Relationships of non-typeable *Haemophilus influenzae* with hemolytic and non-hemolytic

*Haemophilus haemolyticus*

Running title: Relationships of *H. influenzae* and *H. haemolyticus*

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ABSTRACT

*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 non-hemolytic *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 multi-locus sequence analysis (MLSA). All strains hybridizing with a probe to iga, a gene encoding an IgA protease of *H. influenzae*, clustered apart from strains that did not hybridize to 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 MLSA-gene sequences to 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*. 
INTRODUCTION

H. 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 non-typable or 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 content, and both are nearly indistinguishable by their colony and cellular morphology (25). Initial phylogenetic studies using DNA-DNA hybridization revealed that H. haemolyticus associated with H. influenzae in a cluster of the Pasteurellaceae family termed Haemophilus sensu stricto (6, 41, 47), and these results were substantiated using 16s rDNA (8, 9, 43), infB (20), or multilocus sequence analysis (MLSA) (42).

Despite the close relationship of the species, only a β-hemolytic phenotype of H. haemolyticus is routinely used in the clinical setting to distinguish H. haemolyticus from H. influenzae (45, 46, 54). β 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 non-hemolytic H. haemolyticus (38, 40, 62). Murphy and colleagues
found that close to 40% of isolates in a *H. influenzae* collection obtained from the sputum of COPD patients were *H. haemolyticus*, and their identity was confirmed by phylogenetic studies and by amino-acid sequence differences in the P6 OMP (40). Their study also found that more than 20% of a *H. influenzae* collection obtained from the throats of healthy children attending day care was *H. haemolyticus* and this was confirmed on a larger set of strains by Xie et al (62), who also identified non-hemolytic *H. haemolyticus* by their inability to hybridize with a probe made to *iga*, a *H. influenzae* gene encoding the virulence-associated IgA protease (28). Similar to hemolytic *H. haemolyticus*, these studies failed to find an association of the non-hemolytic species with disease: non-hemolytic *H. haemolyticus* were not present in 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 respiratory tract isolates present in the *H. influenzae* 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 first noted strains of *H. haemolyticus* that had lost their hemolytic phenotype) found that only the production of gas during glucose metabolism, H$_2$S 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 non-pathogenic *Haemophilus* species, expressed an IgA protease. In a phylogenetic study of the family *Pasteurellaceae*, Hedegaard et al. (20), however, noted two *H. haemolyticus* strains (HK855 and HK856) that had lost their hemolytic activity but expressed IgA protease. One strain, HK855, produced gas during glucose metabolism and produced H$_2$S (*H. haemolyticus*-associated traits). Phylogenetic analysis using the *infB* gene revealed that HK865 was more related to *H. influenzae* than to *H. haemolyticus*, but that HK855, the gas and H$_2$S 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 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 strain collections of NT H. influenzae and H. haemolyticus (hemolytic and non-hemolytic) 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 lgtC) contributing to virulence-associated lipooligosaccharide (LOS) structures.

MATERIALS AND METHODS

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

Four H. influenzae reference strains were used in this study and represent complete or partially genome-sequenced strains: Rd (KW-20, ATCC 51907), 86-028NP [a NT
nasopharyngeal strain isolated from a child with otitis media], R2846 (strain 12, a NT otitis media strain), and R2866 (INT-1, ATCC 51997, a NT blood isolate). One *H. haemolyticus* type strain, ATCC 33390, was also used. The remaining strains were parts of various collection obtained by this or other laboratories in previous studies (Table 1) (11, 16, 29, 38, 40, 50, 52). Briefly, the *H. influenzae* strains were isolated from the throats of healthy children attending day care, from the throats of healthy adults in a longitudinal carriage study, or from the middle ear aspirates of children with otitis media. The *H. haemolyticus* strains were isolated from either the healthy throats of children attending day care, the adult longitudinal carriage study, or from the sputum of adults enrolled in a COPD exacerbation study (Table 1). *H. haemolyticus* from COPD patients were shown not to be new strain acquisitions leading to COPD exacerbation (40).

*H. influenzae* and non-hemolytic *H. haemolyticus* 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, 52). Hemolysis, used to differentiate hemolytic *H. haemolyticus*, was assessed as zones of β hemolysis surrounding individual colonies grown on horse blood agar (Remel, Lenexa, KS). Working stocks of each strain were made by growing original stocks on chocolate agar (BD Biosciences, San Jose, CA) and then harvesting O/N growth into 2 ml of sterile skim milk. 900 µl aliquots were then placed in duplicate 1-ml 96 well plates. The plates were subsequently sealed and frozen at -80 ºC. 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 bacterial isolates using the Wizard Genomic DNA purification kit from Promega, Co. (Madison, WI). After purification,
DNA from the strains was diluted in half and 1 µl of each was aliquoted to multiple unskirted 96 well thermocycler plates from Bio-Rad Laboratories, Inc. (Hercules, CA). The plates were stored at -20 ºC until needed. PCR amplification was performed using 50 µl reactions comprised of 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, 94 ºC template denaturing; 1 min, 51-55 ºC primer annealing (depending on primer specificity); and 1 min, 72 ºC chain extension.

**DNA sequencing.** Partial DNA sequences of the *adk*, *pgi*, *recA*, and *infB* genes were generated for multilocus sequence analysis (MLSA) according to the strategy used by Nørskov-Lauritsen et al. (42), with the exception that oligonucleotides for PCR amplification of these genes in the present study were based on sequences from the *H. influenzae* strain, Rd (Table 2). Oligonucleotides for partial 16s rDNA sequences for the differentiation of *Haemophilus* species have been previously described (Table 2) (32). After confirming PCR amplification by gel electrophoresis on 1% agarose, the PCR products were purified using the 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 *Pasteurellaceae* family including *H. haemolyticus*, *H. parainfluenzae*, *H. pittmaniae*, *A. lignieresii*, *A. pleuropneumoniae*, *H. segnis*, *H. aphrophilus*, *H. paraphrophilus*, *A. actinomycetemcomitans*, and *P. multocida* have been described for *adk*, *pgi*, and *recA* (42) and for *infB* (20). 16s rDNA sequences for a subset of these strains (shown in Figure 1) are have also been previously
published (8, 9). Relevant sequences from *E. 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 version 7.0 (DNASTar, Madison, WI), and phylogenetic analysis was done with Mega 3.1 (31). Bootstrap consensus, minimum-evolution dendrograms for concatenated or individual sequences were made with 1000 replicates.

**Classical taxonomy.** Biochemical and phenotypic analysis of *H. influenzae* and *H. haemolyticus* traits were performed as previously described by Kilian (26, 25). Briefly, the emission of H$_2$S was assessed with lead acetate strips (Fisher Scientific, Pittsburgh, PA) placed on the lids of chocolate agar plates inoculated with individual bacterial strains. Conversion of the strips from white to black after ON growth was considered positive. Production of gas from glucose metabolism was assessed by gas collected 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 Kovacs reagent (Remel) and observing for a purple color reaction, urease activity was assessed in Rapid Urea Broth (BD) according to the manufacturers instructions by mixing an inoculum loop of bacteria from plate growth and observing for a color reaction after four hours of incubation at 37 ºC, and ODC activity was determined with ODC broth (Remel) according to the manufacturer’s instructions.

**Dot-blot immunoassays for 7F3 mAb recognition of the P6 OMP.** Detection of the P6 OMP by 7F3 mAb in dot-blot immunoassays has been described previously (17). Briefly,
membranes were prepared by harvesting bacteria directly from chocolate agar growth onto nitrocellulose membranes with a sterile pipet tip. The blots were immediately washed in PBS and then blocked in PBS containing 5% non-fat dry milk [blotto (22)] for 1 hour. The mAb, 7F3 was diluted 1:10 in blotto and incubated ON at room temperature. After washing three times in PBS alone, the blots were then incubated in PBS containing goat, anti-mouse IgA antibody conjugated to alkaline phosphatase (Sigma) diluted 1:5000 for 1 hour. Blots were then washed three times in PBS and exposed to NBT/BCIP substrate (Pierce, Rockford, IL) for color development.

**Dot-blot hybridization.** Membranes dotted with single stranded genomic DNA obtained from crude lysates of each *Haemophilus* strains were made using a modification of Foxman et al. (13, 14). Bacteria were grown O/N on chocolate agar and suspended in 4 ml of PBS to O.D. 1.0. Bacteria were then centrifuged and the pellets stored at -20 ºC until all strains were collected. Pellets were then thawed and resuspended in 8 ml of lysis solution (0.4 M NaOH and 10 mM EDTA) and heated for 30 min. in a 70 ºC water bath. One ml aliquots of each extract were placed in multiple, 1-ml 96 well plates. The plates were sealed and frozen at -80 ºC until needed. Dot-blot membranes were made by applying 40 µl of each DNA extract to nylon membrane in a 8 x 12 array, Bio-Dot Microfiltration Apparatus (Bio-Rad) as previously described (13, 14). Generation of probes, hybridization, and analysis were carried out as previously described (44, 63). Oligonucleotides for the amplification of the iga, licA, lic2A, and lgtC gene probes were generated from *H. influenzae* strain Rd, and oligonucleotides for the LOS genes were directed to the non-repeat regions of each gene (Table 2). DNA was amplified by PCR as described above. PCR products were purified from agarose gels using the QIAquick Gel Extraction Kit from
Qiagen, and labeled with the AlkPhos Direct™ Labeling and Detection Systems (GE Healthcare, Piscataway, NJ). Probes were hybridized to the dot-blot membranes under stringent conditions and developed by the ECF detection system (GE Healthcare). Probe signal intensity was read with the Storm™ 860 phosphorimager and analyzed with ImageQuant version 5.0 software (Molecular Dynamics/GE Healthcare).

Statistical analyses. Data were entered in Excel software (Microsoft) in binary form for the presence (1) or absence (0) of any given trait. The prevalence ratios (PR) of traits for *H. influenzae* compared to traits in *H. haemolyticus* or for traits in non-hemolytic compared to hemolytic *H. haemolyticus* were calculated as a ratio of trait proportions among each species or collection; for example, the proportion of all *H. influenzae* having a particular trait over the proportion of all *H. haemolyticus* having that same trait. $\chi^2$ analysis was used to determine the significance differences of trait associations between species or between non-hemolytic and hemolytic *H. haemolyticus*. The Student’s t-Test was used to assess significant differences in trait distributions 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 with accession numbers EU150426-EU150625 (for *adk*), EU150626-EU150825 (for *pgi*), EU150826-EU151025 (for *recA*), EU151026-EU151225 (for *infB*), and EU151226-EU151425 (for 16s rDNA).
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 on partial, concatenated *adk, pgi, recA,* and *infB* gene sequences. Since this approach revealed that both *H. influenzae* and *H. haemolyticus* were present in a *Haemophilus sensu stricto* phylogenetic cluster, separate from other members of the family, we have used it to determine if the NT *H. influenzae* and *H. haemolyticus* (hemolytic and non-hemolytic) strains in our study also maintain the same relationships. A 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 to not be present in other members of the Pasteurellaceae family (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* and one *H. haemolyticus* type strains, and nine other members of the Pasteurellaceae family (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 Pasteurellaceae family (data not shown). The 3 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 re-assessed the X and V growth factor requirements of these 3 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 re-assessed for their dependence on factors X and V and
all strains were found to require both. These results, together with the MLSA data, support the
designation of these 197 strains as either *H. influenzae* or *H. haemolyticus*.

Because 16s rDNA sequences were previously used to define members of the
*Pasteurellaceae* family (43) and to separate the non-hemolytic form of *H. haemolyticus* from *H.
influenzae* (40), partial sequences of 16s rDNA were obtained from the *H. influenzae* and *H.
haemolyticus* test strains described above and concatenated with the four housekeeping gene
sequences. A dendrogram created from the extended sequences of these strains, together with the
*H. influenzae* and *H. haemolyticus* type strains and four members of the *Pasteurellaceae* family
for which published 16s rDNA sequences were available, revealed that all *H. influenzae* and *H.
haemolyticus* strains remained in a *Haemophilus sensu stricto* cluster similar to that described
with the four housekeeping genes alone (Fig. 1 and 2A). Within the cluster, some branches were
found to contain groups of 2-5 strains with identical sequences. 88 of 197 strains (45%) in the
collection had sequences identical to one or more strains and the remaining strains, with 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 containing four other
members of the *Pasteurellaceae* family (Fig. 1). The *Haemophilus sensu stricto* cluster at node I
was composed of 6 sub-nodes designated A-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 emerging from nodes B-E (Fig. 1).

**Phylogenetic distribution of iga gene-probe hybridization and P6 mAb reactivity**

between *Haemophilus* species. In a previous study, *H. influenzae* was defined as X and V
dependent, non-hemolytic strains that both hybridized to an iga gene probe and reacted with a P6 OMP mAb, 7F3, an antibody previously shown to react with the conserved P6 OMP of H. influenzae but not with H. haemolyticus (40, 62). These strains were distinguished from non-hemolytic H. haemolyticus that lacked at least one of these traits (62). Therefore, the 197 strains belonging to the Haemophilus sensu stricto collection above were also examined for their ability to hybridize with an iga gene probe in 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 dendrogram hybridized with the iga gene probe, while the 109 strains in clades and branches emerging from nodes B-F did not hybridize to the probe (Fig. 1). Next, a dot-blot immunoassay was performed on all strains with the 7F3 mAb. The four H. influenzae type strains, but not the H. haemolyticus type strain, reacted with the 7F3 mAb. All but 3 of the 88 strains (97%) that hybridized to the iga gene probe also reacted with 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 of 7F3 antibody reactivity were distributed throughout the dendrogram. Thus, species designation of the strains in this study will be made primarily by the MLSA and iga-gene probe hybridization. In this context, the data suggest that node A of the MLSA dendrogram contains 88 H. influenzae strains that hybridize with an iga gene probe and predominantly react with the 7F3 mAb, while nodes B-F of the dendrogram contain 109 H. haemolyticus strains (hemolytic or non-hemolytic) that lack iga hybridization and mostly lack 7F3 mAb reactivity.
Distribution of classically defined taxonomic traits between the *Haemophilus* species.

Based on this stratification in species, we estimated the distribution of classically defined taxonomic traits such as the production of gas during glucose metabolism, H$_2$S 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 segregate differently between the species.

As expected, the *H. haemolyticus* type strain, ATCC 33390, was found to produce gas during glucose metabolism, emit H$_2$S, and lack ODC activity while the four *H. influenzae* type strains expressed the opposite phenotypes (with the exception of 86-028NP which lacked ODC activity). Gas production and H$_2$S emission were, respectively, 69 and 88% prevalent among the phylogenetically defined *H. haemolyticus* strains and 12 and 6% prevalent in the phylogenetically defined *H. influenzae* strains (Table 3 and Fig. 1), suggesting that these traits were 17 and 6 times more prevalent (*P* < .05 for both traits) in *H. haemolyticus* than in *H. influenzae* (prevalence ratios are the inverse of those shown in Table 3). In contrast, ODC activity was about 4 times more prevalent (*P* < .05) among the *H. influenzae* than the *H. haemolyticus* strains. Indole production and urease activity were significantly different (*P* < .05) but only 1.3 and 1.2 times, respectively, more prevalent in *H. influenzae* than in *H. haemolyticus* (Table 3). Because *H. influenzae* and *H. haemolyticus* are such close phylogenetic relatives, we unconventionally assigned *H. influenzae* biotype profiles (I-VIII) to *H. haemolyticus* strains and compared the prevalence of the profiles between the species. Biotype I was almost 6 times more
prevalent among *H. influenzae* strains and biotype III was about 2 times more prevalent among *H. haemolyticus* strains (*P* < .05 for both traits). The overall prevalence 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 a previous taxonomic study by Kilian that used 9 *H. haemolyticus* strains (25) but none of the traits were able to completely differentiated *H. influenzae* from *H. haemolyticus* in our larger strain collections.

**Distribution of LOS genes with tetranucleotide repeats among *H. influenzae* and *H. haemolyticus***. Because the presence or absence of the virulence-associated iga gene appears to corroborate the MLSA data 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 to facilitate virulence (2, 37). Because *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* compared to *H. influenzae*.

Dot-blot hybridization was performed with probes made to 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%, respectively, of *H. influenzae* strains and to 42, 16, and 2%, respectively, of *H. haemolyticus* strains (Table 3). Prevalence ratios calculated from these values showed that the licA, lic2A, and lgtC genes were about 2, 6, and 54 times,
respectively, more prevalent ($P < .05$ for all associations) in \textit{H. influenzae} than in \textit{H. haemolyticus} (Table 3). Of the three LOS contingency genes, these data suggest that only \textit{lgtC} segregates between the species similar to the \textit{iga} gene. Although the prevalence of the \textit{licA} and \textit{lic2A} genes was high in \textit{H. influenzae}, they were variably present among \textit{H. haemolyticus}. In this regard, the relatively high prevalence of the \textit{licA} gene in \textit{H. haemolyticus} (42\%) may suggest that it facilitates the normal commensal growth of \textit{H. haemolyticus}.

\textbf{Recombination between species.} The \textit{H. influenzae} and \textit{H. haemolyticus} species designation in this study is 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 \textit{H. influenzae} and \textit{H. haemolyticus}, we examined the ability of individual, rather than concatenated, MLSA genes to segregate the species. Compared to the MLSA dendrogram containing the concatenated sequences (shown as a radial view in Fig. 2A), dendrograms constructed from individual \textit{adk}, \textit{pgi}, \textit{recA}, \textit{infB}, and 16s rDNA sequences (Fig. 2B-F, respectively) failed to completely separate \textit{H. influenzae} from \textit{H. haemolyticus}. In addition, only the \textit{recA} sequence was able to maintain a complete \textit{Haemophilus sensu stricto} cluster separate from four other members (5 strains) of the \textit{Pasteurellaceae} family used in the trees. 16s rDNA sequences poorly defined the cluster, and the \textit{H. parainfluenzae} strain, 1.33A, did not segregate from the \textit{H. influenzae} and \textit{H. haemolyticus} strains with individual \textit{adk}, \textit{pgi}, or \textit{infB} sequences.
Next, we investigated the potential for the unequal distribution of species-specific traits among MLSA-based nodes separating the species. For instance, strains that phylogenetically border the species dividing line in the MLSA 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-F containing all *H. haemolyticus* strains. In addition, node A is composed of three clades emerging at sub-nodes A1 (72 strains), A2 (8 strains), and A3 (8 strains). In the *H. haemolyticus* strains of nodes B-F, 14 strains are found in nodes B-E while 95 strains are more distally positioned in the clade emerging from node F. Therefore, trait prevalence was compared between the *H. influenzae* strains at node A1 and the combined strains (to increase sample size) at nodes A2 and A3. Similarly, trait prevalence in *H. haemolyticus* strains was compared between the combined strains at nodes B-E and the strains at node F.

*H*$_2$S production (a *H. haemolyticus*-associated trait) was found to be significantly less prevalent among *H. influenzae* strains at node A1 (6%) than among *H. influenzae* strains at nodes A2-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-A3 (63, 75, and 56%, respectively) (*P* < .05 for all associations) (Table 4). Although not statistically different, *H*$_2$S production was less prevalent in *H. haemolyticus* strains at nodes B-E (50%) than in *H. haemolyticus* strains at node F (72%), and indole production, the *licA* gene, and the *lic2A* gene were more common in strains of nodes B-E (71, 57, and 21%, respectively) than strains of node F (61, 40, and 15%, respectively). In addition, strains at nodes B-E were twice as likely as strains at node F to react with the mAb, 7F3 (an *H. influenzae*-associated trait) (Table 4). In contrast to
the above results, however, possession of an \textit{lgtC} gene and gas production appeared equally
distributed between clades within the same species, and urease and ODC activity did not exhibit
the trends described above (Table 4). Together, the results of these phylogenetic and taxonomic
studies suggest the possibility of recombination between \textit{H. influenzae} and \textit{H. haemolyticus}.

\textbf{Distribution of traits between hemolytic and non-hemolytic \textit{H. haemolyticus}.} The
relationships between hemolytic and non-hemolytic \textit{H. haemolyticus} are poorly understood. In
the MLSA-based dendrogram (Fig. 1), 33 hemolytic \textit{H. haemolyticus} strains were inter-dispersed
among 62 non-hemolytic \textit{H. haemolyticus} strains emerging from node F, suggesting that
hemolytic and non-hemolytic \textit{H. haemolyticus} do not cluster as a separate sub-species. No
hemolytic strains were present in the 14 strains emerging from nodes B-E that bordered the
species dividing line. Stratification of the molecular and classical taxonomic traits described
above between hemolytic and non-hemolytic \textit{H. haemolyticus} strains from all nodes revealed
that the prevalence of H$_2$S emission, gas formation during glucose metabolism, ODC activity,
and possession of an \textit{lgtC} gene were not significantly different ($P > .05$) between hemolytic and
non-hemolytic \textit{H. haemolyticus} (Table 5). Although urease activity was significantly more
prevalent in hemolytic than non-hemolytic strains, reactivity with the 7F3 mAb and possession
of the \textit{licA} and \textit{lic2A} genes were all significantly more prevalent ($P < .05$ for all associations) in
non-hemolytic than in hemolytic strains (Table 5). Of the 13 total \textit{H. haemolyticus} strains that
reacted with 7F3, none were hemolytic, and of the 17 total \textit{H. haemolyticus} strains that
hybridized with the \textit{lic2A} probe, only one was hemolytic. Similarly, the \textit{licA} gene was almost
twice as prevalent in non-hemolytic than in hemolytic \textit{H. haemolyticus}. To decrease the potential
of interspecies recombination contributing to these results, the prevalence of the traits was
recalculated for \textit{H. haemolyticus} strains emerging only from node F. With the exception of
urease (which lost its statistical significance), the associations remained very similar to those
obtained when all \textit{H. haemolyticus} were used (data not shown). Although hemolytic and non-
hemolytic \textit{H. haemolyticus} appear to be phylogenetically inseparable, the results suggest the
possibility that some genes and traits common to \textit{H. influenzae} exist more frequently in non-
hemolytic than hemolytic \textit{H. haemolyticus}.

\section*{DISCUSSION}

Since its first description as “Bacillus X” in 1919, \textit{H. haemolyticus} has been differentiated
from \textit{H. influenzae} predominantly by its ability to produce hemolysis on blood agar plates (46).
Shortly after this description, arguments were made that \textit{H. haemolyticus} was a hemolytic variant
of \textit{H. influenzae}, since the two species appeared closely related by X and V growth-factor
dependency and other criteria (36, 57, 59). In 1953 Dr. Margaret Pittman (45), however,
empirically proposed that a separate \textit{H. haemolyticus} species designation should continue
according to the 1939, 5\textsuperscript{th} edition of \textit{Bergey’s Manual of Determinative Bacteriology} (3).
Numerous phylogenetic studies, including the current study, clearly validate separation of the
species (8, 9, 20, 25, 42, 47). In addition, these same studies have substantiated that \textit{H. influenzae}
and \textit{H. haemolyticus} are close, or perhaps the closest, phylogenetic relatives of each other.
The recent edition of \textit{Bergey’s Manual of Systematic Bacteriology} suggests the possibility of
novel species that are intermediately related to \textit{H. influenzae} and \textit{H. haemolyticus} (27, 43). These
species include \textit{H. intermedius} subsp. gazogenes (a non-hemolytic taxon noted for its ability to
produce gas in glucose broth, a distinctive feature of non-hemolytic \textit{H. haemolyticus} strains in
this study) and *H. intermedius* subsp. *intermedius* (a species that grows independently of factor X). These proposed species, however, are based only on a few strains that have been segregated with single DNA sequences (e.g. 16s rDNA alone). The increased recognition of recombination within and between species, evidenced by the tremendous diversity in the genome sequences of recombinogenic bacteria [including *H. influenzae* (10, 19)], have prompted the recommendation that phylogenetic designation of species should include multiple strains of each species together with MLSA (7, 15, 53). The difficulty of individual strains with individual gene analysis is seen in this current study: the *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, dependent on only factor V, may have been classified as *H. intermedius* subsp. *intermedius* if assessed by only one DNA sequence. Although not the focus of this current study, these observations also highlight the overall poor understanding of interspecies relationships of *H. parainfluenzae* with *H. haemolyticus* and *H. influenzae*.

The hemolytic properties of *H. haemolyticus* are largely uncharacterized. In 1920, Stillmen and Bourn (54) noted that the hemolytic activity of *Haemophilus* species isolated from the throat (referred to as Bacillus X, but most likely *H. parahaemolyticus* or *H. haemolyticus*) was unfilterable and stable on ice but was heat labile at 56 ºC. In addition, strain to strain variation in the hemolytic phenotype was noted. As mentioned above some *H. haemolyticus* strains, including the type strain, ATCC 33390, are known to have lost their β hemolytic phenotype on passage. Interestingly, we have found the ATCC 33390 strain to be consistently hemolytic on 5% horse blood agar. Similar to what was reported by Stillmen and Bourn (54), the hemolytic
activity of this strain was unfilterable and heat labile at 56 ºC. In addition, the zonal size and
clarity of β hemolysis among hemolytic *H. haemolyticus* strains in our collection was highly
variable but 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, concentrations
of specific ions, the type of basal media 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 use for
differentiating some *Haemophilus* species (24, 25).

As mentioned above, neither hemolytic nor non-hemolytic *H. haemolyticus* have been
associated with disease (40, 62). Therefore, distinguishing *H. influenzae* from other bacterial
species infecting normally sterile sites (i.e. middle ear or blood) can be made reliably by
determining X and V growth-factor dependence. 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 β hemolysis, since the prevalence of hemolytic
*H. haemolyticus* in specimen collections is highly variable ranging from 0 to 51% (5, 30, 40, 51).
In contrast, a β hemolytic phenotype would not be useful in differentiating non-hemolytic *H.
haemolyticus* that are now know to be significantly prevalent in the pharyngeal cavities of
healthy children and 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. Data presented in this current study begins to examine this. Significant differences were found between the species in the prevalence of *H. influenzae* LOS genes associated with virulence. The *lic2A* and *lgtc* genes were nearly ubiquitous in *H. influenzae* (≥ 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 serum resistance of the organism by inhibiting C4b deposition 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 formation of the digalactoside structure in *H. haemolyticus*, the *licA* gene of *H. influenzae* was found in close to half (42%) of *H. haemolyticus* strains. In *H. influenzae*, the *licA* gene is part of the *lic1* locus (containing 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 defense against host derived, anti-microbial peptides (33) and to mediate adherence 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 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 lic1 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 as
compared with 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 virulence
and commensalism is more prominent. H. influenzae, although a serious pathogen, is by far more
devoted to commensal, rather than pathogenic, growth in humans. Yet little is clearly understood
about how genetic factors facilitate different growth environments. Comprehensive taxonomic
studies between H. influenzae and H. haemolyticus utilizing modern bioinformatic tools should
be able to generate genotypic and phenotypic blueprints outlining species similarities that
facilitate commensal growth from species differences that facilitate virulent growth in H.
influenzae.

ACKNOWLEDGEMENTS

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Communication Disorders.

We thank Patricia Juliao for assistance with statistical analysis.


of hemagglutinating pili of Haemophilus influenzae type b with similar structures of
Zhong, J. Gipson, M. Gipson, L. S. Johnson, L. Lewis, L. O. Bakaletz, and R. S.
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20. Hedegaard, J., H. Okkels, B. Bruun, M. Kilian, K. K. Mortensen, and N. Norskov-
Lauritsen. 2001. Phylogeny of the genus Haemophilus as determined by comparison of
expression modulates resistance to C4b deposition on an invasive nontypeable


29. **Krasan, G. P., D. Cutter, S. L. Block, and J. W. St Geme, 3rd.** 1999. Adhesin expression in matched nasopharyngeal and middle ear isolates of nontypeable...


**FIGURE LEGENDS**

**FIG 1.** Minimum-evolution dendrogram of the *Haemophilus sensu stricto* cluster. The tree, rooted by *E. coli* strain K12, is based on concatenated *adk, pgi, recA, infB*, and 16s rDNA sequences with ≥ 50% of 1000 bootstraps indicated. Node I contains all *H. influenzae* and hemolytic and non-hemolytic *H. haemolyticus* strains within a *Haemophilus sensu stricto* cluster while nodes II and III contain selected members of the *Pasteurellaceae* family. Strains with identical sequences are listed on the same branch. Red boxes define β-hemolytic *H. haemolyticus* strains. Positive (+) and negative (-) results for the *iga* gene-probe hybridization, the P6 OMP mAb reactivity, the production of gas during glucose metabolism, and H₂S production, are shown to the right of each strain. Results are also relative to the strain order on branches with multiple strains. Based on the stratification of *iga* and other results (see text and Table 2, below) node A is proposed to contain all *H. influenzae* strains while nodes B-F contain all *H. haemolyticus* strains.
FIG. 2. Multiple and single DNA-sequence segregation of \textit{H. influenzae} and \textit{H. haemolyticus}. Radiation tree views of the A) five concatenated sequences, and individual B) \textit{adk}, C) \textit{pgi}, D) \textit{recA}, E) \textit{infB}, and F) 16s rDNA sequences. \textit{H. influenzae} (purple dots) and \textit{H. haemolyticus} (red dots) strains are shown together with other members of the \textit{Pasteurellaceae} family (green triangles).
TABLE 1. Population and ecological stratification of the *H.*

*influenzae* and *H. haemolyticus* collections

<table>
<thead>
<tr>
<th>Epidemiologic segmentation (n)</th>
<th><em>H. influenzae</em></th>
<th><em>H. haemolyticus</em></th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation site</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle ear (44)</td>
<td>44</td>
<td>0</td>
<td>(15, 28)</td>
</tr>
<tr>
<td>Healthy throat (107)</td>
<td>44</td>
<td>63</td>
<td>(10, 36, 49)</td>
</tr>
<tr>
<td>Sputum (46)</td>
<td>0</td>
<td>46</td>
<td>(38, 48)</td>
</tr>
<tr>
<td>Age groups</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult (67)</td>
<td>6</td>
<td>61</td>
<td>(36, 38, 48)</td>
</tr>
<tr>
<td>Child (130)</td>
<td>82</td>
<td>48</td>
<td>(10, 49)</td>
</tr>
</tbody>
</table>
TABLE 2. Oligonucleotides used for PCR or DNA sequencing

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Position in Rd genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>adk</td>
<td>F: GGTGCACCGGGTGAGGTAA</td>
<td>376521</td>
</tr>
<tr>
<td></td>
<td>R: CCTAAGATTTATCTAACTC</td>
<td>375899</td>
</tr>
<tr>
<td>pgi</td>
<td>F: GGTGAAAAATCAATCGTAC</td>
<td>1644657</td>
</tr>
<tr>
<td></td>
<td>R: ATTGAAAGACCAATAGCTGA</td>
<td>1645246</td>
</tr>
<tr>
<td>recA</td>
<td>F: ATGGCAACTCAAGAAGAAAA</td>
<td>622556</td>
</tr>
<tr>
<td></td>
<td>R: TTACCAAACATCAAGCCCTAT</td>
<td>621940</td>
</tr>
<tr>
<td>infB</td>
<td>F: TGAAAATGAGCTTGAAGAAGCGG</td>
<td>1361550</td>
</tr>
<tr>
<td></td>
<td>R: GATAGTTGCCACAGGCGCGACGACC</td>
<td>1362198</td>
</tr>
<tr>
<td>16s</td>
<td>F1: CCAGCAGCAGCGGTAAATACG</td>
<td>624342</td>
</tr>
<tr>
<td>rDNA</td>
<td>R1: ATCGGYTACCTTGGTACGACTTC</td>
<td>625335</td>
</tr>
<tr>
<td></td>
<td>F2: GCCGCACAAGCCTGGGAGCATGTG</td>
<td>624751</td>
</tr>
<tr>
<td></td>
<td>R2: CTCGTAAGGCGCATGACTTGAGC</td>
<td>625039</td>
</tr>
<tr>
<td>iga</td>
<td>F: GTTCCAACAAGCTGCTGCTAC</td>
<td>1050386</td>
</tr>
<tr>
<td></td>
<td>R: GTTATATTGCCCCCTCGTTATTCA</td>
<td>1049130</td>
</tr>
<tr>
<td>licA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>F: GTAGGATTGGTTAAAACCTTACAAAGCC</td>
<td>1608693</td>
</tr>
<tr>
<td></td>
<td>R: GGCAATTTCCTCTCAAAGCTTGCTGC</td>
<td>1609579</td>
</tr>
<tr>
<td>lic2A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>F: ATATTACATAATAGAGGAATCTAG</td>
<td>571382</td>
</tr>
<tr>
<td></td>
<td>R: CTACATAAAAAGGAACATTTTCCTACC</td>
<td>570690</td>
</tr>
<tr>
<td>lgtC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>F: CGGACTGTCAAGTCAGACAATG</td>
<td>289338</td>
</tr>
<tr>
<td></td>
<td>R: CTCAAAATGATCATACCAAGATG</td>
<td>288499</td>
</tr>
</tbody>
</table>
**a** All oligonucleotides based on DNA sequences from *H. influenzae* strain Rd.

**b** Forward primers begin downstream of tetranucleotide repeats.
TABLE 3. Distribution of taxonomic traits among *H. influenzae* and *H. haemolyticus*

<table>
<thead>
<tr>
<th>Traits</th>
<th>H. influenzae^a^</th>
<th>H. haemolyticus^a^</th>
<th>X^2 (P value)^c^</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular based traits</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>iga</em> gene</td>
<td>88 (100)</td>
<td>0 (0.0)</td>
<td>referent</td>
</tr>
<tr>
<td>mAb 7F3</td>
<td>85 (96.6)</td>
<td>13 (11.9)</td>
<td>8.10 &lt;.0001</td>
</tr>
<tr>
<td>Classical traits</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2S</td>
<td>11 (12.5)</td>
<td>75 (68.8)</td>
<td>0.18 &lt;.0001</td>
</tr>
<tr>
<td>gas</td>
<td>5 (5.7)</td>
<td>96 (88.1)</td>
<td>0.06 &lt;.0001</td>
</tr>
<tr>
<td>indole</td>
<td>73 (83.0)</td>
<td>68 (62.4)</td>
<td>1.33 .0015</td>
</tr>
<tr>
<td>urease</td>
<td>74 (84.1)</td>
<td>77 (70.6)</td>
<td>1.19 .0265</td>
</tr>
<tr>
<td>ODC</td>
<td>24 (27.3)</td>
<td>8 (7.3)</td>
<td>3.72 .0002</td>
</tr>
<tr>
<td>I</td>
<td>18 (20.5)</td>
<td>4 (3.7)</td>
<td>5.57 .0002</td>
</tr>
<tr>
<td>II</td>
<td>44 (50.0)</td>
<td>43 (39.4)</td>
<td>1.27 .1382</td>
</tr>
<tr>
<td>III</td>
<td>12 (13.6)</td>
<td>28 (25.7)</td>
<td>0.53 .0366</td>
</tr>
<tr>
<td>IV</td>
<td>0 (0) ^c^</td>
<td>2 (1.8)</td>
<td>0.31 .2015</td>
</tr>
<tr>
<td>V</td>
<td>5 (5.7)</td>
<td>2 (1.8)</td>
<td>3.10 .1470</td>
</tr>
<tr>
<td>VI</td>
<td>1 (1.1)</td>
<td>0 (0) ^c^</td>
<td>2.48 .2645</td>
</tr>
<tr>
<td>VII</td>
<td>6 (6.8)</td>
<td>19 (17.4)</td>
<td>0.39 .0261</td>
</tr>
<tr>
<td>VIII</td>
<td>2 (2.3)</td>
<td>11 (10.1)</td>
<td>0.23 .0280</td>
</tr>
<tr>
<td>LOS gene virulence traits</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>licA</em></td>
<td>84 (95.5)</td>
<td>46 (42.2)</td>
<td>2.26 &lt;.0001</td>
</tr>
</tbody>
</table>
lic2A  80 (90.0)  17 (15.6)  5.83  <.0001
lgtC  88 (100.0)  2 (1.8)  54.50  <.0001

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.

Prevalence ratios (PR) were calculated for *H. influenzae* using *H. haemolyticus* as the referent group.

Logit, 0.5 used in place of 0 for PR and statistical calculations.

*P* < 0.05 is considered statistically significant.
TABLE 4. Trends of species specific traits within a species

<table>
<thead>
<tr>
<th>Traits</th>
<th>$H.\ influenzae$ nodes$^a$</th>
<th>$H.\ haemolyticus$ nodes$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A1 (72)</td>
<td>A2-A3 (16)</td>
</tr>
<tr>
<td>$H_2S$</td>
<td>4 (5.6)</td>
<td>7 (43.8)*</td>
</tr>
<tr>
<td>indole</td>
<td>63 (87.5)</td>
<td>10 (62.5)*</td>
</tr>
<tr>
<td>licA</td>
<td>72 (100)</td>
<td>12 (75.0)*</td>
</tr>
<tr>
<td>lic2A</td>
<td>71 (98.6)</td>
<td>9 (56.3)*</td>
</tr>
<tr>
<td>P6+</td>
<td>70 (97.2)</td>
<td>15 (93.7)</td>
</tr>
<tr>
<td>lgtC</td>
<td>72 (100)</td>
<td>16 (100)</td>
</tr>
<tr>
<td>gas</td>
<td>4 (5.6)</td>
<td>1 (6.3)</td>
</tr>
<tr>
<td>urease</td>
<td>59 (81.9)</td>
<td>15 (93.8)</td>
</tr>
<tr>
<td>ODC</td>
<td>16 (22.2)</td>
<td>8 (50.0)*</td>
</tr>
</tbody>
</table>

$^a$ The nodes and number of strains in each is relative to the dendrogram of Fig. 1.

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

$^*$ Nodes possessing a statistically significant difference ($P < 0.05$) for the trait based on the student’s T-test.
TABLE 5. Distribution of taxonomic traits between hemolytic and non-hemolytic *H. haemolyticus*

<table>
<thead>
<tr>
<th>Traits</th>
<th>Hemolytic n=33 (%)</th>
<th>non-hemolytic n=76 (%)</th>
<th>PR$^{ac}$</th>
<th>$X^2$ ($P$ value)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Molecular based traits</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hemolysis</td>
<td>33 (100)</td>
<td>0 (0)</td>
<td>referent</td>
<td></td>
</tr>
<tr>
<td>mAb 7F3</td>
<td>0 (0)$^b$</td>
<td>13 (17.1)</td>
<td>11.3</td>
<td>.0114</td>
</tr>
<tr>
<td><strong>Traditional traits</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H$_2$S</td>
<td>24 (72.7)</td>
<td>51 (67.1)</td>
<td>0.92</td>
<td>.5605</td>
</tr>
<tr>
<td>gas</td>
<td>31 (93.9)</td>
<td>65 (85.5)</td>
<td>0.88</td>
<td>.2131</td>
</tr>
<tr>
<td>indole</td>
<td>20 (60.6)</td>
<td>48 (63.2)</td>
<td>1.04</td>
<td>.8005</td>
</tr>
<tr>
<td>urease</td>
<td>28 (84.8)</td>
<td>49 (64.5)</td>
<td>0.76</td>
<td>.0319</td>
</tr>
<tr>
<td>ODC</td>
<td>2 (6.1)</td>
<td>6 (7.9)</td>
<td>1.30</td>
<td>.7358</td>
</tr>
<tr>
<td><strong>LOS gene virulence traits</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>licA</td>
<td>8 (24.2)</td>
<td>38 (50.0)</td>
<td>2.06</td>
<td>.0124</td>
</tr>
<tr>
<td>lic2A</td>
<td>1 (3.0)</td>
<td>16 (21.1)</td>
<td>6.95</td>
<td>.0172</td>
</tr>
<tr>
<td>lgtC</td>
<td>0 (0)$^b$</td>
<td>2 (2.6)</td>
<td>1.74</td>
<td>.3469</td>
</tr>
</tbody>
</table>

$^a$ Prevalence ratios (PR) were calculated for non-hemolytic strains using the hemolytic strains as the referent group.

$^b$ Logit, 0.5 used instead of 0 for PR and statistical calculations.

$^c$ $P < 0.05$ is considered statistically significant.