms of the human pharynx and both are subject to the same host or environmental factors facilitating infection, only *H. influenzae* has the genetic constitution necessary to cause disease. Thus, comparative genomic studies at the species population level should be able to differentiate unique genotypes of *H. influenzae* that are associated with disease from common genotypes of both species that are associated with commensal growth. The data presented in the current study begin to examine this. Significant differences in the prevalence of *H. influenzae* LOS genes associated with virulence were found between the species. The *lic2A* and *lgtC* genes were nearly ubiquitous in *H. influenzae* ($\geq 90\%$) but were only 16 and 2% prevalent, respectively, in *H. haemolyticus*. Interestingly, the combined expression of these genes in *H. influenzae* is necessary to form a LOS digalactoside structure (34, 35) that antigenically mimics the pK blood group of humans and increases the serum resistance of the organism by inhibiting the deposition of the C4b released from the classical complement pathway (21, 61). Of the 17 *H. haemolyticus* strains in this study that possessed a *lic2A* gene, none possessed the *lgtC* gene (unpublished data), suggesting that the digalactoside structure is not made in *H. haemolyticus*. In contrast to the absence of genes facilitating the formation of the digalactoside structure in *H. haemolyticus*, the *licA* gene of *H. influenzae* was found in close to half (42%) of the *H. haemolyticus* strains. In *H. influenzae*, the *licA* gene is part of the *lic-1* locus (which contains the *licA*, *licB*, *licC*, and *licD* genes), required for modifying LOS with phosphorylcholine (or ChoP). ChoP structurally mimics phosphatidylcholine, an abundant component in human cell membranes, and in *H. influenzae* ChoP has been shown to provide a defense against host-derived antimicrobial peptides (33) and to mediate bacterium adherence to and invasion of host cells through the platelet-activating factor receptor (55). In addition, ChoP has been shown to enhance the ability of *H. influenzae* to colonize the middle ear and induce otitis media in the chinchilla model of infection (56). The presence of the *licA* gene in the *H. haemolyticus* strains of this study suggests that they may contain a *lic-1* locus capable of expressing ChoP. If this were found to be the case, the relatively high prevalence of strains with this locus would suggest that ChoP facilitates normal commensal growth, since *H. haemolyticus* has not been implicated as a disease agent. Further studies, however, are necessary to demonstrate ChoP expression, its association with LOS, and its function in *H. haemolyticus* compared with the function of ChoP expression in *H. influenzae*.

Although this study has focused on the phylogenetic and taxonomic relationships of *H. influenzae* and *H. haemolyticus*, the question of how these organisms differ in regard to their virulence and commensalism is more prominent. Although *H. influenzae* is a serious pathogen, it is by far more devoted to commensal growth rather than pathogenic growth in humans. Yet, little is clearly understood about how genetic factors facilitate the different growth environments. Comprehensive taxonomic studies between *H. influenzae* and *H. haemolyticus* by the use of modern bioinformatic tools should be able to generate genotypic and phenotypic blueprints contrasting species similarities that facilitate commensal growth from species differences that facilitate virulent growth in *H. influenzae*.

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