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# Pharyngeal Colonization Dynamics of *Haemophilus influenzae* and *Haemophilus haemolyticus* in Healthy Adult Carriers[▽]

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***Haemophilus influenzae*** **is an important cause of respiratory infections, including acute otitis media, sinusitis, and chronic bronchitis, which are preceded by asymptomatic *H. influenzae* colonization of the human pharynx. The aim of this study was to describe the dynamics of pharyngeal colonization by *H. influenzae* and an intimately related species, *Haemophilus haemolyticus*, in healthy adults. Throat specimens from four healthy adult carriers were screened for *Haemophilus* species; 860 isolates were identified as *H. influenzae* or *H. haemolyticus* based on the porphyrin test and on dependence on hemin and NAD for growth. Based on tests for hemolysis, for the presence of the 7F3 epitope of the P6 protein, and for the presence of *iga* in 412 of the isolates, 346 (84%) were *H. influenzae*, 47 (11%) were *H. haemolyticus*, 18 (4%) were nonhemolytic *H. haemolyticus*, and 1 was a variant strain. Carriers A and B were predominantly colonized with nontypeable *H. influenzae*, carrier C predominantly with b$^-$ *H. influenzae* mutants, and carrier D with *H. haemolyticus*. A total of 358 *H. influenzae* and *H. haemolyticus* isolates were genotyped by pulsed-field gel electrophoresis (PFGE) following SmaI or EagI digestion of their DNA, and the carriers displayed the following: carrier A had 11 unique PFGE genotypes, carrier B had 15, carrier C had 7, and carrier D had 10. Thus, adult *H. influenzae* and *H. haemolyticus* carriers are colonized with multiple unique genotypes, the colonizing strains exhibit genetic diversity, and we observed day-to-day and week-to-week variability of the genotypes. These results appear to reflect both evolutionary processes that occur among *H. influenzae* isolates during asymptomatic pharyngeal carriage and sample-to-sample collection bias from a large, variable population of colonizing bacteria.**

*Haemophilus* species constitute approximately 10% of the total bacterial flora in the human upper respiratory tract (27). *Haemophilus influenzae* is an opportunistic pathogen in humans that asymptomatically colonizes the pharyngeal mucosa and occasionally the genital mucosa. The rate of carriage of *H. influenzae* increases from infancy (about 20% in the first year of life) to early childhood (>50% in children 5 to 6 years old), and *H. influenzae* is recoverable from the upper airways of 20 to 80% of healthy children (2, 15, 22, 32).

Colonization of the human respiratory mucosal surface represents a dynamic process in which bacteria are acquired, replaced, and reacquired many times in a lifetime. Past studies have demonstrated that *H. influenzae* colonization of the pharynx is characterized by rapid bacterial turnover (9, 12, 50, 58) and carriage of multiple strains at any one time (18, 37, 43, 52, 58). Previous studies have demonstrated a 62% week-to-week turnover rate of *H. influenzae* isolated from healthy children attending day care; 37 to 43% of throat cultures contained two or more genetically distinct strains (range, zero to five) (14, 56).

In addition to living as a commensal in the respiratory tract, *H. influenzae* may also cause symptomatic infections. A majority of invasive *H. influenzae* infections in children, such as bacteremia, meningitis, pneumonia, epiglottitis, and septic arthritis, are caused by the encapsulated *H. influenzae* type b. Nontypeable (i.e., nonencapsulated) *H. influenzae* (NTHI) rarely causes invasive disease in healthy hosts but is a significant cause of localized respiratory tract infections, such as otitis media, sinusitis, bronchitis, and conjunctivitis (35). NTHI accounts for 30 to 52% of acute otitis media infections in children (11). NTHI is also the most commonly seen bacterium in acute exacerbations of chronic obstructive pulmonary disease (COPD) (3, 20, 38, 51) and appears to contribute to COPD progression (46). In addition, acute exacerbation has been shown to occur with acquisition of a new strain of *H. influenzae* (relative risk, 2.96 [95% confidence interval, 2.39 to 3.67]) (51). Strain-specific *H. influenzae* bactericidal antibodies were seen in 61% of exacerbations associated with new strains compared to 21% associated with preexisting strains (38), further supporting the role of NTHI in COPD exacerbations.

*Haemophilus haemolyticus* is phylogenetically closely related to *H. influenzae* and is also found in the pharynx in healthy adults (31). Pathogenicity of *H. haemolyticus* to humans has not been demonstrated (1), and the organism can be phenotypically differentiated from NTHI in the laboratory by its ability to produce a clear hemolytic zone on horse blood agar. The hemolytic activity of *H. haemolyticus*, however, may be lost on subculture, resulting in difficulty differentiating it from *H. influenzae*. Since discriminating *H. haemolyticus* from *H. influenzae* is important to fully understand the pathogenesis of *H. influenzae* disease, in this study, the nonhemolytic isolates were further tested for the presence of a specific epitope on the outer membrane protein P6 and the presence of *iga*, both of

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which have been shown to be associated with *H. influenzae* and not *H. haemolyticus* (16, 39).

Although *H. influenzae* pharyngeal colonization in the context of disease has been relatively well studied, its carriage and the dynamics of colonization have not been well characterized in healthy adults. This study used pulsed-field gel electrophoresis (PFGE) to describe the genetic diversity and dynamics of colonization of *H. haemolyticus* and *H. influenzae* over 7 months in four healthy adults.

TABLE 1. Distinguishing *H. influenzae* and *H. haemolyticus* isolates

| Species | Porphyrin | Hemolysis | 7F3 epitope of P6 | *iga* |
|---|---|---|---|---|
| *H. influenzae* | Negative | Absent | Present | Present |
| *H. haemolyticus* | Negative | Present | Absent | Absent |
| Nonhemolytic *H. haemolyticus* | Negative | Absent | Absent | Absent |
| Variant | Negative | Absent | Present | Absent |

## MATERIALS AND METHODS

**Study design.** Throat culture samples were obtained from 16 healthy adults after informed consent, as approved by the University of Michigan Institutional Review Board. Screening samples from four participants were positive for NAD- and hemin-dependent *Haemophilus* species, and further culture samples from these four individuals were obtained five workdays a week for the first month, followed by once a week for the next 6 months.

**Bacteriologic methods.** All throat culture samples were collected with a double-tipped swab by the same person to minimize variability in technique. The swabs were streaked on a chocolate agar plate supplemented with 16,500 units/liter bacitracin (BBL, Sparks, MD) and incubated at 37°C with 5% $CO_2$ for 24 h. Up to 30 isolates per throat sample consistent with *Haemophilus* morphology were selected for further analysis. The porphyrin test and hemolysis of horse erythrocytes (28) were used to differentiate *H. influenzae* from heme-dependent (i.e., porphyrin-negative) *Haemophilus* isolates, such as *Haemophilus parainfluenzae*, and from *H. haemolyticus*, respectively. The putative *H. influenzae* and *H. haemolyticus* isolates were further confirmed by growth on brain heart infusion agar (Difco, Detroit, MI) supplemented with factor X (hemin), V (NAD), or both (Sigma-Aldrich, St. Louis, MO). *H. influenzae* and *H. haemolyticus* are both X and V factor dependent. Isolates identified as *H. influenzae* or *H. haemolyticus* were stored in sterile skim milk at −80°C.

**Serotyping.** All suspected *H. influenzae* isolates were analyzed by slide agglutination for capsular serotype using polyvalent *H. influenzae* capsular-serotyping sera (Difco, Detroit, MI). Isolates positive for the capsule were confirmed by agglutination using antisera specific for types a through f. The positive control was *H. influenzae* type b strain Eagan, and the negative control contained no bacteria. The results from carrier C were ambiguous, and therefore, all isolates from this carrier were further studied by PCR for the presence of capsule genes using the method described by Falla et al. (13). Strains that lacked both the type b-specific gene region and *bexA*, required for the export of the capsule to the cell surface, were designated nontypeable. Strains that carried the type b-specific gene region but did not have *bexA* were designated as $b^-$ mutants. The positive control in the PCRs was *H. influenzae* type b strain Eagan, and the negative control contained no DNA.

**P6 protein immunodot assay.** The P6 protein of *H. influenzae*, a 16,000-Da outer membrane protein, demonstrates a high degree of antigenic conservation among *H. influenzae* strains. Monoclonal antibody 7F3 recognizes an epitope on P6 that is highly specific for *H. influenzae* strains, as shown by assays of several hundred *H. influenzae* strains from diverse geographic and clinical origins (42, 45), and this epitope is absent from the P6 of *H. haemolyticus*. We therefore used the 7F3 antiserum (kindly donated by Timothy Murphy of the Buffalo, NY, Veterans Administration Hospital) to characterize isolates as *H. influenzae* (possessing the 7F3 epitope) or *H. haemolyticus* (lacking the 7F3 epitope), based on the method previously described (41). Briefly, 2 μl of bacterial suspension was dotted onto a nitrocellulose membrane and allowed to dry. The membrane was incubated in BLOTTO (5% nonfat dry milk in sterile water) for 1 h at room temperature to block reactive sites. After being washed with phosphate-buffered saline, the membrane was incubated overnight at room temperature in 7F3 antibody diluted 1:1 in BLOTTO. The nitrocellulose was then washed with phosphate-buffered saline and incubated for an hour in goat anti-mouse immunoglobulin G (Sigma A-3562) diluted 1:1,000 in BLOTTO. The immunodots were developed with nitroblue tetrazolium/BCIP (5-bromo-4-chloro-3-indolylphosphate) (Pierce) color developer and visually assessed. *H. influenzae* type b strain Eagan served as the positive control and *H. haemolyticus* strain 11P31 (donated by Timothy Murphy of the Buffalo, NY, Veterans Administration Hospital) as the negative control.

**DNA isolation and dot blot hybridization analysis for *iga*.** More than 90% of *H. influenzae* and less than 10% of *H. haemolyticus* isolates possess the *iga* gene, which encodes the enzyme immunoglobulin A1 protease (26). The presence of *iga* was detected using the dot blot hybridization method previously described (10). Briefly, a DNA probe specific for the conserved β-core region of *iga* was PCR amplified from *H. influenzae* type b strain Eagan as previously described (59) using β-core domain primers (BF1, GCAGAATTCAAAGCACAATTTG TTGCA, and BR1, TTATAACGTTAATTCAACAGGCTT) derived from the published sequence (48) of the *H. influenzae* HK368 *iga* gene (GenBank accession no. M87492). The probe was fluorescein labeled using the ECF random prime labeling system (Amersham, IL). Crude DNA was isolated from *H. influenzae* and *H. haemolyticus* lysates, and the DNA concentrations were standardized by spectrophotometry. DNA samples were then transferred onto nylon membranes using a Bio-Dot Microfiltration Apparatus (Bio-Rad, CA) which generated a fixed 8-by-12 array of DNA dots. The dot blots were hybridized to the fluorescein-labeled DNA probe under stringent conditions using the fluorescein-based ECF detection system (Amersham, IL). The signal intensity of each dot was detected using the Storm system from Molecular Dynamics (Sunnyvale, CA) and reported as a percentage of the positive control after correction for the background signal. Positive controls for the PCR analysis included amplification of 16S rRNA gene sequences (34) and *pepN*, a peptidase-encoding gene found in all *Haemophilus* strains studied (10).

**Distinguishing *H. influenzae* and *H. haemolyticus* isolates.** Based on the *H. influenzae* characteristics described in *Bergey's Manual of Systematic Bacteriology* (26) and the conservation of the *H. influenzae* 7F3 epitope of P6 (42, 45), along with the presence of the *iga* gene, we developed criteria in our laboratory to distinguish *H. influenzae* from *H. haemolyticus* for the purposes of this study (Table 1). *H. influenzae* isolates were defined as NAD and hemin dependent, nonhemolytic on horse blood agar, and positive for both the 7F3 epitope of P6 (42, 45) and the conserved β-core domain of the *iga* gene (59). Isolates defined as *H. haemolyticus* were also NAD and hemin dependent but demonstrated clear hemolysis on horse blood agar (positive control, *H. haemolyticus* strain 11P31; negative control, *H. influenzae* type b strain Eagan), did not recognize the 7F3 epitope of P6, and lacked *iga*. Since *H. haemolyticus* isolates may lose hemolytic activity during subculture and NAD- and hemin-dependent, nonhemolytic isolates that lack the P6 epitope and *iga* are phylogenetically more related to *H. haemolyticus* (16, 39), we designated such isolates nonhemolytic *H. haemolyticus*. The single nonhemolytic isolate that carried the 7F3 epitope of P6 but not the *iga* gene was designated a variant strain.

**USS-PCR.** Uptake signal sequence (USS)-PCR is a repetitive-element genotyping method recently developed in our laboratory that takes advantage of the multiple (1,471 copies in strain Rd) copies of the 9-bp DNA segment (5′-AAG TGCGGT-3′) (54, 55) that promotes double-stranded DNA uptake by the naturally competent *Haemophilus* bacteria. The PCR consisted of 1 μl of chromosomal DNA, 2 μl 25 mM primer (5′-IIIAAGTGCGGT-3′) (Invitrogen), 2 μl 2.5 mM $MgCl_2$, 2 μl each of a 10 mM concentration of the four deoxynucleoside triphosphates (Invitrogen), 0.5 μl platinum *Taq* polymerase (Invitrogen), and water to achieve a final volume of 50 μl. The PCR was initiated with a 2-min incubation at 94°C, followed by 30 cycles of 94°C for 30 s, annealing at 45°C for 1 min, and denaturing at 68°C for 5 min. The PCR products were separated by gel electrophoresis using 1% agarose gel for 2 h at 97 V, stained with ethidium bromide, and visualized with a UV transilluminator. Strain differences were defined by a single band difference in the band patterns.

**PFGE genotyping.** Genomic DNAs from representative USS-PCR genotypes in each carrier (about one for every two isolates that were identical by USS-PCR) were digested with SmaI and tested by PFGE analysis, using techniques established in our laboratory (47, 56). Following separation of the DNA restriction fragments by gel electrophoresis, the gels were stained with ethidium bromide, visualized under UV light, and photographed for analysis. A previous study from our laboratory showed that restriction patterns from bacterial isolates using SmaI and ApaI restriction enzymes cluster identically (56). Thus, we assumed that those isolates that could not be digested with SmaI after two attempts were genotypically different from SmaI-digestible isolates. Subsequently, digestion with EagI was used to genotype non-SmaI-digestible isolates. Cluster analysis

TABLE 2. Characteristics of the *Haemophilus* isolates from the four carriers

| Characteristic | Value in carrier: | | | | Total |
|---|---|---|---|---|---|
| | A | B | C | D | |
| No. of isolates recovered | 1,110 | 1,170 | 1,140 | 1,132 | 4,552 |
| No. (%) porphyrin positive | 889 (80) | 543 (46) | 984 (86) | 956 (84) | 3,372 (74) |
| No. (%) X and V factor dependent | 76 (7) | 559 (48) | 98 (9) | 127 (11) | 860 (19) |
| No. encapsulated/total (%) | 0/74 | 0/542 | 45/65 (69) | 0/126 | 68/840 (8) |
| No. serotype b/total (%) | 0/74 | 0/542 | 45/65 (69) | 0/126 | 68/840 (8) |
| Hemolysis (total no. tested) | 74 | 236 | 74 | 120 | 504 |
| No. (%) positive | 5 (7) | 1 (0.42) | 3 (4) | 102 (85) | 111/504 (22) |
| No. (%) negative | 69 (93) | 235 (99.6) | 71 (96) | 18 (15) | 393/504 (78) |
| P6 epitope (total no. tested) | 74 | 237 | 70 | 118 | 499 |
| No. (%) positive | 63 (85) | 236 (99.6) | 65 (93) | 0 | 364/499 (73) |
| No. (%) negative | 11 (15) | 1 (0.42) | 5 (7) | 118 (100) | 135/499 (27) |
| *iga* gene (total no. tested) | 57 | 237 | 70 | 48 | 412 |
| No. (%) positive | 45 (79) | 236 (99.6) | 65 (93) | 0 | 346/412 (84) |
| No. (%) negative | 12 (21) | 1 (0.4) | 5 (7) | 48 (100) | 66/412 (16) |
| No. of *H. influenzae* isolates/total (%) | 45/57 (79) | 236/237 (99.6) | 65/70 (93) | 0/48 (0) | 346/412 (84.0) |
| No. of *H. haemophilus* isolates/total (%) | 5/57 (9) | 1/237 (0.4) | 3/70 (4) | 38/48 (79) | 47/412 (11.4) |
| No. of nonhemolytic *H. haemophilus* isolates/total (%) | 6/57 (10) | 0 | 2/70 (3) | 10/48 (21) | 18/412 (4.4) |
| No. of variant isolates (%) | 1/57 (2) | 0 | 0 | 0 | 1/412 (0.2) |
| No. of USS-PCR types | 11 ($n$ = 74) | 16 ($n$ = 541) | 5 ($n$ = 98) | 12 ($n$ = 125) | |
| No. of PFGE genotypes | 11 ($n$ = 51) | 15 ($n$ = 205) | 7 ($n$ = 63) | 10 ($n$ = 39) | |

was performed separately for the PFGE band patterns generated by SmaI and EagI using BioNumerics software from Applied Math (Kortrijk, Belgium), and the patterns were confirmed by visual inspection. Band patterns were designated unique on the basis of ≥7 band differences, in accordance with the criteria of Tenover et al. (57) for determining genotypic differences between possible epidemic bacterial strains.

## RESULTS

A total of 4,552 *Haemophilus* isolates from the throat specimens of four healthy adult carriers, collected over 7 months, were analyzed, and the results are shown in Table 2. Briefly, 3,372 (74%) isolates were NAD dependent and hemin independent (i.e., porphyrin positive), namely, *H. parainfluenzae* and related species, and the prevalences in the four carriers ranged from 46 to 86%. Eight hundred sixty (19%) isolates were NAD- and hemin-dependent (i.e., porphyrin-negative) species, namely, *H. influenzae* and *H. haemolyticus*, and the prevalences were 7 to 48%. Four hundred twelve isolates were tested for hemolysis, the presence of the 7F3 epitope of P6, and *iga*; 346 (84%) were *H. influenzae* isolates, 47 (11%) were *H. haemolyticus*, 18 (4%) were nonhemolytic *H. haemolyticus*, and 1 was a variant strain. Two carriers were predominantly colonized with NTHI, another predominantly with $b^-$ *H. influenzae* mutants, and the remaining carrier with *H. haemolyticus*.

**USS-PCR and PFGE genotypes.** Because the USS-PCR gels exhibited relatively few bands, we chose to use the most stringent criteria for identifying unique USS genotypes, and strain differences were defined by a single band difference in the band patterns. USS-PCR genotyping was thus used as an initial screen to identify strain differences among 838 isolates suspected to be *H. influenzae* or *H. haemolyticus* from the four carriers. A subset of 468 isolates representing different USS-PCR genotypes were further analyzed using PFGE.

USS-PCR genotyping of 838 isolates revealed 11, 16, 5, and 12 distinct USS-PCR genotypes in carriers A, B, C, and D, respectively. Four hundred sixty-eight isolates from representative USS-PCR genotypes (about one for every two isolates identical by USS-PCR) were further typed by PFGE, which revealed 11, 15, 7, and 10 distinct PFGE genotypes in carriers A, B, C, and D, respectively (Table 2). One hundred ten isolates could not be digested by either the SmaI or EagI restriction enzyme and were considered to be genotypically different from the rest of the PFGE genotypes identified in each carrier. Based on ≥7 band differences between genotypes, those isolates with similarity indices of >70% clustered into single unique genotypes.

Subsequently, Pearson correlation on the USS-PCR versus PFGE genotypes was done on a subset of isolates from each carrier, and it ranged from 0.5 to 0.7, thus revealing limited correlation between the USS-PCR genotype and the PFGE genotype.

**Carrier A.** Throat samples were taken on 37 days over the 7-month study, leading to culture of 1,110 isolates from carrier A. Eight hundred eighty-nine (80%) isolates were heme dependent (porphyrin positive) and therefore belonged to *H. parainfluenzae* and related species (26). These generally nonpathogenic organisms are oral and pharyngeal commensals (31) and were not analyzed further. Seventy-six (7%) isolates were NAD and hemin dependent and hence belonged to *H. influenzae* or *H. haemolyticus* species. Fifty-seven of these 76 isolates were tested for hemolysis, the presence of the 7F3 epitope of P6, and *iga*; 45 (79%) were *H. influenzae*, 5 (9%) were *H. haemolyticus*, 6 (10%) were nonhemolytic *H. haemolyticus*, and 1 isolate was a variant strain (Table 2). All the *H. influenzae* isolates were NTHI.

Genotyping of 74 isolates using USS-PCR revealed 11 genotypes. Fifty-one isolates from representative USS-PCR genotypes (about one for every two isolates identical by USS-PCR) were further genotyped by PFGE using the SmaI or EagI restriction enzyme. These 51 isolates consisted of 40

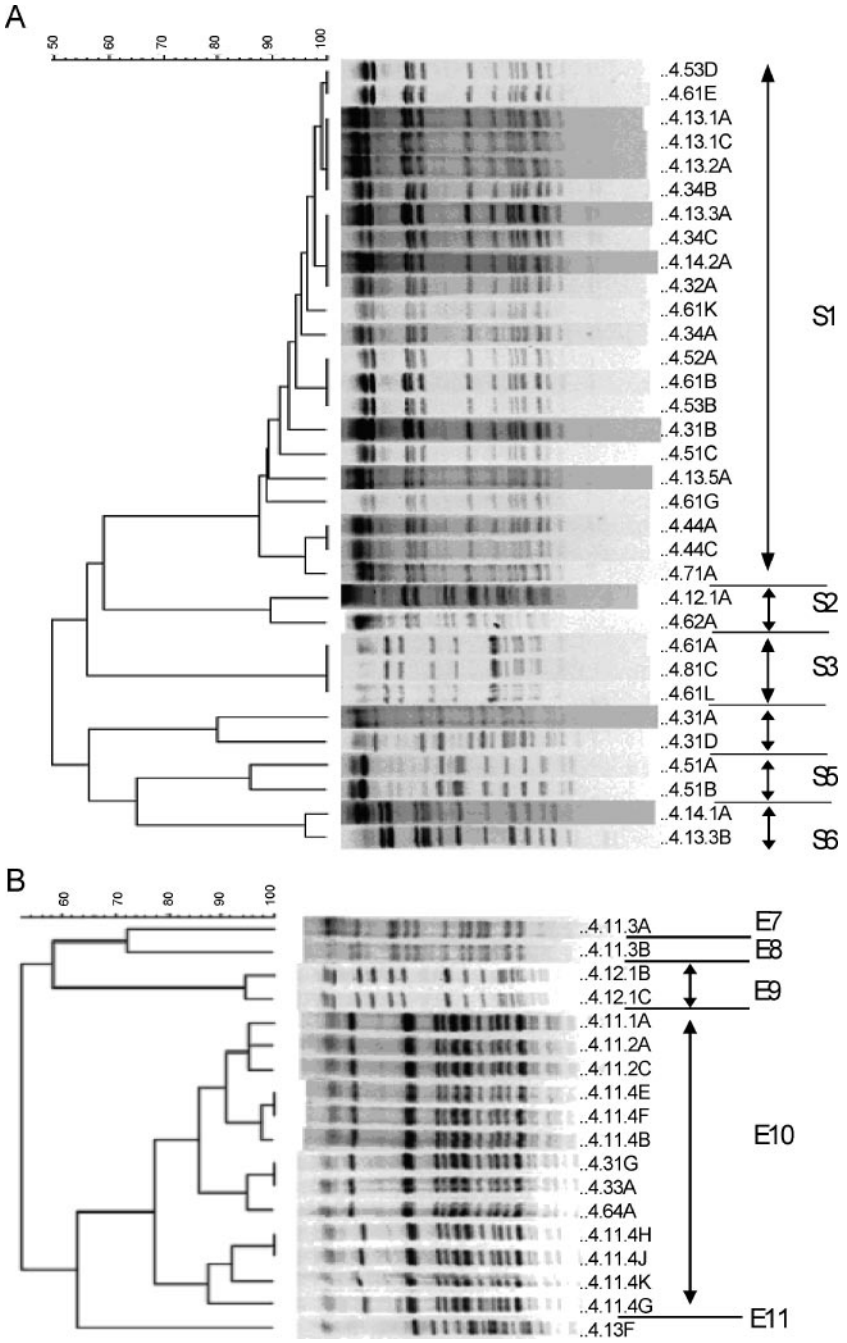


FIG. 1. Dendrogram (generated using BioNumerics software) of isolates from carrier A based on the unweighted pair-group method with arithmetic averages analysis of PFGE band patterns following digestion with SmaI (A) or EagI (B). Isolate labels are to the right of each band pattern; the first number on the left is the carrier number, followed in numerical order by the month, week, and day the sample was collected; each isolate from the same day was further designated by an uppercase letter. The PFGE genotypes shown are S1 to S6 in panel A and E7 to E11 in panel B.

NTHI, 5 *H. haemolyticus*, and 6 nonhemolytic *H. haemolyticus* isolates, and they clustered into 11 PFGE genotypes, 6 based on digestion with SmaI and 5 based on lack of digestion with SmaI but successful digestion with EagI (Fig. 1 and Table 3). The results showed simultaneous colonization with both *Haemophilus* species and with multiple genotypes (Table 3), as well as day-to-day variation in the genotypes and species recovered. The longest duration of carriage of a unique PFGE genotype (S1) was 154 days (Table 3), and this genotype was isolated sporadically on the sampling days during this period.

One sample (day 141) from carrier A demonstrated three isolates exhibiting the S3 genotype (Table 3); two of these isolates were nonhemolytic *H. haemolyticus*, and one was *H. influenzae*. This was confirmed by repeating the PFGE and

TABLE 3. Distribution of unique PFGE genotypes as defined by digestion with SmaI (S1 to S6) or EagI (E7 to E11) restriction enzyme over time in carrier A

| Day | No. of isolates identified with genotype: | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | S1[a] | S2 | S3 | S4 | S5 | S6 | E7[b] | E8 | E9 | E10 | E11 |
| 0 | | | | | | | | | | 1[c] | |
| 1 | | | | | | | | | | 2[c] | |
| 2 | | | | | | | 1[e] | 1[e] | | | |
| 3 | | | | | | | | | | 7[c] | |
| 8 | | 1[e] | | | | | | | 2[c] | | |
| 14 | 2[c] | | | | | | | | | | |
| 15 | 1[c] | | | | | | | | | | |
| 16 | 1[c] | | | | | 1[c] | | | | | |
| 17 | 1[c] | | | | | | | | | | |
| 22 | | | | | | 1[c] | | | | | |
| 23 | 1[c] | | | | | | | | | | |
| 60 | 1[c] | | | 2[d] | | | | | | 1[c] | 1[d] |
| 64 | 1[c] | | | | | | | | | | |
| 71 | | | | | | | | | | 1[c] | |
| 81 | 3[c] | | | | | | | | | | |
| 108 | 2[c] | | | | | | | | | | |
| 116 | 1[c] | | | | 2[d] | | | | | | |
| 122 | 1[c] | | | | | | | | | | |
| 128 | 2[c] | | | | | | | | | | |
| 141 | 4[c] | | 1[c] + 2[e] | | | | | | | | |
| 150 | | 1[e] | | | | | | | | | |
| 161 | | | | | | | | | | 1[c] | |

[a] S genotypes based on digestion of genomic DNA with SmaI.
[b] E genotypes based on failure to be digested with SmaI and successful digestion with EagI.
[c] NTHI.
[d] *H. haemolyticus*.
[e] Nonhemolytic *H. haemolyticus*.

other tests multiple times on the DNA extracted from the original stocks of these isolates.

In summary, carrier A was colonized predominantly with NTHI, had 11 unique PFGE genotypes, was cocolonized with more than one genotype and more than one species with considerable sample-to-sample variation, and was colonized for 154 days with a single genotype.

**Carrier B.** Throat samples were taken on 39 days over the 7-month study, leading to culture of 1,170 isolates from carrier B. Five hundred forty-three (46%) isolates were heme dependent (porphyrin positive) and therefore belonged to *H. parainfluenzae* and related species (26) and were not analyzed further. Five hundred fifty-nine (48%) isolates were NAD and hemin dependent and hence belonged to *H. influenzae* or *H. haemolyticus* species. Two hundred thirty-seven isolates were tested for hemolysis, the presence of the 7F3 epitope of P6, and *iga*; 236 (99.6%) were *H. influenzae*, 1 (0.4%) was *H. haemolyticus*, and none were nonhemolytic *H. haemolyticus* or variant strains (Table 2). All the *H. influenzae* isolates were NTHI.

Genotyping using USS-PCR of 540 NTHI isolates and 1 *H. haemolyticus* isolate revealed 16 genotypes, and 259 NTHI isolates from representative USS-PCR genotypes (about 1 for every 2 isolates identical by USS-PCR) were further genotyped by PFGE using the SmaI or EagI restriction enzyme. Fifty-four (21%) isolates could not be digested by either restriction enzyme after repeated attempts and thus were considered genotypically different from the digestible strains. The remaining 205 NTHI isolates clustered into 15 unique PFGE genotypes (Fig. 2 and Table 4). The results showed simultaneous colonization with multiple genotypes (Table 4), as well as day-to-day variation in the genotypes recovered. The longest duration of carriage of a single unique PFGE genotype (S4) was 129 days, and this genotype was isolated sporadically on the sampling days during this period, with a 106-day interval between the last two isolations.

In summary, carrier B was colonized almost exclusively with NTHI, had 15 unique PFGE genotypes, was cocolonized with more than one genotype on 15 sampling days, and was colonized for 129 days with a single genotype.

**Carrier C.** Throat samples were taken on 38 days over the 7-month study, leading to culture of 1,140 isolates from carrier C. Nine hundred eighty-four (86%) isolates were heme dependent (porphyrin positive) and therefore belonged to *H. parainfluenzae* and related species (26) and were not analyzed further. Ninety-eight (9%) isolates were NAD and hemin dependent and hence belonged to *H. influenzae* or *H. haemolyticus* species. Seventy isolates were tested for hemolysis, the presence of the 7F3 epitope of P6, and *iga*; 65 (93%) were *H. influenzae*, 3 (4%) *H. haemolyticus*, and 2 (3%) nonhemolytic *H. haemolyticus* (Table 2).

Serotyping by slide agglutination revealed mild clumping, so all *H. influenzae* isolates were serotyped using PCR. Forty-five (69%) isolates were found to be $b^-$ mutants. The remaining 20 (31%) isolates failed to generate a PCR product for both *bexA* and the type-specific region of the *cap* genes and were thus considered NTHI.

Genotyping of the 98 isolates using USS-PCR revealed five genotypes, and 71 isolates from representative USS-PCR genotypes (about 1 for every 2 isolates identical by USS-PCR) were further genotyped by PFGE using the SmaI or EagI restriction enzyme. These 71 isolates consisted of 66 *H. influenzae*, 3 *H. haemolyticus*, and 2 nonhemolytic *H. haemolyticus* isolates. Eight isolates (four *H. influenzae*, three *H. haemolyticus*, and one nonhemolytic *H. haemolyticus*) could not be digested by either restriction enzyme on repeated attempts and were thus considered to be genotypically different from the digestible strains. The remaining 63 isolates (43 $b^-$ *H. influenzae* mutants, 19 NTHI, and 1 nonhemolytic *H. haemolyticus*) clustered into seven unique PFGE genotypes (Fig. 3 and Table 5). The results showed simultaneous colonization with both *Haemophilus* species and with multiple genotypes (Table 5), as well as day-to-day variation in the genotypes recovered. Interestingly, genotype S6 contained both NTHI and $b^-$ mutants (Table 5). The longest duration of carriage of a single unique PFGE genotype (S1) was 173 days, and this genotype was isolated sporadically on the sampling days during this period.

In summary, carrier C was colonized primarily with $b^-$ *H. influenzae* mutants, as well as NTHI; had seven unique PFGE genotypes; was cocolonized with more than one genotype; and was colonized for 173 days with a single genotype.

**Carrier D.** Throat samples were taken on 38 days over the 7-month study, leading to culture of 1,132 isolates from carrier D. Nine hundred fifty-six (84%) isolates were heme dependent (porphyrin positive) and therefore belonged to *H. parainfluenzae* and related species (26) and were not analyzed further. One hundred twenty-seven (11%) isolates were NAD and hemin dependent and hence belonged to *H. influenzae* or *H. haemolyticus* species. Forty-eight isolates were tested for he-

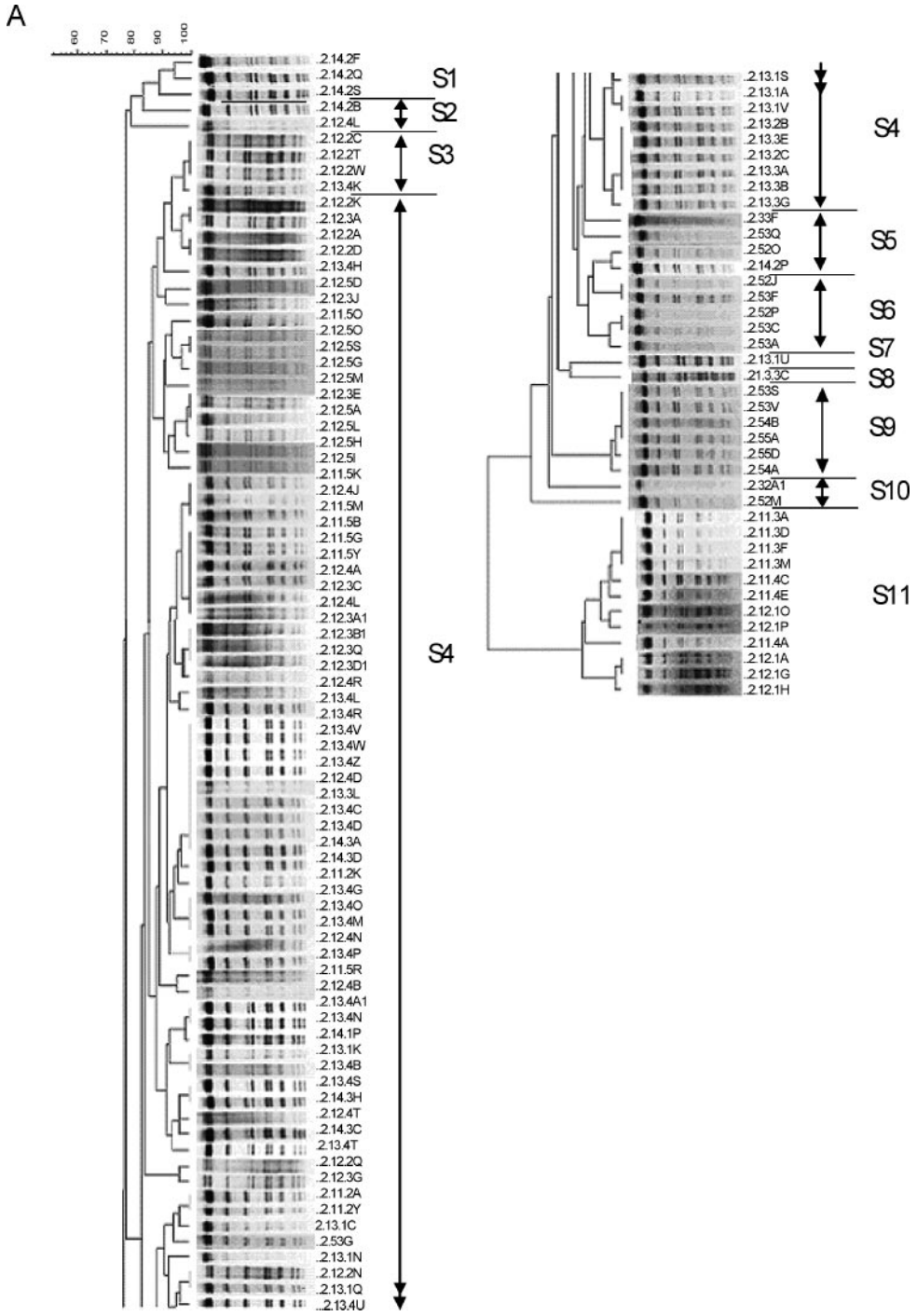


FIG. 2. Dendrogram (generated using BioNumerics software) of carrier B isolates digested with SmaI (A) and EagI (B). See the legend to Fig. 1 for an explanation of the isolate labels.

molysis, the presence of the 7F3 epitope of P6, and *iga*; none were *H. influenzae*, 38 (79%) were *H. haemolyticus*, and 10 (21%) were nonhemolytic *H. haemolyticus*, with no variant strain (Table 2).

Genotyping using USS-PCR of 125 isolates revealed 12 genotypes, and 87 isolates from representative USS PCR genotypes (about 1 for every 2 isolates identical by USS-PCR) were further genotyped by PFGE using the SmaI or EagI restriction enzyme. Forty-eight (55%) isolates could not be digested by either restriction enzyme after repeated attempts and thus were considered different from the digestible strains. The remaining 39 isolates (35 *H. haemolyticus* and 4 nonhemolytic *H. haemolyticus*) were all digested with SmaI and clustered into 10 unique PFGE genotypes (Fig. 4 and Table 6). The results showed simultaneous colonization with multiple genotypes (Table 6), as well as day-to-day variation in the genotypes

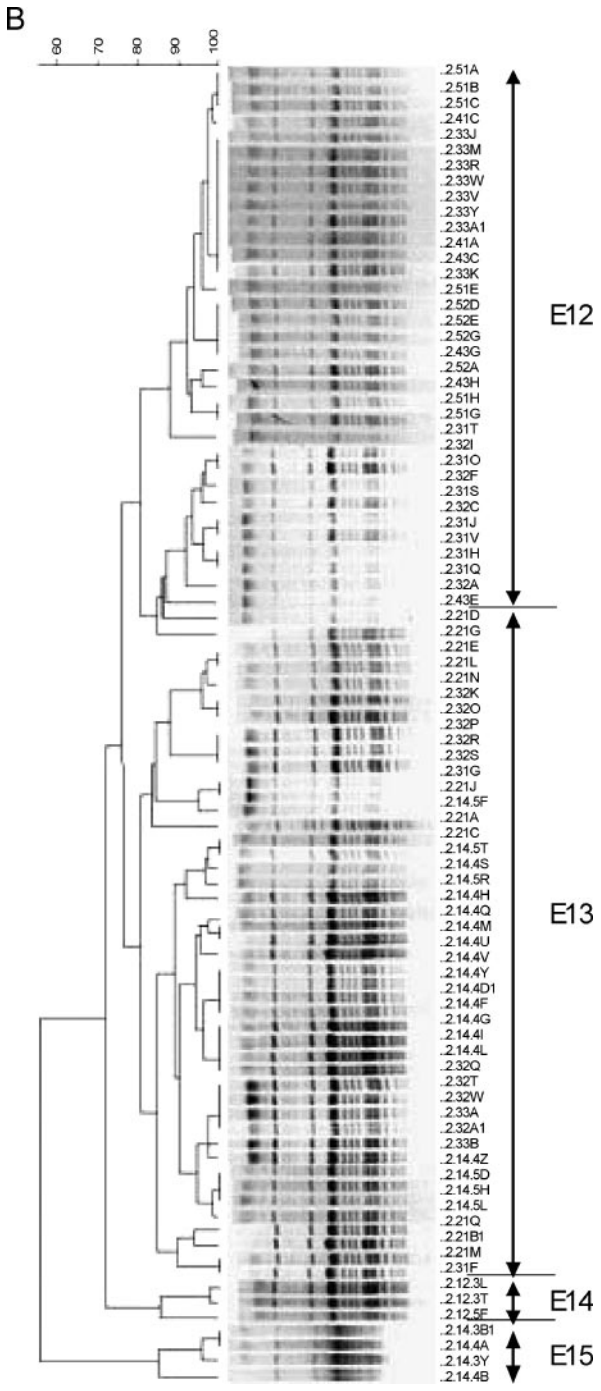


FIG. 2—*Continued.*

recovered. The longest duration of carriage of a single unique PFGE genotype (S4) was 198 days, and this genotype was isolated sporadically on the sampling days during this period, with 174 days between the last two isolations.

In summary, carrier D was colonized predominantly with *H. haemolyticus*, had 10 unique PFGE genotypes, was cocolonized with more than one genotype, and was colonized for 198 days with a single genotype.

## DISCUSSION

Pharyngeal colonization is the initial step in the sequence of events leading to *H. influenzae* infection and disease. Thus, understanding the dynamics of *H. influenzae* colonization forms the foundation for understanding the natural history of diseases caused by this bacterium. While previous studies have investigated *H. influenzae* pharyngeal colonization among healthy adults (4) and among individuals at risk for *H. influenzae* infections, such as children attending day care centers (8, 14, 56), patients with cystic fibrosis (49), and adults with COPD (38, 43), no previous reports have documented colonization with *H. haemolyticus* in population-based studies of healthy adults. Pharyngeal colonization with nonhemolytic *H. haemolyticus* has only recently been described (16, 39). The results of the present study describe the dynamics of *H. influenzae* and *H. haemolyticus* colonization over a 7-month period studied by culturing throat specimens from four healthy adult carriers at frequent intervals.

Multiple *H. influenzae* genotypes are frequently present in the upper respiratory tracts of adults with cystic fibrosis (49) and COPD (43) and children in day care centers (14, 56). In our study, cocolonization with two to five *H. influenzae* or *H. haemolyticus* strains, as defined by PFGE genotypes, was seen on 37 out of 132 (28%) sampling days. Furthermore, from 7 to 15 genotypes of *H. influenzae* and *H. haemolyticus* were cultured from the four carriers over the course of 7 months. Likely explanations for the high yield of positive cultures and for the

TABLE 4. Distribution of unique PFGE genotypes as defined by digestion with SmaI (genotypes S1 to S11) or EagI (genotypes E12 to E15) restriction enzyme over time in carrier B

| Day | No. of isolates identified with genotype[a]: | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | S1[b] | S2 | S3 | S4 | S5 | S6 | S7 | S8 | S9 | S10 | S11 | E12[c] | E13 | E14 | E15 |
| 1 | | | | 3 | | | | | | | | | | | |
| 2 | | | | | | | | | | | 4 | | | | |
| 3 | | | | | | | | | | | 3 | | | | |
| 4 | | | | 6 | | | | | | | | | | | |
| 7 | | | | | | | | | | | 5 | | | | |
| 8 | | | 3 | 5 | | | | | | | | | | | |
| 9 | | | | 9 | | | | | | | | | | 2 | |
| 10 | | 1 | | 7 | | | | | | | | | | | |
| 11 | | | | 10 | | | | | | | | | | 1 | |
| 15 | | | | 8 | | | 1 | | | | | | | | |
| 16 | | | | 2 | | | | | | | | | | | |
| 17 | | | | 5 | | | | 1 | | | | | | | |
| 18 | | | 1 | 18 | | | | | | | | | | | |
| 21 | | | | 1 | | | | | | | | | | | |
| 22 | 3 | 1 | | | 1 | | | | | | | | | | |
| 23 | | | | 5 | | | | | | | | | | | 2 |
| 24 | | | | | | | | | | | | | 13 | | 2 |
| 25 | | | | | | | | | | | | | 6 | | |
| 28 | | | | | | | | | | | | | 11 | | |
| 59 | | | | | | | | | | | | 7 | 2 | | |
| 67 | | | | | | | | | | | | 4 | 9 | | |
| 72 | | | | | 1 | | | | | | | 8 | 2 | | |
| 88 | | | | | | | | | | | | 2 | | | |
| 102 | | | | | | | | | | | | 4 | | | |
| 114 | | | | | | | | | | | | 6 | | | |
| 123 | | | | | 1 | 2 | | | | 1 | | 4 | | | |
| 129 | | | | 1 | 1 | 3 | | | 2 | 1 | | | | | |
| 135 | | | | | | | | | 2 | | | | | | |
| 143 | | | | | | | | | 2 | | | | | | |

[a] All isolates were confirmed to be NTHI.
[b] S genotypes based on digestion of genomic DNA with SmaI.
[c] E genotypes based on failure to be digested with SmaI and successful digestion with EagI.

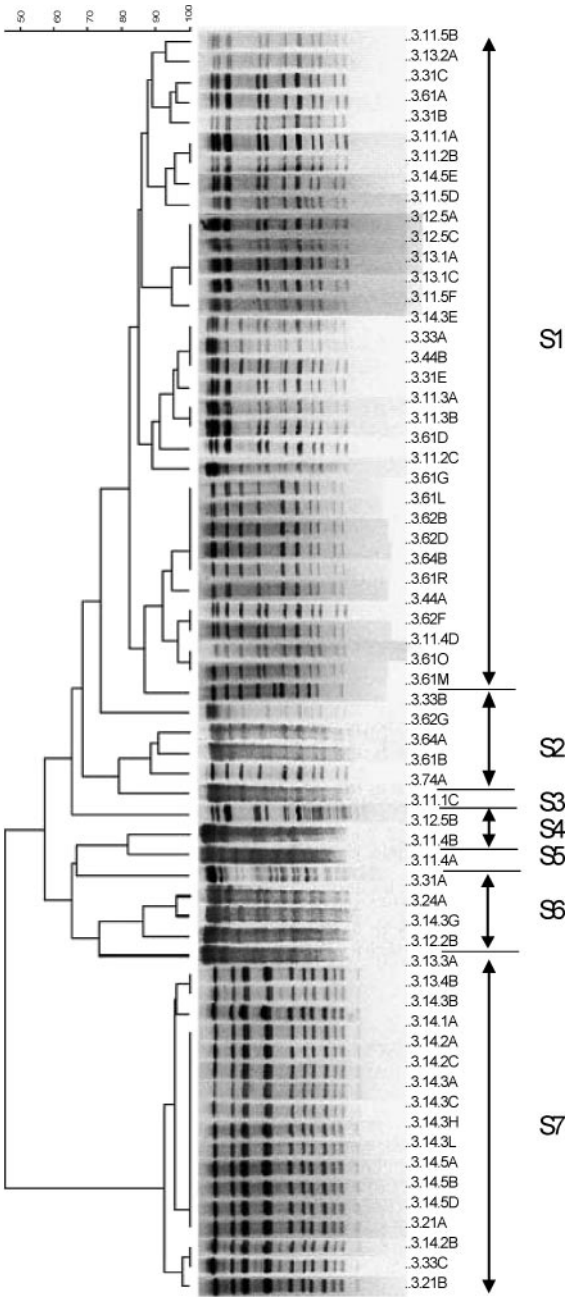


FIG. 3. Dendrogram (generated using BioNumerics software) of carrier C isolates digested with SmaI. See the legend to Fig. 1 for an explanation of the isolate labels.

genetic diversity seen in each individual include the use of selective agar containing bacitracin, which significantly reduces contamination with non-*Haemophilus* species and facilitates isolation of *Haemophilus*; vigorous sampling of the throat, which yields *H. influenzae* isolation rates equal to or greater than sampling the nasopharynx in adults (19, 33); and analysis of multiple isolates with morphology suggestive of *Haemophilus* species from each sample.

USS-PCR genotyping (a technique that is more rapid and resource conserving than PFGE) was used as an initial screen to identify strains showing identical genotypes that would not require further genotyping. The USS-PCR genotyping procedure resulted in relatively few bands compared to PFGE and thus may be less sensitive in identifying unique genotypes. Our results subsequently showed limited correlation between USS-PCR genotyping and PFGE genotyping, which could be expected, as the two procedures assess very different characteristics of the bacteria (patterns generated by PCR amplification products of DNA fragments flanked by *H. influenzae* uptake signal sequences and patterns generated by DNA fragments flanked by restriction enzyme sites, respectively). The lack of good correlation between these two techniques underscores the genetic variability between strains and the many mechanisms of genetic variability. Thus, the results reported in this study represent the minimum genetic diversity among strains. If we had performed PFGE on all 838 isolates cultured from the four subjects, we anticipate we would have documented even more diversity.

We chose to employ the criteria of Tenover et al. (57) to define unique genotypes in the PFGE analysis. These criteria were developed for outbreak investigation to identify the genetic relatedness of epidemiologically clustered strains, with the assumption that bacterial isolates from individual disease outbreaks have undergone few evolutionary changes. These criteria stated that strains whose band patterns differed by seven or more restriction fragments exhibited at least three genetic differences between them and were considered genetically unrelated; strains with zero band differences were considered to be genetically identical, strains with two or three

TABLE 5. Distribution of unique PFGE genotypes as defined by digestion with SmaI (S1 to S7) restriction enzyme over time in carrier C

| Day | No. of isolates identified with genotype: | | | | | | |
|---|---|---|---|---|---|---|---|
| | S1[a] | S2 | S3 | S4 | S5 | S6 | S7 |
| 0 | 1[b] | | 1[b] | | | | |
| 1 | 2[b] | | | | | | |
| 2 | 2[b] | | | | | | |
| 3 | 1[b] | | | 1[b] | 1[d] | | |
| 4 | 3[b] | | | | | | |
| 8 | | | | | | 1[c] | |
| 11 | 2[b] | | | 1[b] | | | |
| 15 | 2[b] | | | | | | |
| 16 | 1[b] | | . | | | | |
| 17 | | | | | | | 1[c] |
| 18 | | | | | | | 1[c] |
| 21 | | | | | | | 1[c] |
| 22 | | | | | | | 3[c] |
| 23 | 1[b] | | | | | 1[b] | 5[c] |
| 25 | 1[b] | | | | | | 3[c] |
| 31 | | | | | | | 2[c] |
| 51 | | | | | | 1[c] | |
| 57 | 3[b] | | | | | 1[b] | |
| 71 | 1[b] | 1[b] | | | | | 1[c] |
| 106 | 2[b] | | | | | | |
| 148 | 7[b] | 1[b] | | | | | |
| 155 | 3[b] | 1[b] | | | | | |
| 172 | 1[b] | 1[b] | | | | | |
| 197 | | 1[b] | | | | | |

[a] S genotypes based on digestion of genomic DNA with SmaI.
[b] Capsule-deficient type b mutant.
[c] NTHI.
[d] Nonhemolytic *H. haemolyticus*.

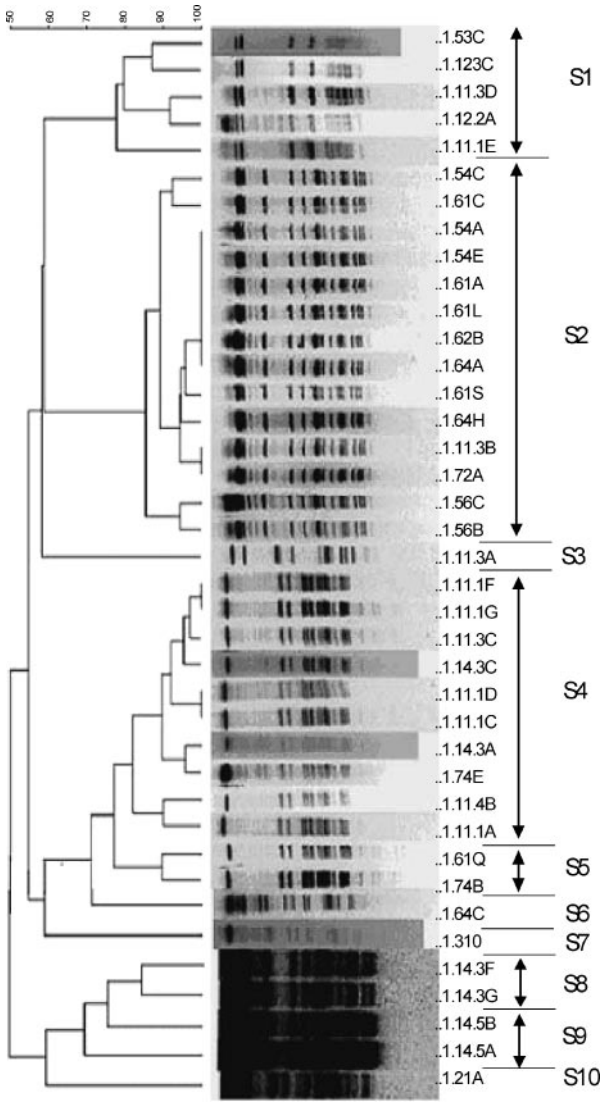


FIG. 4. Dendrogram (generated using BioNumerics software) of carrier D isolates digested with SmaI. See the legend to Fig. 1 for an explanation of the isolate labels.

band differences (one genetic difference) were considered closely related, and strains with four to six band differences (two genetic differences) were considered possibly related. While repeated throat samples from the same individual could be viewed as representing a closed epidemiologic unit, our data support the concept that pharyngeal colonization is a dynamic process during which bacterial evolution may occur.

Examination of the dendrograms in Fig. 1, 2, 3, and 4 reveals that some isolates from a single individual collected within a few weeks were clearly different genotypes while others were closely related but exhibited several band differences. This observation is consistent with a population of organisms in the pharynx in which both acquisition of new strains and evolution of existing strains was occurring, as has been described for *Helicobacter pylori* strains isolated several years apart from single individuals (25). These results are consistent with the recent suggestion that *H. influenzae* exists in the pharynx in the form of a biofilm (17, 40, 60), a structured community of bacterial cells enveloped in a polymeric matrix and adherent to an inert or living surface (6, 7). We anticipate that some of the day-to-day and week-to-week variability of the genotypes and the skips in persistence of unique strains (as also described by Murphy et al. [38]) resulted from sampling bias in that we obtained only a few colonies from a huge, and somewhat diverse, population of bacteria.

*H. influenzae* is a naturally competent organism and possesses approximately 1,400 copies (54) of the 9-bp uptake signal sequence that allows genetic exchange by transformation and homologous recombination. Intraspecies (28, 29) and interspecies (30) gene transfer has been demonstrated in *H. influenzae*. High rates of recombination have also been demonstrated in otitis media isolates of NTHI (5), and hypermutability mutants have been associated with high genetic variability among *H. influenzae* isolates from the airways of patients with cystic fibrosis (49). Thus, *H. influenzae* is well equipped to survive in its natural environment, the human pharynx, by generating genetic mutants or variants that exhibit various fitness characteristics.

*H. haemolyticus*, closely related to *H. influenzae*, is found in the pharynges of healthy humans and often in the bacterial deposits on teeth below the gingival margin. In our study, *H. haemolyticus* was isolated from carrier D throughout the study period of 7 months and clustered into 10 PFGE genotypes, 9 of which contained only hemolytic isolates while genotype S1 contained one hemolytic and four nonhemolytic isolates. Conceivably, these five isolates represent one classical *H. haemolyticus* and four *H. haemolyticus* mutants with either nonfunctional mutations in the hemolysin gene or loss of the gene itself. Further clarification of these strains awaits analysis of their hemolysin genes, which have not yet been defined in *H. haemolyticus*. Carrier A carried seven genotypes of *H. haemolyticus*; three genotypes included only nonhemolytic isolates,

TABLE 6. Distribution of unique pulsed field gel electrophoresis (PFGE) genotypes as defined by digestion with SmaI (S1 to S10) restriction enzyme over time in Carrier D

| Day | No. of isolates identified with genotype: | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | S1[a] | S2 | S3 | S4 | S5 | S6 | S7 | S8 | S9 | S10 |
| 0 | 1[c] | | | 5[b] | | | | | | |
| 2 | 1[b] | 1[b] | 1[b] | 1[b] | | | | | | |
| 3 | | | | 1[b] | | | | | | |
| 8 | 1[c] | | | | | | | | | |
| 9 | 1[c] | | | | | | | | | |
| 23 | | | | 2[b] | | | | 2[b] | | |
| 25 | | | | | | | | | 2[b] | |
| 28 | | | | | | | | | | 1[b] |
| 56 | | | | | | | 1[b] | | | |
| 129 | 1[c] | | | | | | | | | |
| 137 | | 3[b] | | | | | | | | |
| 144 | | 2[b] | | | | | | | | |
| 148 | | 4[b] | | | 1[b] | | | | | |
| 157 | | 1[b] | | | | | | | | |
| 172 | | 2[b] | | | | 1[b] | | | | |
| 185 | | 1[b] | | | | | | | | |
| 197 | | | | 1[b] | 1[b] | | | | | |

[a] S genotypes based on digestion of genomic DNA with SmaI.
[b] *H. haemolyticus*.
[c] Nonhemolytic *H. haemolyticus*.

and three included only hemolytic isolates. On the other hand, genotype S3 from this carrier included two nonhemolytic *H. haemolyticus* isolates and one *H. influenzae* isolate. If these three S3-type strains truly represent variants of the same clone, either a parent *H. influenzae* clone lost both the 7F3 epitope of P6 and *iga* or a parent nonhemolytic *H. haemolyticus* gained both, which is less likely. The taxonomy of bacterial strains attempts to group isolates exhibiting identical phenotypic or genotypic features. The failure of a few strains to fit a strict definition of *H. influenzae* or *H. haemolyticus* is expected, as bacteria, because of random evolution of individual genes (5, 36), form a continuum of strain characteristics rather than a dichotomy. This blurring of the intersection of two species is similar to that described with *Neisseria* strains (21).

This study is, to our knowledge, the first to describe the dynamics of *H. haemolyticus* colonization and to characterize its diversity. Although *H. haemolyticus* is not a common human pathogen (1, 31), its presence on the respiratory mucosa may serve as a source of genetic material for horizontal gene transfer to *H. influenzae* and thus enhance the pool of genes available to *H. influenzae* in sustaining the genetic diversity that fosters its existence in the antibody-laden milieu of the human pharynx.

*H. influenzae* expressing the type b capsule is found in the pharynx less frequently than nontypeable strains but is responsible for the majority of the cases of *H. influenzae* invasive infections in nonimmune individuals. Furthermore, type b strains are more clonal than NTHI (44, 53). Capsule-deficient, or $b^-$, *H. influenzae* mutants carry the b-type *cap* region but do not have the *bexA* gene required for exportation of the capsule to the cell surface (13, 24). To our knowledge, long-term colonization with $b^-$ mutants has not been documented, although $b^-$ mutants have been isolated from the throats of patients with type b infections (23). In our study, carrier C was colonized with $b^-$ *H. influenzae* strains of five different genotypes, one of which (S6) also contained two isolates of NTHI. This observation is difficult to explain; the apparent NTHI strains, which demonstrated absence of *cap* region genes by PCR on repeated testing, may be Hib variants with complete deletion of the *cap* gene region and thus could not be distinguished from NTHI by our techniques. Alternatively, this observation may represent a limitation of PFGE in identifying unique clonal differences among strains. In addition, this carrier may have been colonized with the $b^-$ mutant over enough time for significant evolutionary events to occur that altered the restriction enzyme genes upon which our typing system depends, leading to unique genotypes.

This study has demonstrated immense genetic diversity of *H. influenzae* and *H. haemolyticus* isolated from each of four individuals over 7 months. These results may reflect the extensive recombination and DNA uptake known to occur in *H. influenzae* and are also consistent with bacterial growth in multimicrobial biofilms (17, 38, 40, 60). Identifying host characteristics that increase the risk of colonization with pathogenic strains, discovering the virulence factors in *H. influenzae* that cause it to establish infection, and knowledge of environments that are conducive to *H. influenzae* disease will further our understanding of the pathogenesis of *H. influenzae* disease and will facilitate the development of preventive strategies for diseases caused by these organisms.

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# AUTHOR'S CORRECTION

## Pharyngeal Colonization Dynamics of *Haemophilus influenzae* and *Haemophilus haemolyticus* in Healthy Adult Carriers

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Volume 45, no. 10, p. 3207–3217, 2007. Page 3208, column 2, lines 3–4: "primers (BF1, GCAGAATTCAAAGCACAATTT GTTGCA" should read "primers (BF1, TGAATAACGAGGGGCAATATAAC."