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Complete Deletion of the Fucose Operon in *Haemophilus influenzae* Is Associated with a Cluster in Multilocus Sequence Analysis-Based Phylogenetic Group II Related to *Haemophilus haemolyticus*: Implications for Identification and Typing

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Nonhemolytic variants of *Haemophilus haemolyticus* are difficult to differentiate from *Haemophilus influenzae* despite a wide difference in pathogenic potential. A previous investigation characterized a challenging set of 60 clinical strains using multiple PCRs for marker genes and described strains that could not be unequivocally identified as either species. We have analyzed the same set of strains by multilocus sequence analysis (MLSA) and near-full-length 16S rRNA gene sequencing. MLSA unambiguously allocated all study strains to either of the two species, while identification by 16S rRNA sequence was inconclusive for three strains. Notably, the two methods yielded conflicting identifications for two strains. Most of the "fuzzy species" strains were identified as *H. influenzae* that had undergone complete deletion of the fucose operon. Such strains, which are untypeable by the *H. influenzae* multilocus sequence type (MLST) scheme, have sporadically been reported and predominantly belong to a single branch of *H. influenzae* MLSA phylogenetic group II. We also found evidence of interspecies recombination between *H. influenzae* and *H. haemolyticus* within the 16S rRNA genes. Establishing an accurate method for rapid and inexpensive identification of *H. influenzae* is important for disease surveillance and treatment.

Haemophilus influenzae is an important human pathogen involved in respiratory tract infections, such as sinusitis, acute otitis media, pneumonia, and exacerbations in chronic obstructive pulmonary disease (1–5). The majority of these infections are caused by unencapsulated *H. influenzae*, traditionally designated nontypeable *H. influenzae* (NTHi). *Haemophilus haemolyticus* is a close relative of *H. influenzae*, and the two species colonize the upper respiratory tract of humans. Although *H. haemolyticus* has, on rare occasion, been isolated from invasive infections (6), several lines of evidence indicate that the pathogenicity of *H. haemolyticus* is much reduced compared with *H. influenzae*. While up to 20% of presumptive *H. influenzae* nasopharyngeal isolates can be identified as *H. haemolyticus* by molecular characterization (7–9), *H. haemolyticus* is rarely cultured from middle ear fluid (10, 11), supporting the view that *H. haemolyticus*, in contrast to NTHi, is a respiratory commensal infrequently associated with otitis media. Reinvestigation of presumptive *H. influenzae* isolates, cultured from lower respiratory tract samples from cystic fibrosis patients (12) or from unselected clinical samples submitted to the laboratory on suspicion of lower respiratory tract infection (13), detected that <1% were misidentified strains, further supporting a minor pathogenic role for *H. haemolyticus*.

H. haemolyticus was traditionally identified by its hemolytic action on erythrocytes, but it has become clear that a large proportion of *H. haemolyticus* strains is nonhemolytic (10, 14). Such strains, which are designated as nonhemolytic *H. haemolyticus*, can only be differentiated from *H. influenzae* by DNA sequencing or extended phenotypic testing (15, 16). Although this may be a minor problem when strains are isolated from infections, it constitutes a major problem in colonization and surveillance studies.

The most reliable delineation of *H. influenzae* from *H. haemolyticus* is based on the comparison of near-full-length 16S rRNA gene sequences (17) or concatenated sequences of housekeeping gene fragments (16, 18). However, these methods are labor intensive and too expensive for routine use. A number of studies have, therefore, attempted to identify and evaluate suitable assays for rapid and inexpensive identification of *H. influenzae* (13, 17–23). Recently, an evaluation of 9 PCR screening assays was performed on a challenging sample of 60 nasopharyngeal carriage isolates selected to include approximately equal numbers of NTHi, *H. haemolyticus*, and equivocal strains (23). A drawback of the study was that the species cutoff was selected arbitrarily, while a strict definition based on partial *recA* and 16S rRNA gene sequences (1,142 nucleotides [nt] combined) left 11 isolates unidentified ("fuzzy species"); thus, the true performance of the PCR assays could not be calculated. In the present study, we have reinvestigated the same set of strains by near-full-length sequencing of 16S rRNA genes and multilocus sequence typing (MLST) of *H. influenzae* in order to unambiguously identify the isolates to species

Received 26 July 2015 **Returned for modification** 13 August 2015
Accepted 9 September 2015

Accepted manuscript posted online 16 September 2015

Citation de Gier C, Kirkham L-AS, Nørskov-Lauritsen N. 2015. Complete deletion of the fucose operon in Haemophilus influenzae is associated with a cluster in multilocus sequence analysis-based phylogenetic group II related to Haemophilus haemolyticus: implications for identification and typing. J Clin Microbiol 53:3773–3778. doi:10.1128/JCM.01969-15.

Editor: A. J. McAdam

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level. Unexpectedly, a significant proportion of the previously unidentified isolates were *H. influenzae* that had undergone complete deletion of the fucose operon.

MATERIALS AND METHODS

Bacterial strains. The 60 strains (H01 through H60) included in the study by Binks et al. (23) were selected from a larger study investigating nasopharyngeal carriage isolates collected from otitis-prone and healthy children aged 6 to 36 months (7, 24). A total of 266 *Haemophilus* isolates were collected and subjected to an initial PCR screen of the 16S rRNA gene using the two primer sets that have been described as specific for either *H. haemolyticus* or *H. influenzae* (10). Twenty isolates identified as *H. influenzae*, 20 isolates identified as *H. haemolyticus*, and 20 isolates with equivocal results (bands in both or none of the PCRs or additional or aberrant bands) were then selected for further study. When the dual 16S rRNA gene PCR was repeated for the 60 strains in a different laboratory, 22 were *H. influenzae*, 27 were *H. haemolyticus*, and 11 were categorized as equivocal (23).

The bacterial strains had been propagated in several laboratories prior to the present investigation. To ascertain the identity of the strains for this study, partial *recA* fragments were sequenced and compared with deposited sequences for these strains (23). For 6 strains, the identity could not be confirmed, and these strains were excluded from the present study (H01, fuzzy species; H08, *H. haemolyticus*; H09, *H. haemolyticus*; H22, *H. influenzae*, H38, *Haemophilus parainfluenzae*; and H50, *H. haemolyticus*) (categorization according to the former study [23]).

DNA sequencing. Identification of polymorphic nucleotide positions and 16S rRNA gene sequencing were carried out as previously described (25). A 1,362-nt fragment corresponding to nucleotides 27 to 1,388 of the 16S rRNA gene in *H. influenzae* strain Rd was obtained from all 54 strains and used for analysis.

Fragments of the housekeeping genes *adk*, *atpG*, *frdB*, *fucK*, *mdh*, *pgi*, and *recA* were amplified as recommended (www.mlst.net). In the case of weak or absent bands, PCR amplification was repeated with an annealing temperature of 40°C. The *fucK* fragment could not be amplified from 33 of 54 strains. The remaining 6 housekeeping gene fragments were sequenced, trimmed to appropriate lengths, concatenated into a hybrid sequence of 2,712 nt, and used for multilocus sequence analysis (MLSA).

Phylogenetic reconstruction. The 16S rRNA fragments of the 54 study isolates were compared with sequences from the type strain and 75 other reference strains of *H. influenzae* as well as the type strain and 35 other reference strains of *H. haemolyticus* (including cryptic genospecies biotype IV and other variant strains excluded from *H. influenzae*) (18, 26, 27). Of 166 16S rRNA sequences, 158 were unique.

The complete *H. influenzae* database with 1,294 sequence types was downloaded from the *H. influenzae* MLST website (http://pubmlst.org/hinfluenzae/) on 29 January 2014. After removal of *fucK* fragments and the addition of sequences from 54 study strains, the type strain, 15 other reference strains of *H. influenzae*, and the type strain and 35 other reference strains of *H. haemolyticus* (18, 27, 28), 1,268 sequences were unique.

DNA sequences were edited, assembled, and aligned using CLC Main Workbench 7 (CLC bio, Aarhus, Denmark). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (29) and the neighbor-joining method. Bootstrap tests were performed with 500 (16S) or 200 (MLSA) replications.

Fucose operon analysis. In *H. influenzae*, a fucose operon of approximately 10 kb is located between *folD* and *hepA* (30), while no open reading frames separate *folD* and *hepA* in the available genomes of *H. haemolyticus* (27). The flanking primers HI_0608_1002f and HI_0616_1708r (30) failed to amplify the region in several study strains, and new flanking primers were designed: folD.101Ra (GGTGCACGTTTACCTTG), folD.101Rb (GGAGCGCGTTTTCCTTG), hepA.53Ra (CCTAAAGCGTTTTCACTTTCAC), and hepA.53Rb (CCTAAAGTATTCTCACTTTCAC). The fucose region was amplified from all study strains by PCR (35 cycles consisting of 94°C for 15 s, 55°C for 15 s, and 68°C for 10 min) using a Kapa Long Range HotStart ReadyMix (Kapa Biosystems Ltd., London, United Kingdom) and 0.25 μM each of the four primers. PCRs targeting the other individual genes of the fucose operon, *fucP*, *fucA*, *fucU*, *fucK*, *fucI*, and *fucR*, were performed as previously described (30).

Supplementary PCR assays. A fragment of the copper/zinc-cofactored superoxide dismutase (CuZnSOD) gene *sodC* was amplified by PCR using primers sodC.253f (CCAAGCTGTGATCCAAAAG) and sodC.523r (CAAGTGGAGCTGGATGATC) (numbering with reference to the position in the gene in *H. parainfluenzae* strain T3T1); the primers display one mismatch with the gene in *H. haemolyticus* and with deposited sequences of the pseudogene that is present in some strains of *H. influenzae* (31).

PCR screen for the capsule locus of *H. influenzae* was performed by amplification of a 760-bp fragment of *bexB* using primers bexB.FLF and bexB.FLR as previously described (32). *H. influenzae* serotype e was documented by PCR amplification using primers bexD.128R (GAAGCATC AGCACCTTGGTT) located in *bexD* and Hie_ecs1.366R (CGTGCGCAA ACCAGCTTCAA) located in the serotype e-specific gene *ecs1*, encoding a putative UDP-*N*-acetyl-D-glucosamine 2-epimerase (33). The PCR amplifies an 895-bp fragment from *H. influenzae* serotype e strains; the Hie reference strain PN125 (GenBank accession number FJ939590) was used as a positive control, and the unencapsulated *Haemophilus aegyptius* strain NCTC 8502^T was used as a negative control. PCR products were separated by 1% agarose gel electrophoresis.

RESULTS

Identification based on comparison of 16S rRNA gene sequences. Figure 1A shows a neighbor-joining dendrogram of near-full-length (1,362 nt) 16S rRNA gene sequences comparing 54 study strains with 76 reference strains of *H. influenzae* and 36 reference strains of *H. haemolyticus* (18, 26, 27). Three major clusters can be discerned in the dendrogram: *H. influenzae* 16S rRNA phylogenetic group I (blue), which includes the type strain of the species, 62 other *H. influenzae* reference strains, and 20 study strains; *H. influenzae* 16S rRNA phylogenetic group II (yellow), which includes 13 *H. influenzae* reference strains of serotype e and f as well as other distantly related lineages and 15 study strains; and the *H. haemolyticus* cluster (red), which includes the type strain of the species, 35 other *H. haemolyticus* reference strains, and 16 study strains. Three study strains, H26, H43, and H56, take up an intermediary position in the dendrogram (gray).

The 16S rRNA gene sequences of 83 strains in *H. influenzae* phylogenetic group I were similar with a maximal divergence of 1.8%. The cluster was supported by a high bootstrap value of 74% (not shown). More variation was observed for 16S rRNA gene sequences of 28 strains of *H. influenzae* phylogenetic group II (maximal divergence of 2.4%) and 52 strains of the *H. haemolyticus* cluster (maximal divergence of 3.5%). The *H. influenzae* 16S rRNA phylogenetic group II cluster was supported by a modest bootstrap value of 7%, while the *H. haemolyticus* cluster was divided into several subclusters (Fig. 1A).

Polymorphic nucleotide positions due to intragenomic 16S rRNA gene heterogeneity of the multiple RNA operons were observed in 48/54 study strains. The average frequencies of 16S rRNA gene polymorphic nucleotide positions were 0.26% for 20 study strains of *H. influenzae* 16S rRNA phylogenetic group I, 0.74% for 15 study strains of *H. influenzae* 16S rRNA phylogenetic group II, and 1.44% for 16 study strains of the *H. haemolyticus* 16S rRNA cluster. Of the unclustered strains, H43 and H56 were characterized by a high divergence among their 16S rRNA gene copies (>4% polymorphic sites), and the position in the dendrogram indicates a mixture of *H. influenzae* and *H. haemolyticus* 16S rRNA genes in the genomes of these two strains. The 16S rRNA genes of

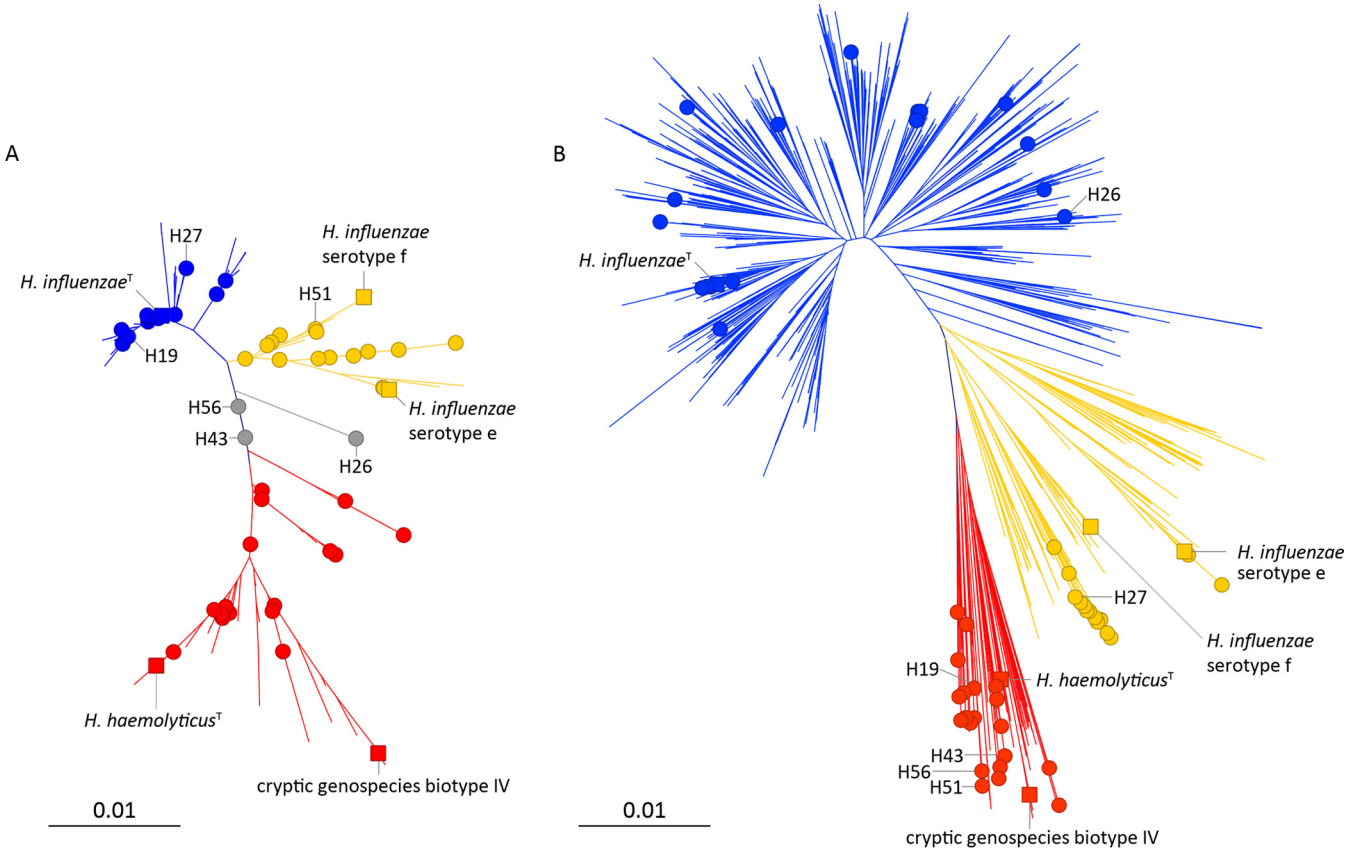

FIG 1 (A) Neighbor-joining dendrogram comparing 16S rRNA gene sequences (1,362 nucleotides) of 54 study strains (filled circles) with those of 76 reference strains of *H. influenzae* and 36 reference strains of *H. haemolyticus* and related organisms. (B) Neighbor-joining dendrogram based on concatenated sequence fragments of housekeeping genes *adk*, *atpG*, *frdB*, *mdh*, *pgi*, and *recA* (2,712 nt) of 54 study strains (filled circles) compared with 1,294 sequences from the *H. influenzae* MLST database and 16 additional reference strains of *H. influenzae* and 36 reference strains of *H. haemolyticus* and related organisms. *H. influenzae* phylogenetic group I, blue; *H. influenzae* phylogenetic group II, yellow; *H. haemolyticus*, red. Selected reference strains (solid squares): *H. influenzae* type strain NCTC 8143, *H. influenzae* serotype e, strain PN125; *H. influenzae* serotype f, strain HK2067; Cryptic genospecies biotype IV, strain S32F2; *H. haemolyticus* type strain, NCTC 10659^T. Selected study strains are indicated and referred to in the text. Bars indicate 1 nt substitution per 100 residues.

strain H26 contained no polymorphic positions, and the sequence was unique; a BLAST search in GenBank showed a maximum of 1,347/1,362 identities with reference strains of *H. influenzae* and 1,344/1,364 identities with reference strains of *H. haemolyticus*.

Identification based on MLSA. Fifty-four study strains were subjected to MLSA using the *H. influenzae* MLST scheme. Thirty-three strains were negative for *fucK*, which is typical for *H. haemolyticus* and related organisms excluded from *H. influenzae* (17, 18). The 6 other housekeeping gene fragments (*adk*, *atpG*, *frdB*, *mdh*, *pgi*, and *recA*) were concatenated into hybrid sequences of 2,712 nt. Figure 1B shows a neighbor-joining dendrogram comparing the 54 study strains with 1,294 sequence types downloaded from the MLST database and the type strains of *H. influenzae* and *H. haemolyticus* and 50 additional reference strains (18, 27, 28). An *H. haemolyticus* cluster (red) was clearly separate from the rest of the strains and was supported by a high bootstrap value of 86% (not shown). All of the *H. haemolyticus* reference strains were located in this cluster with 7 sequence types from the MLST database and 20 study strains. These 20 study strains were all negative for the *fucK* allele.

With an increasing number of sequence types deposited in the MLST database, it has become difficult to make a clear distinction between phylogenetic groups I and II for unencapsulated isolates (15). The continuum of *H. influenzae* MLSA sequences is also apparent from Fig. 1B. Annotation of phylogenetic groups in Fig. 1B is based on the location of capsulated strains, with serotype e and f strains invariably located in phylogenetic group II.

Nineteen study strains belonged to the *H. influenzae* MLSA phylogenetic group I cluster (blue). These strains were located on many separate branches and appear to be a random sample of typical NTHi. Fifteen study strains were located on 2 branches in the *H. influenzae* MLSA phylogenetic group II cluster (yellow). Two strains, H20 and H29, were almost identical with *H. influenzae* serotype e reference strains and were indeed shown to be serotype e as revealed by positive PCRs for capsule export protein B (*bexB*) and serotype e-specific epimerase genes. Sequences from 13 other strains were closely related but not identical; these 13 strains were all negative for *fucK*. In the MLSA tree, they were located on a branch with 9 sequence types from the MLST database (and thus positive for *fucK*). The clustering of 13 of 15 phylogenetic group II study strains on a single branch validates that the strains investigated are a nonrandom sample of *H. influenzae* isolates.

Conflicting identifications by 16S rRNA comparison versus housekeeping gene analysis. Three study strains with ambiguous identification by 16S rRNA gene analysis (H26, H43, and H56)

TABLE 1 Genotypes of 54 *Haemophilus* carriage isolates defined by PCR assays

Species[a]	No. (%) of isolates positive for:								
	fucK[b]	*sodC*[b]	*ompP2*[c]	*ompP6*[c]	*ompP6* (*hrm*)[c]	*lgtC*[c]	*hpd* 1[c]	*hpd* 3[c]	*iga*[c]
H. influenzae	21 (62)	9 (27)	27 (79)	34 (100)	34 (100)	28 (82)	32 (94)	32 (94)	32 (94)
H. haemolyticus	0 (0)	20 (100)	2 (10)	7 (35)	6 (30)	0 (0)	0 (0)	0 (0)	0 (0)

[a] As defined be MLSA (*H. influenzae*, $n = 34$; *H. haemolyticus*, $n = 20$).
[b] This study.
[c] PCR results from reference 23.

were clearly resolved by MLSA. Strain H26, which carried identical copies of a unique 16S rRNA gene, belonged to the core of *H. influenzae* by MLSA. Strains H43 and H56, with 56 and 55 polymorphic positions in their 16S rRNA genes, respectively, were clearly located in the *H. haemolyticus* cluster by MLSA. Furthermore, conflicting identification using the 2 methods was obtained for 3 other strains (H19, H27, and H51). H19 belonged to *H. influenzae* phylogenetic group I by 16S rRNA and to *H. haemolyticus* by MLSA, H27 belonged to *H. influenzae* 16S rRNA phylogenetic group I but to *H. influenzae* phylogenetic group II by MLSA, and H51 belonged to *H. influenzae* phylogenetic group II by 16S rRNA and to *H. haemolyticus* by MLSA (Fig. 1A and B).

With respect to the decisive distinction between *H. influenzae* and *H. haemolyticus*, the distribution of marker genes *hpd*, *iga*, and *sodC* in the 6 strains supported the identification obtained by MLSA (the expected genotype of *H. influenzae* was positive for *hpd* and *iga* and negative for *sodC*; the expected genotype of *H. haemolyticus* was negative for *hpd* and *iga* and positive for *sodC*).

Complete deletion of the fucose operon. In *H. influenzae*, a fucose operon of approximately 10 kb is located between the folate dehydrogenase *folD* and the ATP-dependent helicase *hepA* (30), while no open reading frames separate the genes in the available genomes of *H. haemolyticus* (27). We used a long range PCR to amplify the entire fucose operon in the 54 study strains. All 21 *fucK*-positive strains (19 MLSA phylogenetic group I strains and 2 *H. influenzae* serotype e strains) gave rise to amplicons of approximately 10 kb, while all 33 *fucK*-negative strains (13 strains from a single branch in MLSA phylogenetic group II and 20 *H. haemolyticus*) gave rise to amplicons of approximately 700 nt (not shown).

We also performed PCR targeting the other genes of the fucose operon, *fucP*, *fucA*, *fucU*, *fucI*, and *fucR*. Products of the expected size were obtained with all *fucK*-positive study strains, while all *fucK*-negative strains were negative for all genes of the fucose operon. Thus, the fucose operon was not translocated to other parts of the genome by genomic rearrangements in *fucK*-negative strains.

Assessment of PCR assays for delineation of *H. influenzae*. Unambiguous allocation to species level by MLSA was used to calculate the true performance of PCR screening results previously reported for the study collection (23). The best discrimination of *H. influenzae* from *H. haemolyticus* was obtained with *hpd* 1, *hpd* 3, and *iga*, which were positive for 32 of 34 *H. influenzae* strains and negative for 20 of 20 *H. haemolyticus* strains (Table 1).

DISCUSSION

It has become clear that widely used identification methods are not able to differentiate strains of *H. influenzae* reliably from *H. haemolyticus*. This has important implications for the clinical laboratory because accurate species identification is paramount for treatment and disease surveillance.

Several recent studies have explored the use of PCR screens for the distinction of these species (13, 18–23). The major challenge in the evaluation of these studies is the lack of a universal delineation of *H. influenzae*. Various collections of strains have been investigated, and different tests are used to define *H. influenzae*; thus, isolates that are included in the species in one investigation may be excluded from the species in another. We subjected a challenging collection of carriage strains to MLSA and 16S rRNA gene sequencing in order to clarify the distribution of marker genes in borderline or fuzzy strains. Unambiguous identification to species level enabled accurate evaluation of PCR assays and confirmed the superiority of PCR assays *hpd* 1, *hpd* 3, and *iga*. However, the characterization also revealed a selected study collection, with 13 of 34 *H. influenzae* strains located on a particular branch in *H. influenzae* MLSA phylogenetic group II. The entire fucose operon consisting of 6 genes was absent from the chromosome of these 13 strains. Fermentation of fucose is considered a stable trait of *H. influenzae*, and the *fucK* gene is part of the MLST scheme (34). Consequently, strains lacking *fucK* cannot be assigned a sequence type by the MLST scheme. *fucK*-negative strains of *H. influenzae* appear to be rare, and prior to this study, only 17 such isolates were reported (12, 28, 30). Interestingly, 13 of the 17 previously reported isolates are located on the same branch in phylogenetic group II as the 13 isolates from the present study. Given the biased nature of the present study sample, the performance of the PCR assays will differ when applied to other collections. We found 32 of 34 *H. influenzae* strains to be positive for the *hpd* gene, which is similar to other studies (17, 35). The absence of *hpd* in strains of *H. influenzae* has recently been confirmed by whole-genome sequencing (36).

Unexpectedly, conflicting identifications were obtained for 2 of 54 study strains. These strains clustered with reference strains of *H. influenzae* by 16S rRNA sequencing and with *H. haemolyticus* by MLSA. The presence of the *H. haemolyticus* marker gene *sodC* and the absence of the *H. influenzae* marker genes *hpd* and *iga* supported the identification arising from MLSA. Thus, the strains contain 16S rRNA gene copies representative of *H. influenzae* while 9 protein coding genes, located on separate regions of the chromosome, are compatible with *H. haemolyticus*. We hypothesize that interspecies transfer of 16S rRNA genes has resulted in the development of such hybrid genomes. A mixture of *H. influenzae* and *H. haemolyticus* 16S rRNA genes in the genomes of strains H43 and H56 is indicated by their position in the dendrogram (Fig. 1A), and such mixtures have previously been documented for other strains of *H. haemolyticus* by sequencing of individual RNA operons (25). However, an exclusive presence of 16S rRNA genes from a different species, to our knowledge, has not been

reported before; if corroborated, this would constitute a significant challenge to current hierarchic taxonomy that depends on 16S rRNA comparisons (37).

In conclusion, the present investigation underscores the limitations of marker genes for unambiguous separation of *H. influenzae* and *H. haemolyticus*. Recombination between the two species may not be rare (38, 39) and may even involve rRNA genes. Comparative whole-genome analysis of *H. influenzae* and *H. haemolyticus* is warranted to elucidate the genomic differences between the two species (36, 40). Accurate species identification may necessitate the use of MLSA or at least detection of multiple marker genes.

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